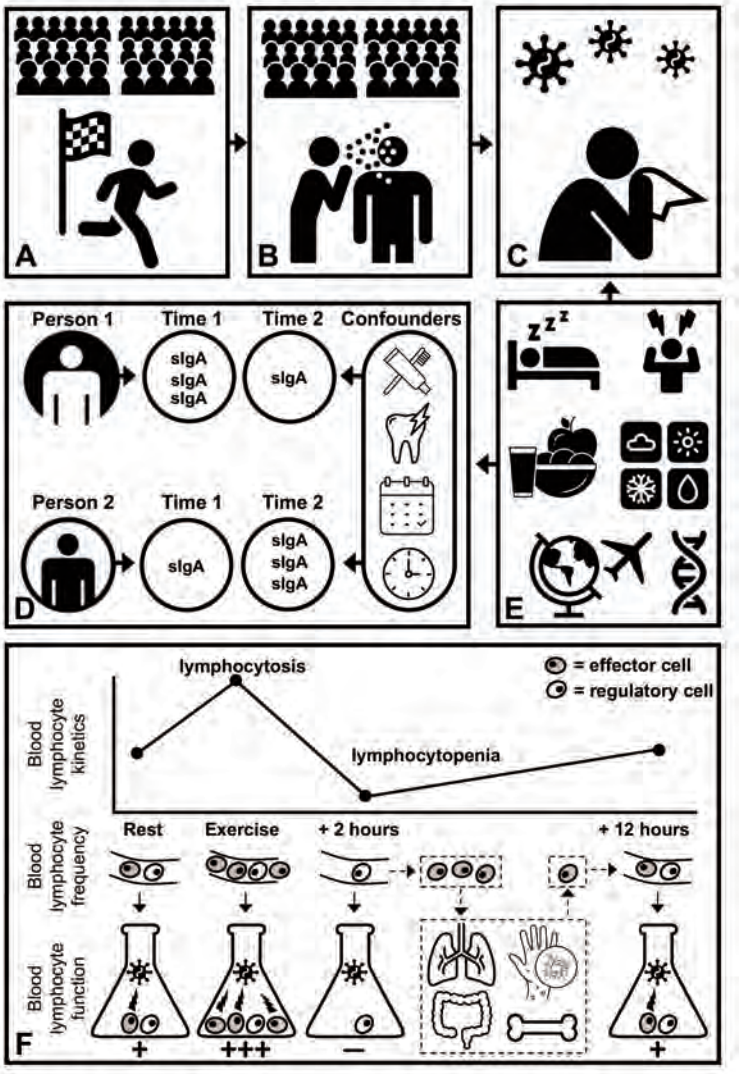
EXERCISE IMMUNOLOGY REVIEW



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EXERCISE IMMUNOLOGY REVIEW

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Exercise Immunology Review

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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 Exercise Immunology Review is indexed in Sport Database, Sport Discus, Sport Search, SciSearch, EMBASE/ Excerpta Medica, Focus on: Sports Science & Medicine, Index Medicus, MEDLINE, Physical Education Index, Research Alert, International Bibliography of Periodical Literature, International Bibliography of Book Reviews, and CINAHL database.

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From the Editors

EIR26 2020 contains eight articles. Our associate editor, Richard Simpson, coordinated a debate article, which represents a new type of article for EIR. The purpose of this article was to solicit opposing arguments centered around a fundamental question in the exercise immunology field: can exercise affect immune function to increase susceptibility to infection. The second article by Joisten et al. gives an overview about the current state of knowledge in exercise and the Kynurenine pathway. Hoekstra et al. discuss the potential and mechanisms of active (e.g. exercise) and passive (e.g. hot water immersion; sauna therapy) heating methods to reduce chronic low-grade inflammation and improve metabolic health in physically inactive people. Mulas et al. examined the effects of exercise on tumor growth and its possible adjuvant effects when combined with anti-PD-1 immunotherapy in a patient-derived xenograft model of non–small-cell lung cancer. A study from Hwang et al. suggests that mobilizing serum factors and immune cells may be a key mechanism by which exercise counteracts cancer in older adults. Colby et al. analyzed and present key viral immune genes and pathways to identify elite athletes with symptoms of upper respiratory illness. Curran et al. demonstrate that the mobilisation of early mature CD56dim/CD16bright NK cells is blunted in response to a single bout of vigorous intensity exercise in people living with Type 1 Diabetes. Finally, Siveira et al. present evidence for the mechanistic role of peroxisome proliferator-activated receptor gamma (PPARγ) in macrophage immunophenotypes differentiation during exercise. We hope you enjoy reading this new issue of EIR and we send out a special thanks to all contributors and reviewers of EIR26.

In 2019, a total of 17 manuscripts were submitted to EIR, which corresponds to an acceptance rate of about 50%. Currently (2018/19), EIR has an official impact factor of 6.455. For EIR27 and beyond, our preference is to always solicit and publish topical review articles. While original research articles are also published in EIR, we encourage potential authors to combine their new data with an extended literature review and/or discussion of the topic. Please note that the submission deadline for EIR27 is 31st July 2020. Thank you, Rickie Simpson, Neil Walsh and Jonathan Peake, for the continuing close, trusting and friendly teamwork as we work towards keeping EIR as one of the top ranked journals in the Sports Science category. We thank all members of ISEI and the EIR Editorial Board for the confidence and trust you have placed in us. We greatly appreciate your ongoing support of EIR.

Finally, we hope to see a large number of ISEI members at the 15th ISEI symposium, will be held in Tucson, Arizona (USA) in November 2021. Please check the ISEI website and LinkedIn page for updates.

On behalf of the Editors,

Karsten Krüger

Can exercise affect immune function to increase susceptibility to infection?

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Abstract

Multiple studies in humans and animals have demonstrated the profound impact that exercise can have on the immune system. There is a general consensus that regular bouts of short-lasting (i.e. up to 45 minutes) moderate intensity exercise is beneficial for host immune defense, particularly in older adults and people with chronic diseases. In contrast, infection burden is reported to be high among high performance athletes and second only to injury for the number of training days lost during preparation for major sporting events. This has shaped the common view that arduous exercise (i.e. those activities practiced by high performance athletes/military personnel that greatly exceed recommended physical activity guidelines) can suppress immunity and increase infection risk. However, the idea that exercise per se can suppress immunity and increase infection risk independently of the many other factors (e.g. anxiety, sleep disruption, travel, exposure, nutritional deficits, environmental extremes, etc.) experienced by these populations has recently been challenged. The purpose of this debate article was to solicit opposing arguments centered around this fundamental question in the exercise immunology field: can exercise affect immune function to increase susceptibility to infection. Issues that were contested between the debating groups include: (i) whether or not athletes are more susceptible to infection (mainly of the upper respiratory tract) than the general population; (ii) whether exercise per se is capable of altering immunity to increase infection risk independently of the multiple factors that activate shared immune pathways and are unique to the study populations involved; (iii) the usefulness of certain biomarkers and the interpretation of in vitro and in vivo data to monitor immune health in those who perform arduous exercise; and (iv) the quality of scientific evidence that has been used to substantiate claims for and against the potential negative effects of arduous exercise on immunity and infection risk. A key point of agreement between the groups is that infection susceptibility has a multifactorial underpinning. An issue that remains to be resolved is whether exercise per se is a causative factor of increased infection risk in athletes. This article should provide impetus for more empirical research to unravel the complex questions that surround this contentious issue in the field of exercise immunology.

Keywords: Exercise immunology, Athletes, Immuno-suppression, Upper respiratory tract infections, Open window of infection risk, stress, physical activity.

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Author Contributions:
The basis of the article and the debate process was devised and moderated by RJS, KK and NPW. The abstract, introduction and summary sections were written by RJS, KK and NPW, with input from JPC, MG, DCN, DBP and JET. The Yes Case and Response to the No Case sections were written by MG, DCN and DBP, who contributed equally to the work. The No case and Response to the Yes Case sections were written by JPC and JET, who contributed equally to the work. All authors approved the final version of the article.

Introduction

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Exercise immunology as a discipline came of age in the latter part of the twentieth century (121). Since 1990, ~5,000 peer-reviewed original research and review papers have been published, cutting across multiple themes including acute/chronic changes in athletic and non-athletic populations, clinical and

translational perspectives, nutritional interactions and immunosenescence (91, 124, 141). Both cross-sectional and longitudinal studies in humans have demonstrated the profound impact that exercise can have on the immune system. Physical fitness and moderate intensity exercise training have been shown to improve immune responses to vaccination, lower chronic low-grade inflammation, and improve various immune markers in several disease states including cancer, HIV, cardiovascular disease, diabetes, cognitive impairment and obesity (39, 56, 67, 130). Conversely, arduous bouts of exercise, typically those practiced by athletes and other high-performance personnel (e.g. the military), have been associated with suppressed mucosal and cellular immunity, increased symptoms of upper respiratory tract infections (URTI), latent viral reactivation, and impaired immune responses to vaccine and novel antigens (15, 64, 91, 98). This body of research has informed the view in the exercise immunology field that regular bouts of short-lasting (i.e. up to 45 minutes) moderate intensity exercise are 'immunoenhancing' whereas repeated bouts of long-lasting (>2hours) arduous intensity exercise can be 'immunosuppressive' (126, 141). The J-curve and open-window hypothesis have been staples of the exercise immunology discipline for almost three decades, providing a set of theoretical frameworks to explain why exercise can apparently exert both enhancing and suppressive effects on the immune system and alter susceptibility to illness (89, 100). While the plethora of beneficial effects provided by regular short-lasting moderate intensity exercise on the immune system of older adults and people with chronic disease are undisputed (91, 126), the empirical research supporting the basis of these frameworks and the idea that any form of exercise can be considered 'immunosuppressive' has recently been challenged (17, 18).

The purpose of this debate article was to revisit a fundamental question in the exercise immunology field – can exercise affect immune function to *increase* susceptibility to illness? Renowned experts in exercise immunology were asked to provide a brief narrative supporting their contention that exercise is/is not capable of affecting immune function to increase susceptibility to illness. Providing the argument for (The Yes Case) are Maree Gleeson (University of Newcastle, Australia), David C. Nieman (Appalachian State University, USA) and David B. Pyne (University of Canberra, Australia). The argument against (The No Case) is provided by John P. Campbell (University of Bath, UK) and James E. Turner (University of Bath, UK). Both groups of authors were also asked to provide a rebuttal to the original narratives. Finally, points of agreement and issues that remain to be resolved are presented by the editorial team to provide impetus for future empirical research studies in the area.

Can Exercise Affect Immune Function to increase Susceptibility to Infection? – The Yes Case

Maree Gleeson, University of Newcastle, Callaghan, NSW, Australia
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Introduction

The impact of exercise on innate and acquired immune parameters (magnitude, direction of changes and recovery time) is dependent on the intensity of exercise, and in high-performance sports, the duration and load of training. Immune function can be compromised at the high-performance end of the spectrum of physical activity, and place an individual at increased risk of infection (Figure 1). These risks are co-dependent on factors that regulate immune function (genetic, nutritional status, psychological stress, interrupted circadian rhythm), environmental stressors (extreme temperatures, allergens, airway irritants), or underlying health conditions that promote inflammatory processes [see reviews (12, 140, 141)]. As upper respiratory illness (URI) is the most common (35-65%) non-injury related presentation in sports medicine (49), there is substantial clinical and laboratory evidence of

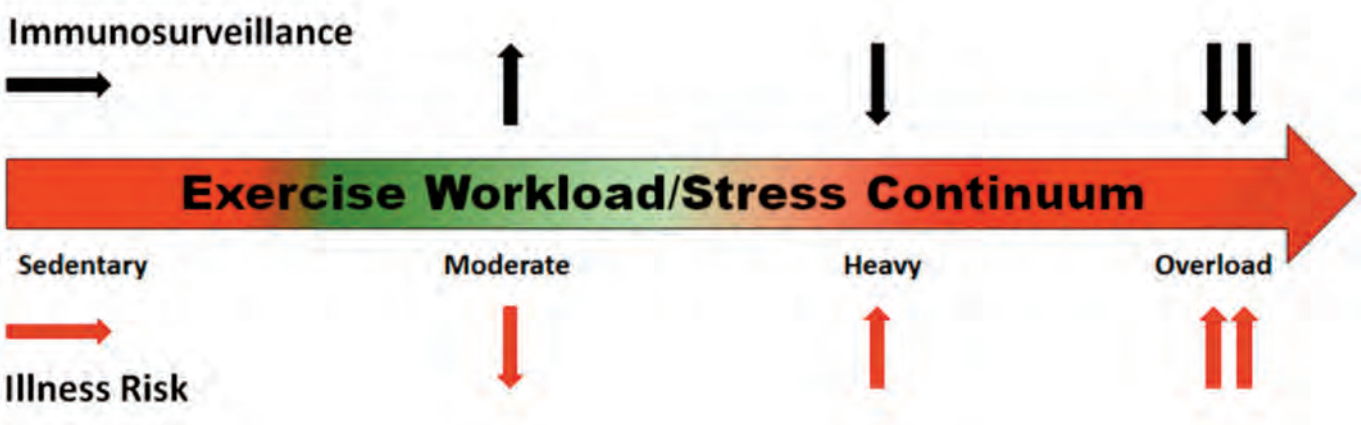

Figure 1:
Schematic model of the exercise workload/stress continuum and the relationship between immunosurveillance measures and risk of illness as the exercise workload is increased to moderate, heavy and overload.

exercise-related immune disturbance and increased susceptibility to URI in athletes undertaking strenuous exercise.

Animal and Cell Culture Data

Animal-based experiments assessing the linkage between muscular fatigue and pathogen resistance date back to the late 1800s. These early studies were reviewed by Baetjer (7) in 1932 who concluded that the data "appear to indicate that exhaustive exercise just preceding or immediately following infection, but more especially the latter, predisposes the animal to a more rapid and fatal attack of the infectious disease." Numerous studies since then have supported this observation and provided insights into how exercise fatigue impacts underlying immune intracellular processes (19, 21-23, 32, 47, 72, 79, 86, 87, 145). A representative murine study indicated higher mortality from herpes simplex type 1 virus (HSV-1) injected intranasally after prolonged strenuous (2.5-3.5 h to fatigue) compared to moderate (30 minutes) exercise (32). Antiviral resistance of lung macrophages from the exercise-fatigued group was suppressed, linking exercise-induced immune dysfunction with increased susceptibility to respiratory infection *in vivo.*

Immunometabolism is an emerging science that highlights connections between the metabolic state of immune cells and the nature of the immune response (44, 68, 76, 81, 90, 92, 102, 106, 120, 134). In response to an acute immunological challenge such as exercise stress, immune cells grow, proliferate, and generate molecules such as cytokines and cytotoxic granules. This immune activation requires metabolic reprogramming to generate sufficient energy to fuel these demands. This relationship between metabolic and immune systems is particularly apparent during recovery from physiologically demanding bouts of intensive exercise (11), and provides a new methodology for future research to better understand exercise-induced immune dysfunction (90).

URI in High Performance Athletes

While the majority of athletes have a similar incidence of URI to the general population, a small proportion (5-7%) experience recurrent episodes at significantly higher rates (48), often associated with persistent fatigue that interferes with training (111) and may affect competition performance (108). The incidence of URI in high-performance athletes can increase during periods of intense training, in association with increases in training load and competitions. Epidemiologic data collected during international competitions reveals that 7% of elite athletes (range 2-16%) experience an illness episode, with respiratory illness the major cause of presentation (30-64%) and infection the most common diagnosis (32-58%) by medical teams (4, 42, 84). Studies using self-reported URI rather than validated illness questionnaires (52, 105) may not as be as accurate, but physician verification is common for elite athletes. Responsive, reliable and validated survey instruments for respiratory illness (8, 10) can be used confidently for research and clinical applications.

The aetiology of URI is rarely examined, with the few studies that included pathology indicating that ~30-40% of URI episodes in athletes have an identified infective origin (28, 128). A single negative test point does not exclude the possibility of infection for other pathogens not included in the tested panels, or timing of the appearance of detectable levels of infections. Allergy is also a common clinical finding in high-performance athletes (28, 42, 84, 107), but regardless of the infectious and/or allergic stimulus (2, 70, 97) that induces an inflammatory cytokine cascade in the airways, a major concern for the athlete is the accompanying fatigue that can limit or prevent training (50, 57, 111) and impair performance (50, 108, 111).

Impact of Exercise on Immunity and URI Risk in High Performance Athletes

Intense exercise induces a well-characterised systemic and mucosal response in innate and acquired immune parameters (141). NK cell and neutrophil function, T- and B-lymphocyte function, salivary IgA output, skin delayed-type hypersensitivity response, major histocompatibility complex II expression in macrophages, and other biomarkers of immune function are altered for several hours to days during recovery from prolonged and intensive endurance exercise (119). Exercise has the potential to transiently alter immune protection, increase the risk of infection, or induce inflammatory processes in the airways (91). Suppression of immune parameters can occur in elite athletes over years of training (25, 111). This may result in temporary or sustained reactivation of viruses (61, 111), most likely due to an exercise-induced decline in cytotoxic T-cells (141).

Despite extensive laboratory studies of immune parameters in response to exercise, parallel examination of URI is often not included in the study design. Elite athletes prone to recurrent URI have altered/adverse cytokine responses to exercise in comparison with healthy athletes (29), and an underlying genetic predisposition to pro-inflammatory cytokine responses (27, 147). Differences in IFN-γ and IL-10 polymorphisms are known to affect illness severity, cytokine protein levels and duration/recovery time from various viral infections (136). A reversible defect in IFN-ɣ has been associated with illness-prone athletes experiencing fatigue (25). Viral reactivation of EBV is also a common finding (22-50%) in athletes experiencing recurrent URI (61, 111), and expression of EBV DNA in saliva is associated with a prior reduction in salivary IgA levels (61, 146), which is part of mucosal protection against viral infections (114).

Measurement of secretory IgA (SIgA) in saliva has shown consistent associations with URI in athletes. The consensus for studies of elite athletes is that low levels of salivary IgA and/or secretion rates (55, 58, 59), low pre-season salivary IgA levels (59), declining levels over a training period (57, 88), and failure to recover to pre-training resting levels (57), are associated with an increased risk of URI. Longitudinal studies have identified the impacts of intense training over both short (months) (50, 59) and long (years) (25, 111) periods on immune suppression and increased incidence of URI. Low levels of SIgA can occur prior to the symptoms (59, 61, 88). However, the best predictive use of salivary IgA is monitoring immune status in individual athletes with a history of URI (50, 57).

Concluding statement

A large body of evidence supports the proposition that elite athletes undertaking prolonged heavy intensive exercise can exhibit immune changes, in association with physiological, metabolic, and psychological stressors, and pathogen/allergen exposure, that increase the risk of infection and/or airway inflammation. Individual responses to different exercise workloads vary widely (46), and the changes in immune parameters reflect the magnitude of the stressors experienced by the athlete (Figure 1). A "survivor" effect exists for elite athletes whose immune system can be trained to adapt and attenuate responses to greater workloads than the general public. But athletes too have their limits, and their underlying genetic profile, in association with other stressors and environmental factors, will determine their risk profile for URI. We assert that multiple lines of laboratory-, field- and clinically-based evidence converge in support of the viewpoint that exercise at a high-performance level can affect immune function, increasing susceptibility to infection.

Can Exercise Affect Immune Function to increase Susceptibility to Infection? – The No Case

John P. Campbell, University of Bath, Bath, UK
James E. Turner, University of Bath, Bath, UK

Is there evidence that exercise impairs the normal functioning of the immune system?

A central dogma of exercise immunology has incontrovertibly persisted that strenuous exercise bouts, or periods of intensified training, impair aspects of cellular and humoral immunity, leading to an 'open window' of infection risk. Consistent and reliable evidence in support of this assertion is lacking (17, 18) (Figure 2).

Measurement of blood leukocyte frequency and functional competency in response to strenuous exercise is common in the literature (141). Exercise induces a bi-phasic response, whereby leukocyte frequency in blood increases, and then, upon exercise cessation, the frequency of some cells decreases below resting levels to a nadir one or two hours later (113) (Figure 2F). Coinciding with changes in cell number, parallel alterations to cell function are consistently reported (e.g., cytokine production, proliferation, migration capability, cytotoxicity), whereby increases are observed during exercise, followed by decreases shortly afterwards (77), leading to speculation that immune function is compromised. Evidence indicates that the fall in cell number after exercise does not reflect mass apoptosis. Instead, cells are redistributed out of the bloodstream to tissues and organs (Figure 2F). This phenomenon has been demonstrated in rodents with fluorescent cell tracking (74) and in humans by the proportional reduction of cells expressing homing receptors for tissue and organ sites (16, 75, 125). This redistribution effect is largely comprised of highly functional sub-populations of T cells and NK cells (16, 75, 125), and seems to confer host benefits, for example, by enhancing the identification and eradication of tumour cells in tissues (101). Following exercise, a small number of apoptotic lymphocytes accumulate in bone marrow and blood, coinciding with mobilisation of haematopoietic stem cells (83). These observations support the proposal that exercise might reverse T cell immunosenescence (123), partly by selective apoptosis of senescent T cells, and by promoting the development and / or survival of naïve T cells, facilitated by myokine release from contracting skeletal muscle (39).

A mainstay of exercise immunology that is used to assess whether exercise impairs humoral immunity is the measurement of salivary IgA (Figure 2D). Some studies have reported a decline (e.g., 20-25%) in saliva IgA following exercise (132), yet, other studies do not show this effect (13). A reason for discordant findings is that IgA measured within-day and between-days is highly variable within a person. Such intra-individual differences – exacerbated at an inter-individual level – are likely orchestrated by multiple factors that include sleep and circadian rhythms, psychological stress, diet, and oral health (Figure 2D). Use of salivary IgA as a single measure of immune competency in the hours and days after exercise should be interpreted with caution. At a systemic level, it has never been demonstrated that exercise suppresses plasma cell immunoglobulin production. This could be due to the long half-life (1-3 weeks) and high concentration of immunoglobulins in blood, which together, may mask any subtle suppression of plasma cell immunoglobulin synthesis.

Is there evidence that exercise increases susceptibility to illness?

Observational studies have reported that symptoms of upper respiratory tract infections were more common in competitors of mass-participation endurance sporting events (103) (Figure 2A). However, a limitation of studies from this era was that infection symptoms were not confirmed by laboratory analysis. Subsequently, a study using molecular techniques showed that only one-third of illness symptoms reported by athletes over five-months represented genuine infections (128). Although more recent research showed three-quarters of illness symptoms reported by athletes were infectious (133), it is likely that a substantial number of perceived illnesses are caused by factors such as allergy, asthma or non-specific mucosal inflammation, and not infection due to exercise-induced immuno-suppression. Among the genuine infections, it seems speculative to isolate exercise as a sole factor as other non-exercise factors contribute, including: long-haul air travel, sleep disruption, altered diet, and psychological stress (54, 131) (Figure 2E). Importantly, attending any mass participation event – whether exercising or not – increases the risk of encountering pathogens due to crowds (Figure 2B-C). Indeed, it has been shown that one-third of people attending a mass-participation religious gathering reported infections (24). Thus, guidelines to reduce infection risk (e.g., hygiene practices) are relevant (138). Finally, we are not aware of robust evidence showing that endurance athletes develop more infections annually than the general population.

Will future research demonstrate that exercise is capable of impairing immune competency?

Research over four decades has examined whether strenuous exercise suppresses immunity. The lack of compelling evi-

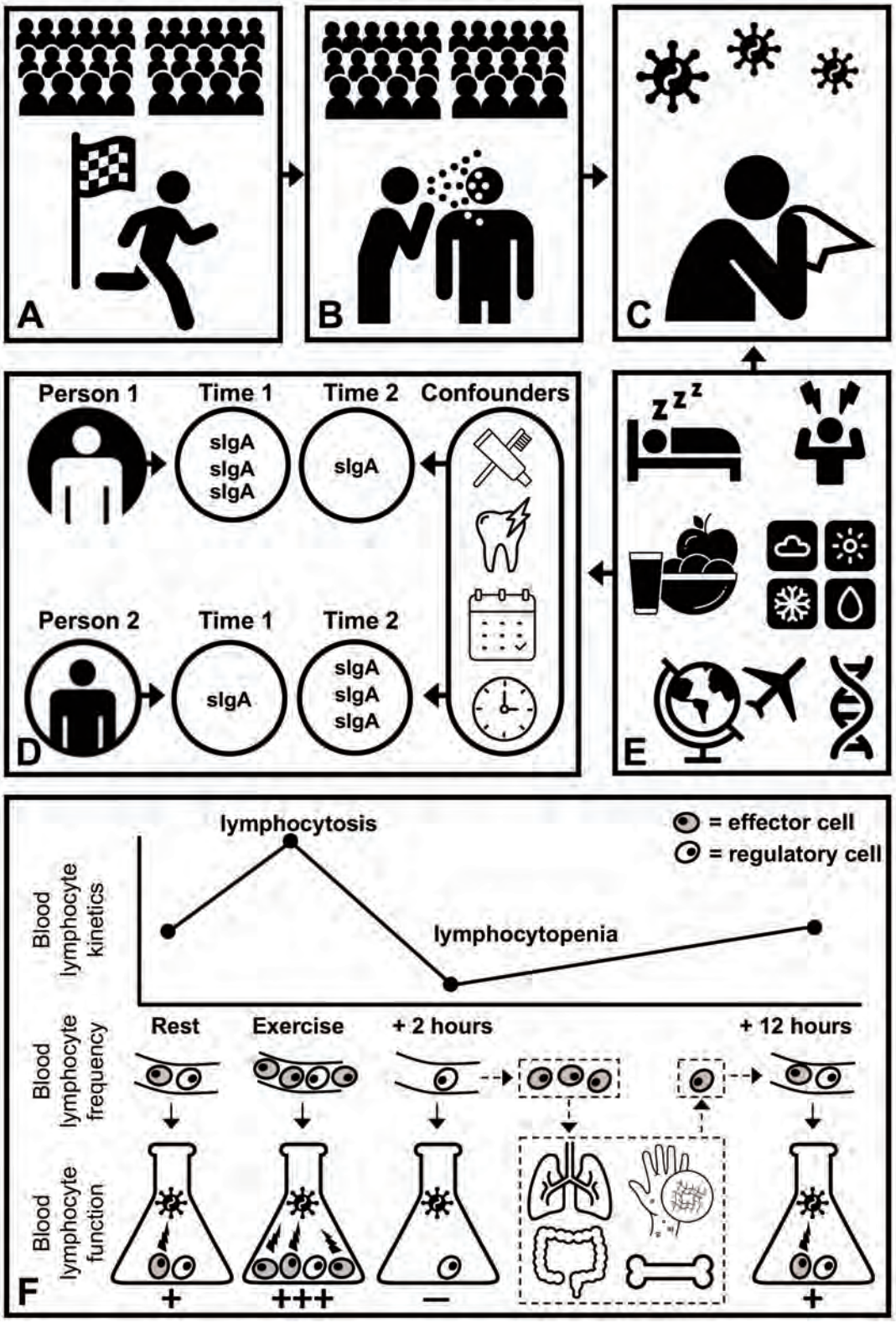

Figure 2:

A-C: Athletes competing in mass-participation events, and even spectators, are at an increased risk of infection due to heightened pathogen exposure from crowds because some people will be harboring infections. **D:** In saliva, secretory immunoglobulin-A (sIgA) concentration and secretion rate exhibit profound inter-and intra-individual variation, likely due to oral health, psychological stress or sleep, and diurnal or seasonal changes respectively. **E:** Non-exercise factors influence infection risk, including poor sleep quality and quantity, psychological stress, inadequate nutrition, extreme environmental conditions, air travel (particularly across multiple time zones) and single-nucleotide polymorphisms in critical immune defence genes. **F:** Acute bouts of exercise mobilise lymphocytes into peripheral blood, characterised by a selective mobilisation of effector T cells and NK cells. Following exercise, these effector cells extravasate to tissues such as the lungs, peyers patches, bone marrow or inflammatory sites (e.g. in skin) for immune-surveillance. The number of effector and regulatory cells in blood typically returns to pre-exercise values within 12 hours. Assessing the functional capacity of lymphocytes (or major sub-types such as T cells and NK cells) in blood samples collected at rest, during exercise, or afterwards, is confounded by the proportions of effector and regulatory cells, even when accounting for total cell number. Functional capacity (e.g. proliferation, cytokine production, cytotoxicity) is directly related to the number of effector cells in samples.

dence suggests that: (i) the detrimental effects of exercise on immunity are negligible, and / or (ii) research has not been designed optimally to assess immune competency. As we have discussed elsewhere (17, 18), it could be speculated that exercise is capable – in principle – of impairing aspects of cellular immune function due to the energy cost of exercise and metabolic perturbations that can occur in the absence of appropriate nutrition. However, to date, immuno-metabolic stress has not been investigated at a single cell level in the context of exercise. It could also be speculated that heightened steroid hormone production, or adrenaline exposure during exercise, may impair cell function. However, cell function can be regulated both positively and negatively by stress hormone exposure (93, 94) and different cell sub-types can respond differently to the same hormone (127). Thus, it is an over-simplification to denounce exercise as pan-immunosuppressive.

Given the aforementioned complexities, clinically relevant models of primary or secondary antigenic challenge should be used to examine the effects of exercise on immunity. Pertinently, previous research has examined the effects of a marathon on the response to vaccination (43). In this study, participants were vaccinated with tetanus toxoid approximately 30 minutes after exercise. This post-exercise time-point coincides with elevated cortisol levels, 'reduced' blood lymphocyte frequency, 'impaired' lymphocyte function and metabolic perturbations. Antibody titres were measured 15 days later and compared to those from a control group who did not compete in the marathon. Although the sample size included 4 runners and 59 controls, the results indicated that antibody titres were *higher* in those who received the vaccine following the marathon (43). Similar methodology was employed by administering diphtheria and tetanus toxoid and a pneumococcal polysaccharide vaccine to 22 athletes 30 minutes after a triathlon (15). After 14 days antibody titres from athletes were compared with those from 33 control participants who received the vaccine without prior exercise, and there were no differences between groups (15). If energy depletion or stress hormones were capable of hindering immunity following exercise, then one would expect impaired rather than *enhanced* or *unchanged* vaccine responses following exercise. These findings align with an emerging theorem in the field, that, on the balance of available evidence, exercise may in fact enhance immune competency and regulation.

Response to the No Case

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There is agreement between the debating groups that regular bouts of moderate-to-vigorous physical activity (MVPA) enhance the exchange of immune cells between the circulation and peripheral lymphoid tissues (1, 16, 36, 125, 126). The net effect is enhancement of immune surveillance, improved health, and decreased risk of illness (91, 123, 126).

Despite the large number of studies describing changes in immune parameters, this debate centers on the effect of acute and chronic exercise on the immune system and risk of illness and infection. This issue was the focus of investigation by the early pioneers in exercise immunology, including Pedersen and Hoffman-Goetz, who in 1994 and again in 2000, reviewed the literature and reasoned that "exercise-immune interactions can be viewed as a subset of stress immunology"(66, 99). These investigators emphasized that "many clinical physical stressors (e.g. surgery, trauma, burn, and sepsis) induce a pattern of hormonal and immunological responses that have similarities to that of exercise"(66).

This interpretation has withstood the test of time, especially when evaluation of animal and human studies is made in the full context of the exercise workload - stress continuum (15, 22, 32, 43, 50, 61, 72, 86, 91, 98, 108, 121, 131, 140, 141, 145). Unfortunately, the argument by the opposing debate group that "consistent and reliable evidence in support of this assertion is lacking" was supported using many references that included MVPA workloads well within recommended levels for the general community (16, 39, 74, 75, 83, 100, 101). However, high-performance athletes and other personnel (e.g. elite military groups) undertake workloads well beyond the recommended upper levels over extended periods. Individuals in these cohorts can have an increased risk of respiratory infection (4, 42, 48, 52, 85, 96, 107, 111, 131) associated with altered immune biomarkers (25, 27, 50, 55, 57, 59, 61, 88). The consensus among investigators is that exercise-induced immune changes reflect the physiological and metabolic stress experienced by the individual (12, 34, 91, 121, 140, 141).

Several lines of evidence across animal and human studies support the paradigm that illness risk may be elevated during periods of unusually heavy exertion, especially when other stressors are present. These factors include mental depression or anxiety, international travel across several time zones, participation in competitive events, lack of sleep, temperature extremes, and low dietary energy intake and nutritional deficiencies (31, 34, 38, 54, 60, 65, 73, 84, 91, 111, 112, 131, 136, 144).

The opposing debate group disregarded these findings because of mistrust in self-reported acute respiratory illness (ARI) symptom data. However, Barrett and others (8, 10) have shown carefully that individuals are capable of reporting ARI symptoms that agree with physician-based diagnosis. There are no perfect tools or gold standard for assessing ARI episodes and symptoms. Studies show that etiological pathogens cannot reliably be detected using laboratory methods in 20-40% of people with classic ARI symptoms (9, 28, 128, 144). ARI episodes caused by viral infection can be asymptomatic at the time of testing in 25-35% of people (9, 28, 128), while symptoms can also be linked to a non-viral cause (28, 70, 84, 111). Defining ARI is part of the challenge, but epidemiological and clinical trial ARI data contributes to the discussion and should not be discarded.

Until recently, exercise-induced immune responses were measured using a few targeted biomarkers, but increasingly the focus has shifted to multi-omics approaches (91). Advances in measurement technologies and bioinformatics will improve our capacity to measure both the beneficial effects of moderate exercise on immunity, and the downturn in immunity that can occur during periods of heavy exercise training. In a representative study, an integrative omics approach was used to explore immunosuppression in female physique athletes undertaking prolonged periods of intense training coupled with low-energy availability (116). Several molecular pathways were elucidated and included dysregulated hematopoiesis, suppressed immune cell proliferation, and loss of immune cell function by reduced antibody and chemokine secretion. Most of these measures of dysregulated immune function were reversed during an 18-week weight regain period.

Data generated from multi-omics approaches will reshape our future understanding of how exercise influences immune function and the complex interactions with neuroendocrine systems in individuals to either enhance protection, or increase the risk of illness and infections in susceptible individuals.

Response to the Yes Case

John P. Campbell, University of Bath, Bath, UK
James E. Turner, University of Bath, Bath, UK

There is limited evidence supporting the proposition that exercise suppresses immunity. The Yes case claims *'substantial evidence'* exists because *'upper respiratory illness (URI) is the most common non-injury presentation in sports medicine'*. URI is also the most common health problem in primary care (45) and is therefore not a problem unique to sport. Given the ubiquity of URI, the incidence among athletes is not in question. At the crux of this debate is whether exercise causatively suppresses immunity to a clinically meaningful degree, and in doing so, increases the risk of URI in a sub-group of athletes. We posit herein, that it is misleading to conclude from existing evidence, that exercise is the causative factor of URI among athletes.

Immune competency is strongly influenced by non-exercise factors

The Yes case states that, in the context of exercise, infection *'risks are co-dependent on factors that regulate immune function (genetic, nutritional status, psychological stress, interrupted circadian rhythm)'*. Crucially, this statement acknowledges that infection risk is *'co-dependent'* on other factors. Our standpoint is that infection risk is fundamentally dependent on non-exercise factors given the evidence that polymorphisms in critical immune-defence genes, inadequate nutrition, psychological stress, poor sleep quality or quantity, and environmental conditions dysregulate immunity (54, 117, 131). Importantly, these *'factors'* are rarely controlled in exercise studies. Thus, without measuring these non-exercise factors, and in the absence of mechanistic human evidence that exercise causatively suppresses immunity at a humoral, cellular and systems level, the *a priori* assumption that exercise is a *de facto* cause of immune suppression is unsubstantiated. Immunological idiosyncrasies, unrelated to exercise, most likely explain why, as highlighted in the Yes case, that *'the majority of athletes have a similar incidence of URI to the general population'* (51).

The primary risk factor for infections is exposure

Evidence cited in the Yes case relating to infection incidence predominantly relies on studies surveying athletes around the time of competitions, which are often attended by large groups of athletes or spectators (4, 42). Other studies, assessing illness symptoms over longer periods, may not accurately capture time spent by athletes in settings where they are in close proximity to other people or crowds. Our standpoint is that public travel to, or attendance at, any mass-participation event is likely to increase exposure to pathogens (17, 24). Thus, anyone attending a sporting event, whether a competitor or spectator, is at a heightened risk of infection (Figure 2A-C). Indeed, this risk is likely to be exacerbated in subgroups of people – both athletes and non-athletes alike – due to aforementioned inter-individual differences in immune competency.

If exercise causatively suppresses immunity increasing infection risk, what is the mechanism?

Firstly, it is stated in the Yes case that *'measurement of sIgA has shown consistent associations with URI in athletes',* however, numerous well-conducted studies have found no associations (5, 20, 53, 95, 104, 118, 135). It is stated that *'low levels of sIgA can occur prior to the symptoms'* of infection, but it is just as likely that sIgA does not decline at this time because of the profound intra-individual variability of sIgA (109). Moreover, in studies linking sIgA to infections, confounding factors known to impact sIgA secretion, are rarely considered (14) (Figure 2D). Pertinently, many studies show that exercise does not alter sIgA levels, bringing into question the relevance of correlating sIgA with infections assumed to be brought about by exercise (3, 33, 71, 110, 115, 139).

Secondly, some evidence cited in the Yes case as supporting exercise-induced immune-suppression must be interpreted carefully. For example, conclusions drawn from a systematic review (119), do not account for temporal changes in the cellular composition of blood as explained previously (17) (Figure 2F). Separately, the observational studies which examined immunological features of *'illness prone'* individuals lack important methodological controls (25, 111), and rather than signposting exercise-induced immune-suppression, the results highlight inter-individual differences in basal immune function. The most robust evidence shows that transient changes in cell numbers and function after exercise represent immune-surveillance (35) (Figure 2F), and evidence that exercise suppresses immunity at a systems level is lacking (17). Further, lifelong exercise (40) and physical fitness (129) might facilitate the deletion of senescent immune cells, theoretically maintaining global immune competency (123).

Thirdly, results from animal models are inconsistent due to methodological heterogeneity; some show exercise

improves responses to infectious (80, 122, 142) and neoplastic challenge (6). For example, a study cited in the Yes case, showed that rodents intranasally infected with HSV-1 after strenuous exercise exhibited 36% lower morbidity and 61% lower mortality than non-exercise controls (86). In addition, important confounders must be considered. For example, in one study cited by the Yes case, cold exposure, was the key factor – rather than exercise – driving morbidity and mortality (79). Other work has shown that forced exercise, compared to voluntary exercise, induces psychological stress which is the cause of immune dysregulation rather than exercise per se (26). Further, it must be considered whether experimental infections in animal studies provide a representative dose and route or method of pathogen entry. Conclusions must also be interpreted considering exercise timing: if already infected, some studies show exercise can be detrimental, but this does not represent *risk* of becoming infected.

Finally, the Yes case does not appraise the most robust evidence in humans showing that immunity is enhanced or at least unchanged with pathogenic challenge after exercise (15, 43, 78), supporting systematic reviews that exercise in general does not suppress immunity or increase risk of infections (62, 63).

Summary

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The purpose of this debate article was to solicit opposing arguments centered around the following question: can exercise affect immune function to increase susceptibility to infection. After reviewing the original narratives and responses from each camp, we asked the debating groups to highlight points of agreement and issues that remain to be resolved. These were then coalesced by the editorial team (Simpson, Kruger and Walsh) and summarised as follows:

Points of Agreement:

- Regular bouts of moderate to vigorous intensity exercise are beneficial for the normal functioning of the immune system and likely help lower the risk of respiratory infection/illness and some cancers. The frequent exchange of immune cells between the blood and the tissues with each bout of moderate to vigorous intensity exercise likely contributes to enhanced immune surveillance, improved health and a lower risk of illness.

- Infection susceptibility has a multifactorial underpinning. Both groups acknowledge that factors such as stress, sleep, nutrition, circadian misalignment and infection/vaccination history could directly impact or contribute to impaired immunity and infection risk, particularly in situations when pathogen exposure is more likely.

- There is a critical need for more research to help unravel the immune modulating effects of exercise, with multi-omics and immunometabolism-based studies being pivotal to further our understanding. The Yes camp suggest that further studies using these techniques will provide experimental support for the concept that reductions in immune competency reflects the physiological stress imposed from excessive exercise workloads. The No camp have called for more systems-level (e.g., immune response to vaccination) studies that control for confounding factors (e.g., nutrition, sleep, hygiene and prior exposure from infections and/or vaccinations) to determine if very large volumes of exercise (e.g., ultra-endurance activities and prolonged period of training) impair global immune competency.

- Both camps acknowledge that the field has moved on substantially from salivary IgA and total blood lymphocyte counts after acute exercise as measures of immune competency. While the lymphocytopenia observed after acute high-intensity/prolonged exercise was identified as a biomarker supporting the concept of an 'open-window' in the 1990's, the contemporary interpretation is that this particular measure reflects a redistribution of lymphocytes from the blood to the tissues after exercise, albeit experimental data in humans is still currently lacking.

Issues to be resolved:

- Whether or not athletes are more susceptible to illness/infection than the general population continues to be debated between the Yes and No camps. The No camp identify the reliance on self-reported measures of upper respiratory illness symptoms as a limitation. They also contend that exposure to pathogens is the major cause of upper respiratory infection, is the most common health problem in primary care, and is therefore not unique to sport. The Yes camp counter that the questionnaires used for these studies have been extensively validated and agree with physician-based diagnoses. They also posit that there is no substantial evidence that post-race infectious episodes among athletes are linked to increased exposure from spectators at major sporting events. Moreover, the Yes camp asserted that most illnesses in humans are based on multiple risk factors, and to exclude arduous exercise as one of the important risk factors is highly selective.

- The Yes camp argue that several lines of evidence across animal and human studies support the paradigm that illness risk may be elevated during periods of heavy exertion that go beyond recommended physical activity guidelines, especially when other stressors are present. The No camp contend that, even if athletes are more susceptible to infection than the general population, it is difficult to discern exercise (regardless of volume) as the causative factor independently of the non-exercise factors that are potential confounders (e.g. nutrition, anxiety, travel, sleep disturbances, tem-

perature variations, genetic polymorphisms and prior exposure due to infection and/or vaccination).

- There continues to be disagreement on the use of salivary IgA as a biomarker to determine infection risk in athletes. The Yes camp point to the clinical evidence linking low salivary IgA levels as a biomarker of recurrent mucosal infections regardless of exercise status. They also highlighted that the most effective use of salivary IgA was monitoring individual athletes with a history of URI. The No camp argue that salivary IgA is profoundly influenced by an array of factors including diurnal variation, psychological stress and oral health and therefore has limited clinical use as a single marker of infection risk in athletes.

- Both camps challenged statements made by the other regarding the scientific evidence that is available to substantiate their claims. The No camp highlighted several experimental design features they feel should be taken into consideration when interpreting results from animal studies, suggesting that misleading conclusions could be drawn due to experimental heterogeneity (e.g. forced versus voluntary exercise, and the timing of exercise relative to infection/neoplastic challenge). The Yes camp argue that most of the evidence cited by the No camp to show immune enhancing effects of exercise are in response to bouts of moderate to vigorous intensity exercise and of relatively short duration (e.g. 30-45 minutes), are in special populations who likely have lowered immunity to begin with (e.g. older adults), and that these volumes/intensities of exercise are well within the recommended physical activity guidelines for the general population. The Yes camp contend that undertaking exercise workloads beyond these recommendations for extended periods of time is what can impair immune competency and increase infection risk.

- Finally, both camps point to markers of global immunity to measure immune competency (e.g. vaccination, latent viral reactivation) in people exposed to different volumes/intensities of exercise. The No camp argue that the most robust evidence in humans indicates that immunity is enhanced or at least unchanged with pathogenic challenge after even arduous exercise. This is countered by the Yes camp who cite evidence of latent viral reactivation among high performance athletes as a marker of reduced immune competency after periods of intense exercise.

Conclusion:

While the debating groups were able to find areas of agreement on this topic (e.g. that infection susceptibility has a multifactorial underpinning), the idea that exercise, be it arduous or otherwise, can affect immune function and increase susceptibility to infection remains a contentious issue. Although the question at hand was to focus on whether exercise can affect immunity to increase susceptibility to infection, the lack of laboratory-controlled studies resulted in both camps addressing the issue of whether participation in high-performance events (e.g. elite sport, military activities) and not exercise *per se* alters immunity and infection risk. On reflection, this might have been a more pertinent question to ask as it would take into consideration not only arduous exercise (i.e. exercise that far exceeds the recommended physical activity guidelines), but also the multi-factorial aspects that share pathways for the immune response to challenges including life events, exposure, personal hygiene, sleep, travel, anxiety, mental fatigue, rumination, nutrition, etc. Moreover, this debate process has perhaps exposed the field of being too focused on the exercise component, while the multitude of other factors that could directly affect and/or interact with exercise to alter immunity and infection susceptibility may have been overlooked (41, 138). While the assertion that changes in immune function measures following acute bouts of strenuous exercise or periods of heavy training account for URI symptoms in athletes remains open for debate, URI symptoms will nevertheless hinder athletic training and competition regardless of the aetiology (138). Taking a multifactorial approach may allow us to develop evidence-based recommendations and countermeasures to better prepare athletes and military personnel for the multiple challenges they will face to their immune health during training and competition/deployment (137, 138). Indeed, this multi-factorial approach is currently taken in space immunology research to help understand why astronauts experience increased episodes of latent viral reactivation and altered hypersensitivity reactions during missions, with microgravity, radiation exposure, isolation/confinement stress, altered nutrition, circadian misalignment, exertion and physical deconditioning (e.g. muscle and bone loss) all believed to play a role (30).

If exercise is directly capable of altering immunity to increase susceptibility to infection then the duration/volume of exercise will likely be a key factor. While natural infection rates are always difficult to incorporate as endpoints in highly controlled studies, there is a critical need for more controlled comparative studies centered around exercise duration (e.g. bouts lasting <45 minutes to bouts lasting >2h) as a key variable, particularly those using reliable *in vivo* endpoint measures of immune function. To this end, vaccine or experimental infection models in humans that elicit both primary and recall immune responses combined with multi-omics approaches would be highly informative for future studies. While experimental rhinovirus models have been used with exercise in humans previously (143), these early studies lacked the appropriate technology to document changes in reliable *in vivo* endpoints such as viral replication and immune responses to re-exposure. Thus, experimental pathogen/antigen challenge studies in humans could be revisited (37, 143), incorporating more cutting edge technology such as RT-PCR, RNA sequencing, proteomics and metabolomics to determine if exercise (with volume/duration as a key variable) can increase susceptibility to infection and alter immunological control of pathogens in the host. While *in vitro* assays can be useful to determine the impact of exercise on certain aspects of immune function, it is important that the limitations and potential confounding factors (e.g. cell trafficking) of these methods are adequately appraised to avoid potentially flawed interpretations.

To address the number of issues that remain to be resolved, we suggest that more robust longitudinal studies are needed to determine, firstly, if athletes or other high-performance personnel are at greater risk of laboratory-confirmed infections compared to the general population. Secondly, we should determine if arduous physical exercise *per se* is a direct cause and/or a co-factor responsible for any potential increases in infection susceptibility among athletes/military personnel. Thirdly, further work is required to clarify the underlying causes of respiratory illness and whether they are infectious in origin or initiated by other inflammatory stimuli such as allergy or epithelial trauma; and finally, what are the immunological components/pathways (including genetic predisposition, multi-omics and immunometabolism-based studies) involved if arduous exercise increases infection susceptibility directly. Studies focused on laboratory-confirmed infections as an endpoint should consider seasonal variations, as confirmed infectious URI's are more prevalent during the autumn and winter months (~70%) compared to spring and summer (~35%) (128, 133). It would also be useful to compare athletes of different sports (e.g. endurance versus strength/power activities) who, presumably would be exposed to similar confounding factors (e.g. travel, stress, exposure), but differ in the key variable of interest, which is prolonged 'heavy' exercise. It will also be important for future experimental studies to control for these confounding variables (e.g. sleep, nutrition, life stress, anxiety, etc) that could influence the selected endpoint measures, even in the laboratory setting. This will present some challenges, as even laboratory-controlled studies have to contend with a number of immune-modulating psychological variables that come into play more so during prolonged compared to shorter bouts of arduous exercise (41). Moreover, psychological traits such as emotional intelligence and mental toughness can affect the individual's ability to regulate mood and psychological strain during prolonged exercise (69, 82).

While this research continues to evolve, it is important in the interim to emphasise the context of the message that is projected to the public and scientific community. On the one hand, if performing frequent and arduous bouts of exercise that far exceed recommended physical activity guidelines is projected to have no negative impact on immunity or infection rates, then the immune health of athletes and other high-performance personnel could be unjustly ignored and regarded as insignificant. On the other hand, if exercise is portrayed as being 'immunosuppressive' then this might discourage patients and clinicians from participating in and recommending exercise, and could also project the wrong message to the vast majority of the population who would benefit from increasing their physical activity levels to improve, not only immune function, but also general health and wellness. We anticipate this debate article will provide impetus for more empirical research in the area to unravel the complex questions that surround this contentious issue in the field of exercise immunology.

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Exercise and the Kynurenine pathway: Current state of knowledge and results from a randomized cross-over study comparing acute effects of endurance and resistance training

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Abstract

Introduction: *The essential amino acid tryptophan (TRP) is primarily degraded through the kynurenine (KYN) pathway, which is dysregulated in several chronic diseases. KYN pathway metabolites have immune- and neuro-modulatory properties and are involved in the de novo synthesis of nicotinamide adenine dinucleotide (NAD^+). Currently, little evidence exists demonstrating that physical exercise may influence this pathway. However, differences between acute and chronic stimuli as well as the influence of exercise modalities remain to be investigated. Here, we provide an overview of existing studies and present results of a randomized cross-over trial on acute effects of a single-bout of resistance and endurance exercise.*

Methods: *24 healthy male adults conducted both an acute endurance exercise (EE) and resistance exercise (RE) session. Blood samples were collected before, immediately after and one hour after cessation of each exercise session. Outcomes comprised serum levels of TRP, KYN, kynurenic acid (KA), quinolinic acid (QA) and calculated ratios. Gene expression of the enzymes indoleamine 2,3 dioxygenase (IDO) 1 and kynurenine aminotransferase (KAT) 4 was measured in peripheral blood mononuclear cells (PBMCs). Moreover, serum concentrations of the potential KYN pathway mediators interleukin (IL)-6 and cortisol were determined. Finally, we investigated baseline correlations between immune cell subsets, potential mediators and initial KYN pathway activation outcomes.*

Results: *The KYN/TRP ratio correlated positively with IL-6 and $CD56^{bright}$ NK-cells and negatively with $CD56^{dim}$ NK-cells. Expression of IDO1 in PBMCs correlated positively with IL-6, regulatory T-cells and $CD56^{bright}$ NK-cells, whereas negative correlations to cytotoxic T-cells and $CD56^{dim}$ NK-cells were revealed. A significant time effect on KYN/TRP ratio was detected for RE. Regarding KA and KA/KYN ratio, an increase after exercise followed by a decrease at the follow-up measurement was revealed in EE. KAT4 expression also increased after exercise in EE. Moreover, elevated QA levels were observed after the EE session.*

Conclusion: *In contrast to chronic exercise interventions, single-bouts of endurance exercise provoke acute alterations on KYN pathway outcomes in humans. Our results indicate that EE induces stronger alterations than RE. Enhanced conversion of KYN to both, KA and QA suggest a peripheral KYN clearance, thereby preventing pathological accumulation within the CNS. Future acute and chronic exercise studies are needed to examine the role of NAD^+ synthesis starting with TRP and the interplay between KYN pathway activation and mid- to long-term immunological modulations.*

Keywords: Exercise, Kynurenine Pathway, Endurance Exercise, Resistance Exercise, Immune system

Introduction

Only one percent of the essential amino acid tryptophan (TRP) is used for protein synthesis under physiological conditions. While merely a small portion is metabolized via the serotonergic pathway, the vast majority of available TRP (over 95%) is metabolized through the kynurenine (KYN) pathway (8). During the past two decades, the pathogenesis and progression of various chronic diseases have been linked to metabolic disturbances of the KYN pathway. Dysregulations were revealed in diseases involving the central nervous system (CNS) (e.g. Alzheimer's disease, Multiple Sclerosis or Parkinson's disease (14, 68)) and several internistic pathologies (e.g. diabetes mellitus (16) and cancer (57)).

The tryptophan 2,3 dioxygenase (TDO) and its isoenzymes indoleamine 2,3 dioxygenase (IDO) 1 and 2 catalyze the degradation of TRP to KYN, thereby representing the initial step of the KYN pathway. In contrast to TDO, which is mainly expressed in hepatic tissue and primarily stimulated by TRP itself or glucocorticoids (8), IDO can be expressed in almost all types of human cells (40). IDO1 and IDO2 have demonstrated differing levels of expression, effects on peripheral

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blood metabolites, and enzyme activity in response to specific physiological conditions (58). However, knowledge about the more recently discovered IDO2 is still sparse and the differential roles of both enzymes are not yet fully understood (51, 58). Interestingly, IDO activity closely interacts with the immune system. Especially peripheral blood mononuclear cells (PBMCs) have been shown to be potent producers of IDO (35). Based on immune homeostasis, IDO mediated conversion of TRP to KYN is suspected to be constant and almost entirely mediated by TDO under basal conditions. In contrast, local and systemic inflammatory stimuli provoke a dramatic increase in IDO1 mediated conversion (57). Particularly, elevated levels of interferon-gamma (IFN-y) (66, 70), but also increases in other pro-inflammatory cytokines, such as interleukin (IL)-6 (58) and tumor necrosis factor (TNF)-α (27), seem to be decisive stimuli for IDO induction. KYN itself possesses immunomodulatory effects comprising the suppression of cytotoxic T-cell, NK-cell activity (43, 51, 52) and mediating the differentiation of regulatory T-cells (T_{regs}) (18). Evidence suggests that the IDO1-mediated conversion to KYN activates the aryl hydrocarbon receptor (AHR), which plays a key role in T-cell differentiation (46). Since IDO1 is upregulated in tumor microenvironment (58), it is not surprising that IDO1 has recently attracted extended attention as promising immunotherapeutic target in cancer research (57). Furthermore, a chronic inflammation-induced activation of the KYN pathway has been described as pathological mechanism of depression and might also be of relevance for mood disorders in general (6, 67). A consistent decrease in the bioavailability of TRP that is essential for the neurotransmitter serotonin seems to be one causal factor. If chronic inflammatory conditions are present, the degradation of available TRP through the KYN pathway is pathologically increased, which consequently leads to an impaired synthesis of the neurotransmitter serotonin (6).

Apart from its effects on immune function, KYN pathway metabolites also influence CNS homeostasis. KYN itself can either be converted to the neuroprotective kynurenic acid (KA) or (over several intermediate steps) to quinolinic acid (QA), which is closely linked to neuronal excitotoxicity. Within the CNS, KA and QA act as antagonist and agonist of N-methyl-d-aspartate (NMDA) receptor activation, respectively, thereby mediating either neuronal protection or excitotoxicity. Specifically, KA has been described to act as NMDA receptor inhibitor, either at the strychnine-insensitive glycine-binding site (at low concentrations) or at the glutamate site (at higher concentrations) (17). Furthermore, KA is also able to inhibit α7 nicotinic acetylcholine receptors (nAChRs) that are expressed by glutamatergic axon terminals and involved in increased glutamate release (68). Since NMDA receptor mediated excitotoxicity represents a common pathological mechanism in different neurodegenerative diseases (68), the anti-glutamatergic properties of KA may have beneficial effects on neurodegenerative processes. Regarding QA, the predominant and most-investigated neurotoxic effect is undoubtedly its activation of the NMDA receptor, leading to excitotoxicity. However, several other neurotoxic aspects of QA have been described. These aspects include the inhibition of glutamate uptake by astrocytes, the generation of reactive oxygen intermediates or the suppression of astroglial glutamine synthetase (30, 68), emphasizing its strong association with CNS damage. Interestingly, neurons in certain brain regions, specifically the hippocampus, striatum and neocortex, seem to be more susceptible to QA than others (30).

The homeostasis of this neuroactive branch largely depends on the activity of the enzymes kynurenine aminotransferases (KATs) and kynurenine-3-monooxydase (KMO), which catalyze the degradation of KYN to KA or QA, respectively. More precisely, the conversion to KA can be mediated by four different isoforms of KAT (KAT 1-4), whereas the conversion to QA proceeds through two metabolic intermediate products, namely the 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid (3-HAA). Under non-pathological conditions, the vast majority of KYN is metabolized to QA, yielding the preferred end product nicotinamide adenine dinucleotide (NAD^+), which is highly relevant for oxidative energy production. Based on specific environmental conditions or if higher levels of TRP or KYN are present, the metabolic flux towards KA can increase (16). Considering the neurotoxic potential along the KYN pathway, it is not surprising that central and/or peripheral dysregulations in metabolites and enzyme activities are associated with various neurodegenerative disorders, such as Parkinson's disease, Huntington's disease or Multiple Sclerosis (68). Drawing a parallel between all these neurological diseases, an over-activated KYN pathway leading to an accumulation of QA in the CNS as well as a decrease in the neuroprotective KA has been ubiquitously linked to pathogenesis or disease progression (65, 68). As a consequence of pathological QA accumulation, induced neurodegenerative processes implicate neuro-inflammatory conditions, representing a hallmark of CNS damage. Accordingly, KMO-Inhibitors represent a common therapeutic drug target in various neurological disorders (68).

Exercise and the kynurenine pathway

Evidence suggests that physical exercise has beneficial effects on diseased populations such as persons with Cancer (21), Parkinson's disease (28) or Multiple Sclerosis (49). To date, our understanding of the underlying mechanisms is limited. Some studies in both, human (2, 4, 10, 11, 31, 32, 39, 41, 45, 47, 50, 60, 63, 64) and rodent (1, 38) have shown that physical exercise influences the KYN pathway, thereby representing a potential link between an active lifestyle and primary and tertiary disease prevention. Although studies in rodents investigating the impact of exercise on KYN pathway activation may represent a suitable approach to examine potential underlying mechanisms highly standardized, especially in view of enzyme expression in different tissues, it is worth mentioning that discrepancies in metabolism between humans and rodents impair the transferability of results. In contrast to humans, high output of nitric oxide by inducible nitric oxide synthase (iNOS) has been reported in rodents (71). Since nitric oxide radical is known to supress IDO expression (71), different responses in KYN pathway activation mediated by IDO in immune cells can be assumed. Therefore, among others, studies in rodents may predominantly focus on skeletal muscle and the further course of the KYN pathway. Overall, existing studies in both human and rodent strongly differ in regard to exercise modalities, outcome measures and study populations.

Training studies with humans were inspired by a promising preclinical investigation of Agudelo et al. (1). The authors have shown that endurance exercise training increases muscle expression of KATs through an activation of the transcription coactivator PGC-1α1 in a mouse model of depression. Subsequently, increased serum/plasma KYN levels, as they can also be observed in depressed humans, were degraded to KA. In contrast to KYN, KA is not able to pass the blood brain barrier. Thereby, exercise reduced neurotoxic effects of KYN and its downstream metabolite QA. In fact, exercise-induced reductions of CNS KYN levels were associated with decreased inflammatory stress, increased levels of neurotrophic/protective factors and reduced symptoms of anxiety and depressions. In another mouse model, Kim et al. (38) revealed that exercise counteracts a decline in cognitive performance in QA-induced Huntington's disease.

In contrast to the promising results of animals studies, a clinical trial by Millischer et al. (47) comparing the impact of three non-supervised 12-week exercise interventions varying in intensity (yoga vs. moderate aerobics vs. vigorous aerobics and strength training) in depressed humans did not show any effects on KYN and KA levels. These results are in line with those of Hennings et al. (31) who found no impact of one week of non-supervised "increased physical activity" (daily fitness/stretching exercise) on KYN levels and markers of inflammation in persons suffering from major depressions. Unfortunately, Hennings et al. (31) did neither report KYN/TRP ratios nor what and how much "activity" was performed. Interestingly, both studies revealed reduced self-reported levels of depressions after the exercise interventions. Similar to Agudelo et al. (1), Allison et al. (2) described elevated KAT expression in muscle tissue of healthy older men after a 12-week supervised multimodal (resistance and endurance) exercise program. Reductions in plasma KYN and increases in KA levels did not reach statistical significance. Unfortunately, again no ratio was calculated. A recent study by Herrstedt et al. (32) investigated the impact of a chemotherapy-accompanying 12-week combined exercise in persons with gastro-esophageal junction cancer on TRP metabolites in blood plasma and enzyme expression in skeletal muscle. While the QA precursor 3-HK increased in the control group, no significant changes were observed in the exercise group. Furthermore, KMO expression in skeletal muscle was significant higher in the control group post intervention. These results indirectly suggest that regular exercise has the potential to alleviate treatment-induced alterations along the KYN pathway. However, conclusions should be drawn cautiously since strong methodological limitations exist in this study as recently pointed out by Zimmer et al. (73). Moreover, Küster et al. (39) investigated the effects of a ten-week intervention of physical training with older adults at risk of dementia on a broad range of TRP metabolites. No significant changes were revealed for any outcome in the physical training group. Absent changes might result from low training adherence or selected exercise modalities, since important information on both is insufficiently described. Finally, a study by Bansi et al. (10) has focused on the KYN/TRP ratio in persons with different subtypes of MS. Two different supervised three-week endurance training programs led to a significant increase in the KYN/TRP ratio in persons with relapsing remitting MS, whereas no effects were observed in persons suffering from secondary progressive MS (10).

Regarding acute exercise, an initial activation of the KYN pathway, indicated by a significant increase in KYN/TRP ratio has been demonstrated in healthy trained adults (63, 64) and in persons with relapsing remitting MS (10). Keeping in mind that IDO, as responsible mediator for the conversion of TRP to KYN is sensitive to inflammatory cytokines, it has been proposed that its activation is caused by inflammatory stimuli as they appear during and after acute exercise (69). Nevertheless, since high levels of cortisol were suggested to stimulate TDO activity (9) and acute exercise is known to increase peripheral cortisol levels (29, 34, 54), it cannot be ruled out that activation of the exercise-induced KYN pathway is caused exclusively by increased IDO activity. In view of the immunomodulatory properties of KYN itself, an activation of the KYN pathway is of major research interest due to two reasons. Firstly, repetitive short-term increases in KYN levels represent a potential explanation for the mechanistic interaction between acute exercise-induced responses and chronic adaptions of the immune system. Secondly, persons with inflammation-mediated diseases (e.g. MS, Parkinson's disease) could benefit from short-term elevated KYN levels regarding its long-term anti-inflammatory effects. However, this hypothesis remains to be addressed since no further KYN metabolites of the neuroactive branch, which are highly relevant in MS, were measured in the study by Bansi et al. (10). Beside an initial activation of the KYN pathway, some studies also suggest acute exercise-induced alterations on the further regulation of the KYN pathway. These studies consistently indicate, primarily in healthy populations, that acute endurance exercise upregulates the concentrations of KA and QA (41, 50, 60). Similar to IDO, increased KMO activity is suspected to be mediated by inflammatory stimuli (72), representing a potential explanation for increased QA levels after acute exercise. However, it remains unclear whether KMO activity is influenced by inflammatory stimuli directly or as a consequence of an overall metabolic activation of the KYN pathway as described above. As potential underlying mechanism of increases in KA, it was hypothesized that not only chronic training, but also acute exercise can upregulated gene expression of KATs through PGC-1α1 in skeletal muscle (50). PGC-1α represents a well-studied coactivator that plays a pivotal role in oxidative metabolism through its impact on mitochondrial biogenesis and angiogenesis and is further involved in adaptions in skeletal muscle fiber type composition (3). Especially acute endurance exercise (7, 53) but also resistance exercise is known to upregulate PGC-1α (59, 62).

In summary, current evidence (detailed overview is provided in Table 3) proposes a modifying effect of acute and chronic exercise on different sections of the KYN pathway due to distinct underlying mechanisms with promising perspectives on health benefits (see Figure 1). Nevertheless, knowledge about specific exercise modalities to impact the KYN pathway is strongly restricted by the methodological heterogeneity of existing studies. Limitations of the current state of research also comprise a lack of studies that directly compare acute effects of different exercise modalities (type, duration, inten-

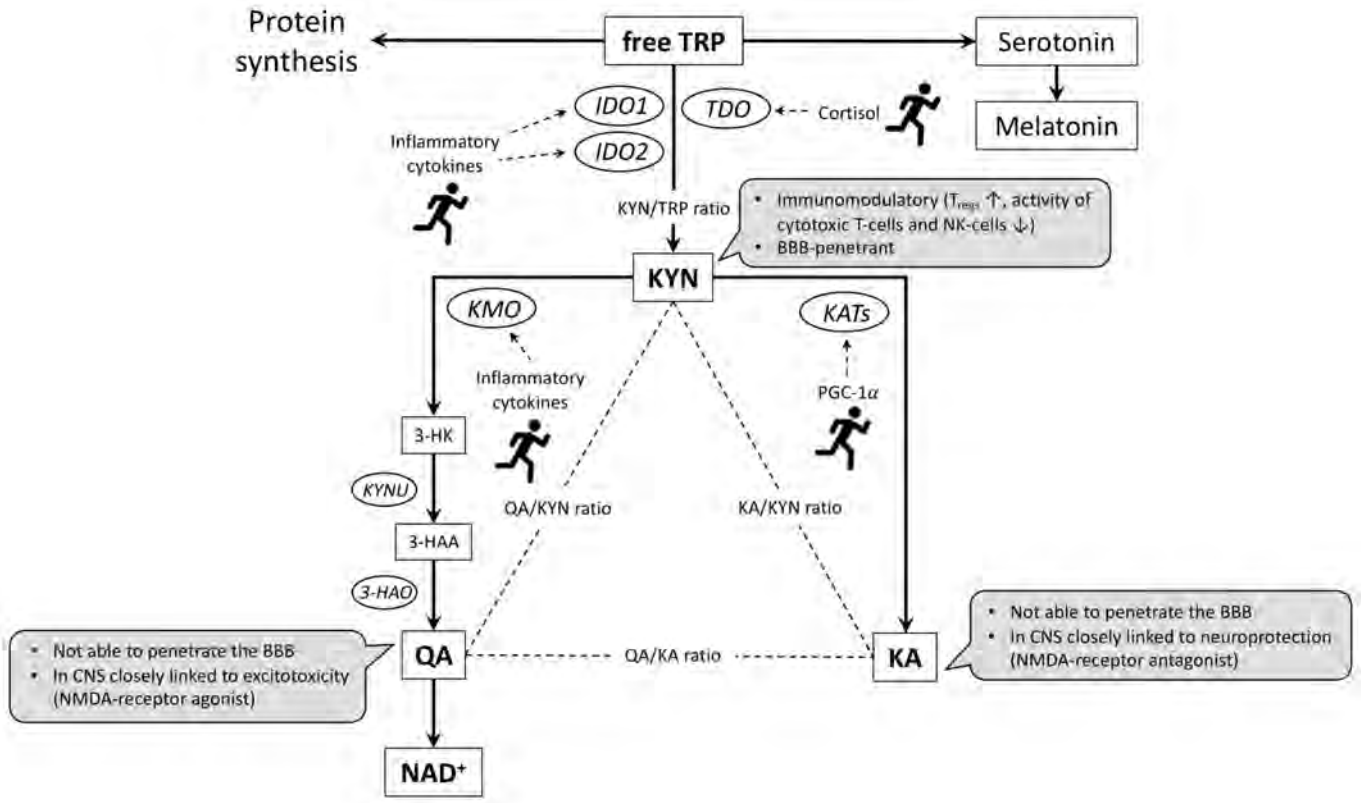


Figure 1. Potential acute exercise-induced mechanisms impacting the kynurenine pathway.
🏃 Modulatory effect of acute exercise; *TRP* tryptophan; *KYN* kynurenine; *KA* kynurenic acid; *QA* quinolinic acid; *3-HK* 3-hydroxykynurenine; *3-HAA* 3-hydroxyanthranilic acid; *NAD+* Oxidized form of nicotinamide adenine dinucleotide; *IDO* indoleamine 2,3-dioxygenase; *TDO* tryptophan 2,3-dioxygenase; *KMO* kynurenine 3-monooxygenase; *KATs*, kynurenine aminotransferases; *KYNU* kynureninase; *3-HAO* 3-hydroxyanthranilic acid oxygenase; *PGC-1α* proliferator-activated receptor-gamma coactivator-1alpha; T_{regs}, regulatory T-cells; *NK-cells* natural killer cells; *CNS* central nervous system; *BBB* blood-brain barrier; *NMDA* N-methyl-d-aspartate.

sity and frequency) under physiological conditions. Furthermore, no data on follow-up measurements exists to evaluate the kinetics of exercise-induced alterations in the KYN pathway. More detailed information about the effects of acute bouts of exercise could help to appropriately design long-term intervention studies. Future randomized controlled trials seem to be of major relevance to achieve sustainable modulation or normalization within the KYN pathway and its consequences, especially in view of populations with inflammation-mediated chronic diseases and dysregulations within the KYN pathway.

Here, we investigated the impact of acute resistance (RE) and endurance exercise (EE) on the KYN pathway. We hypothesize that KYN pathway alterations will be greater following endurance exercise compared to resistance exercise. A broad range of metabolites along the KYN pathway, IL-6 and levels of cortisol of healthy participants were determined in blood serum at pre, post and 1h follow-up measurement. Additionally, gene expression of IDO1 and KAT4 in circulating PBMCs was measured to provide further knowledge about underlying mechanisms of exercise-induced modulations of the KYN pathway. To determine potential relations between initial KYN pathway activation parameters and IL-6, cortisol and immune cell subsets during a resting condition, we performed baseline flow cytometry analysis.

Methods

Participants

A total of 24 healthy males (N=24) were recruited for participation. The inclusion criteria comprised being male, between 20 and 35 years of age, no contraindications to physical exercise and no drug intake during the last six weeks. Inclusion criteria were checked before study participation using the german version of the Physical Activity Readiness Questionnaire (PAR-Q) (22) and an additional question on drug intake during the last six weeks. All participants signed a written informed consent prior to participation. The study was approved by the local ethics committee of the German Sport University Cologne and was prospectively registered at the German clinical trial register (DRKS00014286).

General procedure

A randomized cross-over study design including two intervention arms was applied. An initial baseline testing was conducted one week prior to the intervention. At baseline, participants' maximal endurance and strength capacity were measured in order to standardize and control exercise intensity. Subsequently, all participants performed a single bout of endurance exercise (EE) and resistance exercise (RE) with a wash-out period of at least 48 hours between both exercise

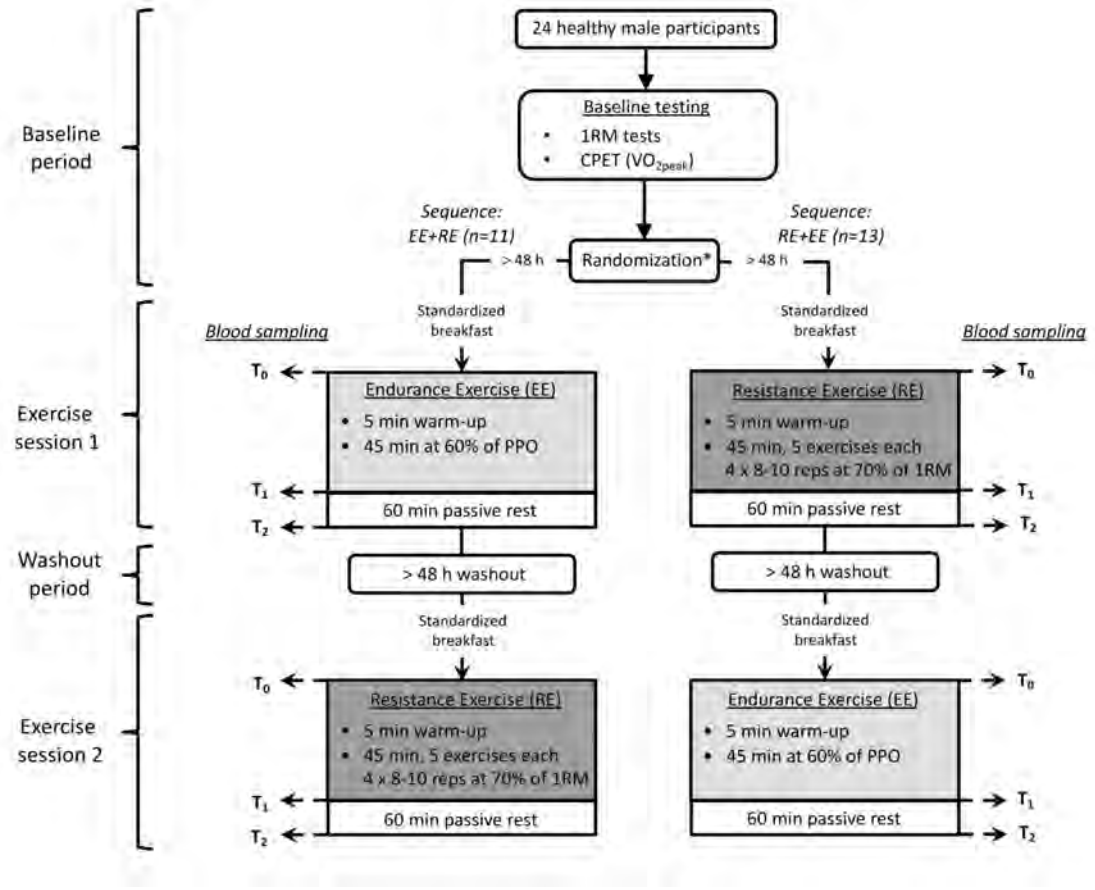


Figure 2. Schematic overview of the study design.
* Participants' smoking status, VO_{2peak}-levels and total strength values were used as stratification factors.
EE endurance exercise; *RE* resistance exercise; *1RM* one-repetition maximum; *CPET* cardiopulmonary exercise test; VO_{2peak} maximal oxygen uptake; *PPO* peak power output; *Reps* repetitions; T_0 resting condition (baseline); T_1 immediately after exercise cessation; T_2 one hour after exercise cessation.

sessions, since acute effects on cytokines and cellular immune components do usually not persist longer than 24 hours. The sequence of the interventions (EE+RE or RE+EE) was randomly allocated (see Randomization). Venous blood samples were collected immediately before (T_0), immediately after (T_1) and one hour after (T_2) both exercise sessions, respectively. The follow-up measurement time point was set at one hour after completion of the exercise sessions in order to provide outcome kinetics and to potentially define a time frame of occurring alterations. One hour prior to each session participants received a standardized breakfast. Participants were told to avoid the consumption of nicotine, supplements and alcohol and to avoid any amount of physical exercise for 48 h prior to baseline testing and each exercise session. Exercise sessions started between 8 and 11 AM. The study design is depicted in Figure 2.

Sample Size Calculation

Sample size calculation was conducted to examine different responses of EE and RE in the change of KYN/TRP ratio post intervention using G*power software (Version 3.1.9.2) (25) in a baseline-adjusted ANCOVA model. Sample size calculation was performed according to the formula $(1-\rho^2)*n$. In detail, ρ represents the estimated correlation between pre and post measurements and n represents the calculated sample size by using G*power software that would have been appropriate for an independent t-test comparing post measurements of both groups. We determined sample size calculation parameters based on the results of the study of Strasser et al. (63) investigating the acute effect of an exhaustive endurance exercise on KYN/TRP ratio in healthy athletes. The data of Strasser et al. (63) revealed an effect size (Cohen's *d* (19)) of -.804 and a correlation between pre and post measurements of r = .856. In contrast to Strasser et al. (63), our study was designed to compare acute responses of two different exercise modalities. Therefore, sample size was calculated using a more conservatively estimated effect size of d = -.65. Since we implemented two post measurement time points in our study (T_1, T_2), estimated correlation between pre and post measurements were reduced to ρ = .7 and alpha (α) was adjusted to α = .025. Test power was set at 80%. Calculation revealed a total sample size of N = 48. Due to the applied cross-over design implicating that both intervention arms are completed by all participants, the required sample size resulted in N = 24. Sample size calculation was performed on the assumption that no carry-over effects exist.

Randomization

Randomization was carried out immediately after baseline testing using Randomization In Treatment Arms (RITA, Evi-

dat, Germany) software. A stratified randomization procedure according to Pocock and Simon's minimization method was used (for Review see (61)). Stratification factors comprised participants' smoking status, relative oxygen uptake maximum (VO_{2peak}) and a total strength value (see Baseline testing). Randomization was conducted by an independent research associate who was not involved in data collection.

Baseline testing

The baseline testing consisted of one-repetition maximum (1RM) tests followed by a cardiopulmonary exercise test (CPET) on a bicycle ergometer after a one hour resting break. Participants were asked to refrain from eating during the hour before the baseline testing. During the testing procedure participants were allowed to drink water ad libitum.

One-repetition maximum (1RM) tests

Following a five min warm-up on a cycle ergometer at 50 W, participants performed five machine-based (Cybex International, Medway, US) 1RM tests conducted in the following order: chest press, lat pull, leg curl, leg extension, back extension. The 1RM test at each machine started with a warm-up set consisting of 15 repetitions of self-selected resistance through a full range of motion. Following a 2-min break, participants were given five attempts to attain their 1RM at each machine. Within each attempt, the maximum amount of repetitions was conducted until exhaustion. The attempts at one machine were separated by a 2-min break, respectively. The 1RM was reached if exactly one repetition was feasible with high quality of movement execution. If the 1RM at one machine was not reached within the five attempts, the weight of the last attempt counted as maximum. Between the 1RM tests of each of the five machines a break of 1.5 min was implemented, respectively. A total strength value was calculated for each participant using the mean of all five 1RM weights. The total strength value was used for stratification.

Incremental cardiopulmonary exercise test (CPET)

An incremental CPET on a bicycle ergometer (Ergoline, Germany) with spirometry (Cortex, Meta-Lyzer 3B-R2, Germany) was conducted to determine participants' relative maximum oxygen uptake (VO_{2peak} [ml/kg/min]), maximum heart rate (HRmax [beats per minute (bpm)]) and peak power output (PPO). Participants started with a five-minute warm-up period at 25 W. Subsequently, workload was set to 100 W and was increased by 20 W/min until the participant was not able to maintain pedal cadence above 60 rounds per minute (rpm). Participants' heart rate, respiratory quotient (RQ) and rate of perceived exertion (RPE (13)) were recorded at exhaustion. PPO was defined as the average of the highest ten second interval, whereas VO_{2peak} was calculated as the average of the highest ten second interval. Additionally, lactate levels were measured at rest (La_{pre}) and at exhaustion (La_{post}).

Interventions

Participants received a standardized breakfast consisting of a cereal bar and a banana one hour prior to each exercise intervention. Each exercise session lasted 50 minutes in total and started at the same time of day. Exercise duration of 50 minutes was chosen to represent an extended and realistic training stimulus that could be transferred to clinical populations. Participants were allowed to drink water ad libitum.

Endurance Exercise (EE)

Following a five min warm-up at 50 W, participants cycled for 45 min at a power output corresponding to 60% of PPO. Participants were asked to refrain from standing during the trial and asked to maintain a cadence between 60 and 80 rpm.

Resistance Exercise (RE)

Following a five min warm-up at 50 W on a cycle ergometer, participants conducted four sets at each of the five resistance machines used in the baseline testing for 1RM determination in equal order (chest press, lat pull, leg curl, leg extension, back extension). A specific warm-up set comprising 15 repetitions at 30% of 1RM was implemented immediately before the exercise at each resistance machine. Each set consisted of 8-10 repetitions at 70% of the attained 1RM. Resting time between the sets and between the five resistance machines was set at 1 min.

Outcomes and measurements

All outcomes were assessed at each measurement time point (T_1, T_2, T_3). Concentrations of IL-6 were determined using enzyme-linked immunosorbent assay (ELISA) according to manufactures instructions (R&D Systems, Human IL-6 Quantikine ELISA Kit; detection limit: 0.70 pg/ml, coefficient of variability for intra-assay precision: 4.2%). Concentrations of cortisol were measured using chemiluminescence immunoassay (CLIA) by contract laboratory (Labor Dr. Wisplinghoff, Cologne, Germany). TRP and its metabolites KYN, KA and QA were analyzed by high performance liquid chromatography (HPLC). To obtain detailed information on changes in KYN pathway balance, ratios of KYN/TRP, KA/KYN, QA/KYN and QA/KA were calculated. Moreover, gene expression of central enzymes along the KYN pathway (IDO1 and KAT4) were measured in PBMCs. In addition to the outcomes, flow cytometry analysis was performed at baseline (T_0) to evaluate potential relationships between numbers and proportions of immune cell subsets (lymphocytes, NK-cells, NK-cell subsets (dim/bright), cytotoxic T-cells, T-helper cells and regulatory T-cells) and initial KYN pathway activation parameters (KYN, KYN/TRP, IDO1). Detailed information on the procedure of haematological analyses is provided in the following.

Blood sampling

Blood samples were drawn at each measurement time point in supine position from a medial cubital vein. Blood samples were used (i) to isolate serum and (ii) to isolate PBMCs. Regarding serum isolation, blood was rested at room temperature for 10 min for clotting and thereafter centrifuged at 3500 rpm for 10 min. Serum samples were aliquoted and stored at -80°C until analyses with HPLC, ELISA and CLIA were per-

formed. EDTA blood was used for PBMC isolation by density gradient centrifugation using a lymphocyte separation medium (PromoCell, Heidelberg, Germany). In brief, blood was layered on top of the lymphocyte separation medium and centrifuged for 30 min at 800xg. Thereafter, the PBMC containing interphase was separated and washed with PBS. PBMCs were aliquoted and frozen at -150°C until analyses with real-time quantitative polymerase chain reaction and flow cytometry were performed.

High performance liquid chromatography (HPLC) and mass spectrometry

Chemicals and materials

For the preparation of aqueous solutions and eluents, ultrapure water obtained from a Sartorius Stedim Arium® pro UV apparatus (Guxhagen, Germany) was used. Acetonitrile (ACN), ammonium acetate, acetic acid, and ammonium hydroxide solution (25% NH3) of analytical grade were from Merck (Darmstadt, Germany). Reference compounds (purity ≥98%) including TRP, KYN, KA and QA as well as pooled human serum (male donors) were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). As internal standards (ISTDs), multiply deuterium-labelled analogues L-Tryptophan-d5 (TRP-d5), Kynurenic acid-d5 (KA-d5) and 2,3-Pydridinedicarboxylic acid-d3 (QA-d3) were obtained from Toronto Research Chemicals (North York, ON, Canada).

Sample preparation/analyte extraction

In accordance to previously published protocols (5), aliquots of 50 µL of serum were spiked with 5 µL of the ISTD [10 µg/mL TRP-d5, 1 µg/mL KA-d5 and 10 µg/mL QA-d3] and were further diluted by the addition of 25 µL of water and 70 µL of ACN. After 5 min of sonication, serum proteins were precipitated by centrifugation at 14.000xg at room temperature (RT) for another 5 min. The supernatant was separated from the obtained pellet and transferred to a fresh polypropylene vial. Subsequently, 5 µL of 25% NH3 was added and samples were ready for analysis.

Liquid chromatography (LC)

Liquid chromatographic separation was achieved by using an ACQUITY UPLC® system (Waters GmbH, Eschborn, Germany) equipped with a Gemini C6-Phenyl analytical column (100 mm x 2 mm ID, 3.0 µm particle size, 110 Å) from Phenomenex (Aschaffenburg, Germany). Gradient elution was carried out using 5 mM ammonium acetate solution acidified with 0.1% acetic acid as solvent A and acetonitrile fortified with 5% of Eluent A as solvent B. The overall gradient program lasted 20 min and the flow rate was set to 250 µL/min. Initiated by the injection of 2 µL of the sample into the LC system, the analytical run started with 1 min of constant flow of 100% A. Subsequently, the organic phase was increased to 40% within 6 min and to 100% within another 2 min. Finally, the initial conditions of 100% A were restored followed by re-equilibration for 10 min.

Mass spectrometry

The mass spectrometric analysis was performed on a Xevo® TQ-XS triple quadrupole mass spectrometer (QqQ/MS) (Waters GmbH, Eschborn, Germany) equipped with a UniSpray™ (US) ion source. The system was operated in positive ionization mode (US+) with activated soft transmission mode. The ion source was set to a desolvation temperature and cone voltage of 400°C and 45 V, respectively. Data were generated by multiple reaction monitoring (MRM) experiments after optimization of the method and individual tuning of all analytes of interest. Argon (purity grade 5.0) was used as damping gas for collision-induced dissociation (CID) experiments. Waters' Mass-Lynx software (V4.2 2016) was used for result interpretation. Further information is provided in supplement 1.

Method evaluation and result interpretation

During the evaluation of the detection method, parameters such as specificity, linearity of calibration curves, lower limit of quantification (LLOQ), recovery and intraday precision were assessed. To ensure the method's specificity, 12 blank serum samples from different female and male volunteers (n = 6 + 6) were analyzed after activated charcoal stripping as published elsewhere (48). Samples were successfully tested for the absence of potentially interfering signals at expected retention times. Moreover, analogously purified commercially available pooled human serum was used for the determination of further validation parameters. Calibration curves for quantitative result interpretation of all target analytes were prepared individually and were found to be linear within appropriate concentration ranges [TRP: 0.5, 2, 5 and 20 µg/mL; KYN: 20, 50, 200, 5000 and 2000 ng/mL; QA: 5, 20, 50, 200, 500 and 2000 ng/mL and KA: 0.5, 2, 5, 20, 50 and 200 ng/mL] with coefficients of correlation of greater than 0.98. Linear correlation was utilized to calculate concentrations of the measured analytes by their peak areas (peak area ration of analytes and corresponding ISTDs). The analytical performance between different sample batches on consecutive days was monitored by measuring quality control samples with defined concentrations. The LLOQ, defined as the lowest concentrations measurable via a signal-to-noise ratio (S/N) greater than 9 was estimated for each substance at 10 ng/mL (QA and KA), 25 ng/mL (KYN), and 50 ng/mL (TRP), and was confirmed by six sample replicates (n = 6) spiked at the aforementioned concentrations. With regard to the recovery during the sample work up, two sets of six charcoal stripped serum aliquots (n = 6 + 6) were fortified with 1 µg/mL of TRP and QA, 100 ng/mL of KYN and 50 ng/mL of KA. In the first set, the analytes were added simultaneously with the ISTDs before sample preparation while for the second set spiking was carried out just before injection to the LC-MS system. Resulting recoveries were between 66-76%. Moreover, six sample replicates at 3 concentration levels (high, medium and low, n = 6 + 6 + 6) were prepared and analyzed to determine the intraday imprecision. Values ranged between 2-18% for TRP, 2-12% for KYN, 2-15% for QA and 3-13% for KA. Further details are summarized in supplement 1.

Real-time quantitative polymerase chain reaction (qRT-PCR)

For gene expression analyses PBMCs were thawed and RNA was isolated using TRIzol reagent (Merck, Darmstadt, Germany) according to the manufacture´s protocol. In brief, phenol chloroform phase separation was used and the aqueous

phase containing the RNA was further processed. RNA was precipitated with isopropanol, washed with ethanol and resuspended in RNAse free water. cDNA synthesis and qRT-PCR were performed using the GoTaq 2-Step qRT-PCR Kit (Promega GmbH, Mannheim, Germany) according to the manufacture´s protocol. For cDNA synthesis a maximum of 1µg RNA per reaction was applied and 4 reactions per sample were performed. The cDNA of these 4 reactions per sample were pooled for further qPCR analysis. Primer for IDO1 and KAT4 were described previously by Dewi et al. (23) and Agudelo et al. (1), respectively. As reference genes HPRT1 and UBE2D2 were chosen, since Oturai et al. (55) found them to be stably expressed in PBMCs. Primers for the housekeeping genes were generated to span Exon-Exon junctions (HPRT1: forward GCGTCGTGATTAGTGATGATG, reverse GTTCAGTCCTGTCCATAATTAGTC; UBE2D2: forward ACTAACTATTTCAAAAGTACTCTTGTCCATCT, reverse CGAGCTATTCTGTTGTACTTTTCTCTA). Gene expression was analyzed by comparison of the delta Ct values of the target genes.

Flow cytometry

PBMCs were thawed for flow cytometry analyses. Two different panels were analysed. In the first panel PBMCs were stained with anti-CD3 PE-Cy7, anti-CD8 PE, anti-CD4 APC, anti-CD16 PE (BD Bioscience, Heidelberg, Germany) and anti-CD56 APC-Cy7 (BioLegend, San Diego, CA, USA). Lymphocytes were gated by size and granularity. Cytotoxic T-cells were gated as $CD3^+CD8^+$, whereas T-helper cells were gated as $CD3^+CD4^+$. NK-cells were gated as $CD3^-CD16^+$ and further divided into $CD56^{dim}$ and $CD56^{bright}$ NK-cells. In the second panel PBMCs were stained with anti-CD3 PE-Cy7, anti-CD4 APC-Cy7, anti-CD127 PE and anti-CD25 APC (BD Bioscience, Heidelberg, Germany). T_{regs} were gated as $CD3^+CD4^+CD25^+CD127^{dim}$. Flow cytometry was performed on a FACS Array (BD Bioscience, Heidelberg, Germany) and gating was performed using the BD FACS Diva Software.

Statistical analyses

To investigate relationships between IL-6, cortisol, immune cells subsets and initial KYN pathway activation outcomes (KYN, KYN/TRP, IDO1) at baseline (T_0), Pearson's correlation coefficient (r) was calculated. To examine the hypothesis of this study, within and between differences in EE and RE post intervention were tested in TRP metabolites, IL-6, cortisol and KYN pathway enzyme expression measured in PBMCs. Therefore, outcome data was first tested for potential significant interaction effects of intervention sequences (EE+RE vs. RE+EE) or intervention periods (trainings session day 1 vs. training session day 2) using a baseline-adjusted analysis of covariance model (ANCOVA) for each outcome (necessary pre testing for cross-over design). If no effects of intervention sequence or periods were revealed, data of equal training modalities were pooled for further analyses. Separated baseline-adjusted ANCOVA models were conducted for all outcomes to examine time and interaction (time*group) effects of EE and RE. Mauchly's test of Sphericity was applied to detect potential violations and Greenhouse-Geisser correction was used if necessary. In case of significant ANCOVA results Bonferroni corrected post-hoc analyses were conducted. In addition, Cohens' *d* effect sizes were calculated for significant results. For all statistical analyses parametric procedures were conducted using SPSS statistics 25 (IBM®, Armonk, NY, USA). Level of significance was set at $p \leq .05$.

Results

Baseline characteristics for all participants and each intervention sequence (RE+EE and EE+RE) for anthropometric data, CPET and 1RM tests are provided in Table 1. The investigated sample revealed an overall mean age ± standard deviation (SD) of 24.6 ± 3.9 years, an overall mean VO_{2peak} ± SD of 48.3 ± 7.4 ml/kg/min and an overall mean of the total strength value ± SD of 101.9 ± 16.2 kg.

Table 1. Baseline characteristics of study participants separated by exercise sequence.

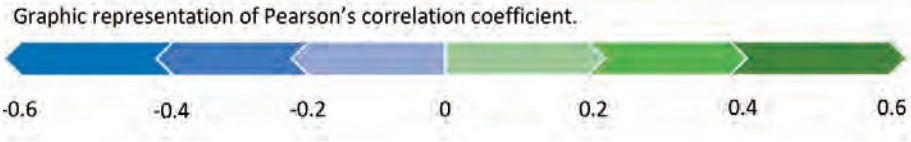
	RE+EE (n=13)	**EE+RE** (n=11)	**Overall** (n=24)
Anthropometric and demographic characteristics			
Age (years)	24.8 ± 4.8	24.4 ± 2.7	24.6 ± 3.9
Height (cm)	182.3 ± 6.8	182.5 ± 5.4	182.4 ± 6.2
Weight (kg)	81.6 ± 10.7	86.6 ± 10.1	83.9 ± 10.5
BMI (kg/m²)	24.5 ± 2.1	26 ± 3.3	25.4 ± 2.7
Smoking status (yes / no)	2 / 11	4 / 7	6 / 18
Performance characteristics			
$\dot{V}O_{2peak}$ (ml/kg/min)	48.8 ± 8.0	47.8 ± 6.9	48.3 ± 7.4
HR_{pre} (bpm)	78.8 ± 8.0	78.8 ± 8.6	78.8 ± 8.1
HR_{max} (bpm)	186.8 ± 7.3	184.5 ± 6.6	185.6 ± 7.0
La_{pre} (mmol/l)	1.1 ± 0.6	0.8 ± 0.2	1.0 ± 0.5
La_{post} (mmol/l)	10.0 ± 2.0	10.2 ± 1.3	10.1 ± 1.7
RER_{max} ($\dot{V}CO_2/\dot{V}O_2$)	1.14 ± 0.0	1.16 ± 0.0	1.15 ± 0.0
PPO (W)	327.7 ± 68.1	336.4 ± 44.6	331.7 ± 57.5
Watt/kg	4.0 ± 0.6	4.0 ± 0.7	4.0 ± 0.6
1RM Chest press (kg)	109.7 ± 25.1	116.8 ± 17.6	113.3 ± 22.1
1RM Lat pull (kg)	99.5 ± 22.2	103.5 ± 21.4	102.0 ± 21.5
1RM Leg curl (kg)	72.5 ± 10.1	76.8 ± 16.7	74.1 ± 13.2
1RM Leg extension (kg)	115.0 ± 26.1	122.7 ± 23.8	120.9 ± 24.8
1RM Back extension (kg)	100.3 ± 21.6	100.0 ± 20.1	100.9 ± 20.4
Total strength value (kg)	99.7 ± 17.9	104.5 ± 14.2	101.9 ± 16.2

Values are presented as mean ± standard deviation. *RE* resistance exercise; *EE* endurance exercise; *BMI* body mass index; VO_{2peak} peak oxygen uptake; HR_{pre} heart rate before incremental exercise test; HR_{max} maximal heart rate; La_{pre} lactate level immediately before cardiopulmonary exercise test; La_{post} lactate level immediately after cardiopulmonary exercise test; RER_{max} maximal respiratory exchange ratio; *PPO* peak power output; *Watt/kg* relative power output; *1RM* one repetition maximum; *Total strength value* mean of all 1RM tests.

Results of bivariate correlational analyses between IL-6, cortisol, immune cell subsets and initial KYN pathway activation outcomes (KYN, KYN/TRP, IDO1) at baseline are presented in Table 2. Significant correlations were revealed between IL-6 and IDO1 (dCt HPRT1: r = -.318, p = .031; dCt UBE2D2: r = -.389, p = .008), IL-6 and KYN/TRP (r = .473, p = .001). Regarding cortisol, concentrations significantly correlated with KYN/TRP (r = -.366, p = .012). Furthermore, proportions of T_{regs} significantly correlated with IDO1 (dCT HPRT1: r = -.356, p = .014). Concerning proportions of cytotoxic T-

Table 2. Baseline correlations (Pearson's coefficient) between IL-6, cortisol, immune cell subsets and KYN pathway outcomes.

	KYN	KYN/TRP	IDO1 (dCt HPRT1)	IDO1 (dCt UBE2D2)
IL-6	.288	.473**	-.318*	-.389**
Cortisol	-.204	-.366*	-.094	-.115
T-helper cells (p)	-.070	-.180	.147	.196
T-helper cells (n)	-.121	-.231	.261	.254
T_{regs} (p)	.023	.082	-.356*	-.282
T_{regs} (n)	-.096	-.131	-.076	-.071
Cytotoxic T-cells (p)	.173	.002	.532**	.464**
Cytotoxic T-cells (n)	.071	-.069	.529**	.439**
NK-cells (p)	.199	.306*	-.193	-.220
NK-cells (n)	.098	.227	-.114	-.153
NK-cells ($CD56^{dim}$) (p)	-.209	-.357*	.353*	.282
NK-cells ($CD56^{dim}$) (n)	.068	.188	-.090	-.133
NK-cells ($CD56^{bright}$) (p)	.208	.356*	-.348*	-.277
NK-cells ($CD56^{bright}$) (n)	.248	.396**	-.234	-.230



* p ≤ .05; ** p ≤ .01; *(p)* proportions *(n)* numbers. Proportions of T-helper cells, cytotoxic T-cells and NK-cells refer to Lymphocytes. Proportions of T_{regs} refer to $CD3^+$ lymphocytes. Proportions of NK-cells ($CD56^{dim}$) and NK-cells ($CD56^{bright}$) refer to NK-cells.

cells, significant correlations to IDO1 (dCt HPRT1: r = .532, p < .001; dCt UBE2D2: r = .464, p = .001) were revealed. In addition, numbers of cytotoxic T-cells correlated significantly with IDO1 (dCt HPRT1: r = .529, p < .001; dCt UBE2D2: r = .439, p = .002). For proportions of NK-cells, analysis indicated significant correlations to KYN/TRP (r = .306, p = .039). Moreover, proportions of $CD56^{dim}$ NK-cells significantly correlated with IDO1 (dCt HPRT1: r = .353, p = .015) and KYN/TRP (r = -.357, p = .015). Regarding proportions of $CD56^{bright}$ NK-cells, significant correlations to IDO1 (dCt HPRT1: r = -.348, p = .016) and KYN/TRP (r = .356, p=.015) were detected. Finally, analysis revealed a significant correlation between numbers of $CD56^{bright}$ NK-cells and KYN/TRP (r = .396, p = .007). A comprehensive overview of correlation coefficients is provided in Table 2.

To examine necessary assumptions considering the cross-over study design, outcome data were pretested for potential significant interaction effects of intervention sequences (EE+RE vs. RE+EE) or intervention periods (trainings session day 1 vs. training session day 2) using baseline-adjusted ANCOVAs for each outcome. Regarding KAT (HPRT1), a significant interaction effect of intervention periods was detected (p < .005, F = 7.365, df = 1.299). For all other outcomes, neither a significant effect of intervention sequences nor of intervention periods was revealed. Therefore, outcome data was pooled before conducting ANCOVA models for each parameter with training modality (EE vs. RE) as between subjects factor.

ANCOVA results of IL-6 showed a significant time (p < .001, F = 12.282, df = 1,355) and interaction (p < .001, F = 12.090, df = 1,355) effect. Bonferroni corrected post-hoc test revealed a significant increase from T_0 to T_1 (p < .001, d = 1.322), from T_0 to T_2 (p = .006, d = .549) and a significant decrease from T_1 to T_2 (p < .001, d = -.951) in EE. Significant interaction effects were detected at T_1 (p < .001) and T_2 (p = .015) with levels of EE being higher than those of RE, respectively. Concerning cortisol, a significant time (p < .001, F = 20.870, df = 1.648) and interaction effect (p < .001, F = 14.642, df = 1,648) was found. In EE, levels of cortisol were significantly increased at T_1 compared to T_0 (p < .001, d = 1.069). Furthermore, levels at T_2 were significantly decreased compared to levels at T_1 (p < .001, d = -.906). In RE, levels of cortisol were significantly decreased at T_2 compared to T_0 (p < .001, d = -.862) and T_1 (p < .001, d = -.752). Significant interaction effects were revealed at T_1 (p < .001) and at T_2 (p < .001) with levels of EE being higher than those of RE, respectively. In view of TRP, ANCOVA results showed a significant effect over time (p < .001, F = 19.894, df = 2). Bonferroni post-hoc test detected a significant decrease from T_0 to T_1 (p = .034, d = -.368) in RE. A significant effect over time was also revealed for KYN (p = .016, F = 4.359, df = 2), but Bonferroni post-hoc test did not show any significant differences between measurement time points in EE or RE. Regarding KYN/TRP ratio, ANCOVA resulted in a significant time effect (p = .041, F = 3.332, df = 2). In RE, values were significantly increased at T_1 compared to T_0 (p = .001, d = .474) and significantly decreased at T_2 compared to T_1 (p = .005, d = -.437). ANCOVA results of KA showed a significant time (p = .007, F = 5.631, df = 1.748) and interaction (p = .028, F = 3.987, df = 1.748) effect. Bonferroni post-hoc test

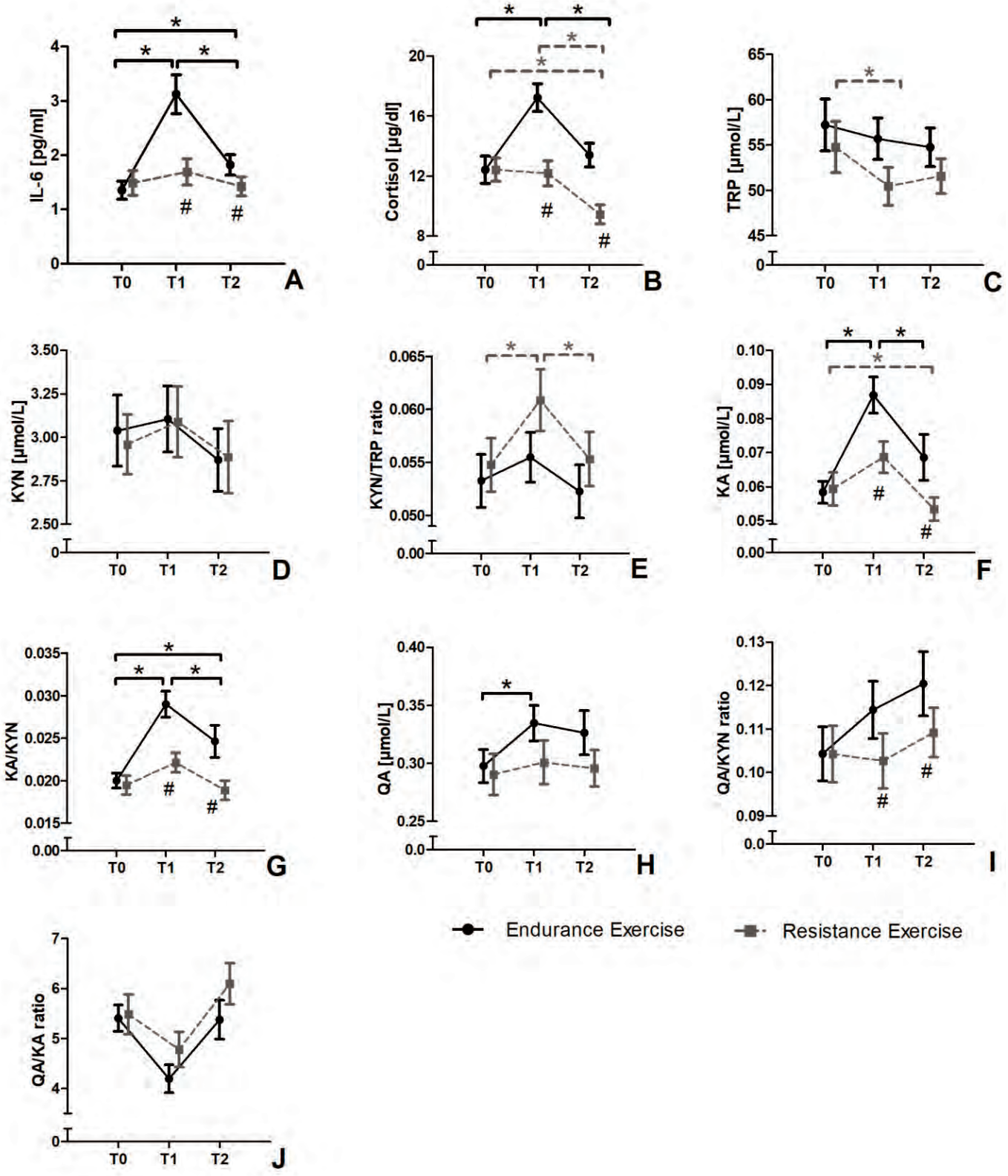


Figure 3. Kinetics of TRP metabolites separated by training intervention.
* significant time effect (p≤0.05); # significant interaction effect (p≤0.05); black shading indicates endurance exercise; grey shading indicates resistance exercise; *T0* baseline; *T1* post exercise; *T2* 1 hour follow-up; values are presented as mean ± standard deviation; *(A)* interleukin-6 concentrations; *(B)* cortisol concentrations; *(C)* tryptophan concentrations; *(D)* kynurenine concentrations; *(E)* kynurenine-tryptophan ratio; *(F)* kynurenic acid concentrations; *(G)* kynurenine-kynurenic acid ratio; *(H)* quinolinic acid concentrations; *(I)* quinolinic acid-kynurenine ratio; *(J)* quinolinic acid-kynurenic acid ratio.

revealed significantly increased levels at T_1 compared to T_0 ($p < .001$, $d = 1.384$) and significantly decreased levels at T_2 compared to T_1 ($p = .008$, $d = -.646$) in EE. Additionally, significantly decreased levels of KA were found at T_2 compared to T_1 ($p = .041$, $d = -.819$) in RE. In regard to the interaction effect, values of EE were significantly higher at T_1 ($p = .002$) and at T_2 ($p = .037$) than values of RE. Considering KA/KYN ratio, a significant time ($p = .024$, $F = 3.925$, $df = 2$) and inter-

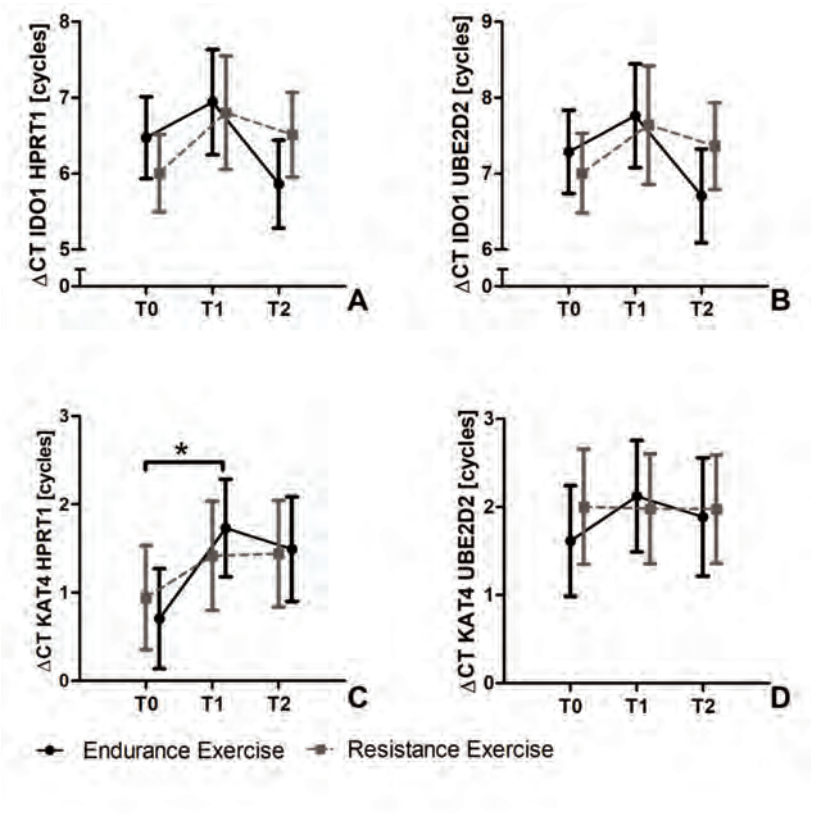


Figure 4. Kinetics of enzyme gene expression in PBMCs.
* significant time effect (p≤0.05); black shading indicates endurance training; grey shading indicates resistance training; *T0* baseline; *T1* post exercise; *T2* 1 hour follow-up; values are presented as mean ± standard deviation; *(A)* ΔCT of IDO1 expression in PBMCs referring to HPRT1 housekeeping gene; *(B)* ΔCT of IDO1 expression in PBMCs referring to UBE2D2 housekeeping gene; *(C)* ΔCT of KAT4 expression in PBMCs referring to HPRT1 housekeeping gene; *(D)* ΔCT of KAT4 expression in PBMCs referring to UBE2D2 housekeeping gene.

action effect (p = .002, F = 6.961, df = 2) was detected. In EE, values compared to T_0 were significantly increased at T_1 (p < .001, d = 1.565) as well as at T_2 (p = .004, d = .666). Moreover, values in EE were significantly decreased at T_2 compared to T_1 (p = .01, d = -.554). Significant interaction effects were found at T_1 (p < .001) and at T_2 (p = .007) with values of EE being higher than those of RE, respectively. For QA, a significant time effect was revealed (p = .014, F = 4.529, df = 2). Post-hoc test showed a significant increase from T_0 to T_1 (p = .008, d = .076) in EE. Furthermore, a significant interaction effect was detected for QA/KYN ratio (p = .016, F = 4.335, df = 2). Values of EE were significantly higher than those of RE at T_1 (p = .006) and at T_2 (p = .015), respectively. In view of QA/KA, no significant effects were revealed. Kinetics of all TRP metabolites separated by training interventions are illustrated in Figure 3.

Concerning ANCOVA results of IDO1 gene expression measured in PBMCs, no significant effects were detected, neither for HPRT1 nor for UBE2D2 calculated ΔCT values. ANCOVA results of ΔCT KAT4 HPRT1 gene expression in PBMCs revealed a significant effect over time (p = .001, F = 10.441, df = 1.254). Bonferroni post-hoc test showed significantly increased values at T_1 compared to T_0 (p = .019, d = .374) in EE. No significant effects were detected for ΔCT KAT4 UBE2D2. Kinetics of enzyme gene expression in PBMCs are shown in Figure 4.

Discussion

This is the first study investigating the acute impact of two different exercise modalities on a broad range of KYN pathway outcomes with simultaneous consideration of the potential mediators IL-6 and cortisol. Moreover, we examined associations between KYN pathway outcomes and immune cell subsets during a resting condition in humans and provide first information on outcome kinetics due to the implemented follow-up measurement time point one hour after exercise cessation. Overall, our results indicate a stronger activation along the KYN pathway induced by EE compared to RE. However, the KYN/TRP ratio was only significantly increased in RE immediately after exercise, representing an initial activation of this metabolic pathway. In line with previous investigations, our results emphasize an increased conversion of KYN to KA in response to EE (41, 50, 60).

Kynurenine pathway activation (TRP → KYN)

IL-6 serum concentrations positively correlated with both, the KYN/TRP ratio and IDO1 expression in PBMCs at baseline. These results support findings from previous studies reporting a stimulating role of IL-6 in the activation of the KYN pathway (20). Moreover, proportions of total NK-cells as well as numbers and proportions of cytokine producing $CD56^{bright}$ NK-cells positively correlated with the KYN/TRP ratio. In contrast, cytotoxic $CD56^{dim}$ NK-cell proportions negatively correlated with the KYN/TRP ratio. These associations between KYN pathway metabolites and NK-cell phenotypes support previously described immunomodulatory properties of KYN pathway activation (16). Frumento et al. (26) already showed that IDO activity inhibits NK-cell proliferation. However, NK-cell subsets were not investigated. In addition to KYN pathway metabolites, our results suggest a positive association between IDO1 expression in PBMCs and cytokine producing $CD56^{bright}$ NK-cells and a negative association with proportions of cytotoxic $CD56^{dim}$ NK-cells. These results give a first hint that NK-cell subsets may be differentially regulated by IDO1. In line with Frumento et al. (26) IDO1 expression showed a negative correlation to numbers and proportions of cytotoxic T-cells. Apart from an inhibition of the cytotoxic potential of different lymphocyte subsets, we also found an association between IDO1 expression and proportions of T_{regs} supporting earlier results (18, 33). In brief, the baseline correlations of KYN/TRP ratio and IDO1 gene expression in PBMCs with different NK- and T-cell subsets strongly emphasize the

Table 3a. Overview of existing studies investigating the impact of acute exercise on kynurenine pathway outcomes.

Authors	Study type	Study population	Exercise intervention	Outcome Measurements		
				Metabolites in blood serum/ plasma	Gene expression of enzymes in	
					Skeletal muscle	PBMCs
Lewis et al. (2010)	longitudinal study	n = 25 (19♂; 6♀) healthy	**Marathon race** (42.2 km)	TRP ↓ KA, AA, QA ↑	–	–
Melancon et al. (2014)	longitudinal study	n = 16 (16♂) elderly	**60 min continuous treadmill exercise** 67-70% $\dot{V}O_{2peak}$	TRP ↑	–	–
Areces et al. (2015)	longitudinal study	n = 26 (26♂) experienced triathletes	**Half-ironman triathlon** (1.9 km swimming, 75 km cycling, 21.1 km running)	TRP ↔	–	–
Mudry et al. (2016)	controlled longitudinal study	n = 12 (12♂) healthy	**30 min cycling exercise** 85% HR_{max}	TRP, KYN ↓ KA, KA/KYN ↑	KAT 1-4 ↔	–
		n = 12 (12♂) Type 2 diabetes	**30 min cycling exercise** 85% HR_{max}	TRP, KYN ↓ KA, KA/KYN ↑	KAT 1-4 ↔	
Schlittler et al. (2016)	longitudinal study (results of 3 different sub-studies)	n = 9 (9♂) endurance trained	**150 km road cycling time trial**	KA, QA ↑ QA/KA ↓	–	–
		n = 11 (10♂; 1♀) recreationally active	**Half marathon race** (21.1 km)	KA ↑		
		n = 9 (9♂) healthy	**100 drop jumps**	KA, QA, QA/KA ↔		
Strasser et al. (2016a)	longitudinal study	n = 33 (16♂; 17♀) trained athletes	**Incremental cycle ergometer exercise** until exhaustion	TRP ↓ KYN, KYN/TRP ↑	–	–
Strasser et al. (2016b)	randomized controlled study#	n = 29 (13♂; 16♀) trained athletes	**Incremental cycle ergometer exercise** until exhaustion	TRP ↓ KYN ↔ KYN/TRP ↑	–	–
Bansi et al. (2018)	longitudinal study (with subgroup analysis of MS phenotype)	n = 24 (9♂; 15♀) Secondary Progressive Multiple Sclerosis	**Incremental cycle ergometer exercise** until symptom limited maximum	TRP, KYN, KYN/TRP ↔	–	–
		n = 33 (10♂; 23♀) Relapsing Remitting Multiple Sclerosis		TRP ↓ KYN ↔ KYN/TRP ↑	–	–
Baxter-Parker et al. (2019)	randomized controlled crossover study	n = 43 (20♂; 23♀)	**Incremental cycle ergometer exercise vs. passive control**	TRP, KYN, KYN/TRP ↔	–	–
Joisten et al. (2019)	randomized crossover study	n = 24 (24♂) healthy	**Resistance vs. endurance exercise** *Resistance exercise:* 5 exercises, each 4 x 8-10 repetitions at 70% 1RM	TRP ↓ KYN/TRP ↑ KYN, KA, QA, KA/KYN, QA/KYN, QA/KA ↔	–	IDO1, KAT 4 ↔
			Endurance exercise: 45 min cycling exercise at 60% PPO	KA, KA/KYN, QA ↑ TRP, KYN, KYN/TRP, QA/KYN, QA/KA ↔	–	IDO1 ↔ KAT 4 ↑

Table 3b. Overview of existing studies investigating the impact of chronic exercise training on kynurenine pathway outcomes.

Authors	Study type	Study population	Exercise intervention	Outcome Measurements		
				Metabolites in blood serum/ plasma	Gene expression of enzymes in	
					Skeletal muscle	PBMCs
Hennings et al. (2013)	controlled longitudinal study	n = 38 (15♂; 23♀) major depressive disorder	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
		n = 27 (7♂; 20♀) somatization syndrome	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
		n = 48 (16♂; 32♀) healthy	**1 week of daily moderate physical activity** 30 min fitness/stretching exercise	TRP, KYN ↔	–	–
Agudelo et al. (2014)	longitudinal study	n = 8-10 (N/A♂; N/A♀) healthy	**3 weeks of intensive endurance training (6x/week; 2x/day)** *Session 1:* 30 min of cycling at a HR corresponding to lactate threshold *Session 2:* 25 min of running at a HR corresponding to lactate threshold	–	KAT 1-4 ↑	–
Melancon et al. (2014)	longitudinal study	n = 16 (16♂) elderly	**16 weeks of treadmill training** 3x/week; 45 min at 80% HR_{max}	TRP ↔	–	–
Küster et al. (2017)	non-randomized controlled study#	n = 47 (20♂; 27♀) elderly at risk of dementia n = 17 (6♂; 11♀)	**10 weeks of physical training vs. cognitive training vs. waitlist control** *Physical training (5x/week):* endurance, coordination, balance, flexibility and strength exercises; 2x/week 60 min supervised and 3x/week 20 min home-based	KYN, KA, 3-HK, QA ↔	–	–
Millischer et al. (2017)	randomized longitudinal study	n = 117 (42♂; 75♀) mild-to-moderate depression	**12 weeks of yoga/aerobic training; 3 groups** *1. light exercise (3x/week):* 60 min; mean intensity: 54.1% HR_{max}	KYN, KA ↔	–	–
			2. moderate exercise (3x/week): 60 min; mean intensity: 70.3% HR_{max}	KYN, KA ↔	–	–
			3. vigorous exercise (3x/week): 60 min; mean intensity: 76.2% of HR_{max}	KYN, KA ↔	–	–
Bansi et al. (2018)	randomized controlled study (with subgroup analysis of MS phenotype)	n = 24 (9♂; 15♀) Secondary Progressive Multiple Sclerosis n = 33 (10♂; 23♀) Relapsing Remitting Multiple Sclerosis	**3 weeks of cycling; 2 groups** *1. HIT (3x/week):* 5 x 3 min at 80% $\dot{V}O_{2peak}$; 1.5 min active recovery between intervals	TRP ↓ (RRMS only) KYN ↔ KYN/TRP ↑ (RRMS only)	–	–
			2. moderate continuous training (5x/week): 30 min at 65% $\dot{V}O_{2peak}$	TRP ↓ (RRMS only) KYN ↔ KYN/TRP ↑ (RRMS only)	–	–
Allison et al. (2019)	longitudinal study	n = 25 (25♂) elderly	**12 weeks of combined HIT and RT** *HIT (1x/week):* 30 min cycling; 10 x 1 min at 90% HR_{max}; 1 min active recovery between intervals *RT (2x/week):* progressive; 3 x 6-12 repetitions at 65-80% 1RM; 4 resistance exercises	KYN ↔ KA, QA ↔ QA/KA ↔	KAT 1-4 ↑	–

Herrstedt et al. (2019)	non-randomized controlled study	n = 43 (38♂; 5♀) gastro-esophageal junction cancer receiving chemotherapy	**12 weeks of combined HIT and RT (2x/week) vs. usual care**			
		n = 18 (15♂; 3♀)	*HIT:* 30-45 min cycling; 4 x 4 min at 85-95% HR_{max}; 3 min recovery between intervals *RT:* following HIT; progressive; 3 x 8-12 repetitions at 50-80% 1RM	TRP ↓ AA ↑ KYN, KA, 3-HK, XA, HAA, QA, 3-HK/KYN ↔	KAT 1-3 ↔	–
		n = 25 (23♂; 2♀)	*Usual care*	TRP ↓ 3-HK, AA, QA, 3-HK/KYN ↑ KYN, KA, XA, HAA ↔	KAT 1-3 ↔	–

study was designed to investigate a different research question. For the purpose of this overview data was extracted from the original study.
Outcome measurements are separated by intervention group. ↑ significant increase ($p \leq .05$); ↓ significant decrease ($p \leq .05$); ↔ no significant change; *PBMCs* peripheral blood mononuclear cells; *TRP* tryptophan; *KYN* kynurenine; *KA* kynurenic acid; *3-HK* 3-hydroxykynurenine; *XA* xanthurenic acid; *HAA* 3-hydroxyanthranilic acid; *QA* quinolinic acid; *KYN/TRP* kynurenine-tryptophan ratio; *KA/KYN* kynurenic acid-kynurenine ratio; *QA/KYN* quinolinic acid-kynurenine ratio; *3-HK/KYN* 3-hydroxykynurenine-kynurenine ratio; *QA/KA* quinolinic acid-kynurenic acid ratio; *KAT* kynurenine-aminotransferase; *IDO* indoleamine 2,3-dioxygenase; *HIT* high intensity training; *RT* resistance training; VO_{2peak} maximal oxygen uptake; *PPO* peak power output; *HR* heart rate; HR_{max} maximal heart rate; *1RM* one-repetition maximum; *MS* Multiple Sclerosis; *RRMS* Relapsing Remitting Multiple Sclerosis.

suppressive effects of IDO-mediated KYN pathway activation on the immune system in humans.

In view of the kinetics of KYN pathway activation mediators IL-6 and cortisol, EE provoked a significant increase from baseline (T_0) levels to post exercise (T_1) followed by a significant decrease from post exercise to the 1h follow-up measurement (T_2) (Figure 3). These results are in agreement with several previous studies (34, 56, 69). In contrast, RE had no effects on IL-6 and reduced cortisol serum concentrations at the 1h follow-up measurement. Overall, EE induced a stronger effect compared with RE on both outcomes not only immediately after exercise but also at 1h follow-up measurement. Thus, EE appears to be the more appropriate stimuli to impact two potential mediators of KYN pathway activation. However, serum levels of TRP, KYN and KYN/TRP ratio do not support the hypothesis of an IL-6 / cortisol mediated activation of the KYN pathway. In regard to TRP, KYN and KYN/TRP ratio, EE did not show any significant changes whereas RE provoked significant changes over time. To our knowledge, this is the first study demonstrating a transient KYN pathway activation in response to acute RE as indicated by an increased KYN/TRP ratio immediately after cessation that is followed by a decrease to baseline level at the 1h follow-up measurement. To date, most studies on exercise-induced KYN pathway activation only investigated the effects of EE. These studies revealed an increased KYN/TRP ratio following exercise, if calculated (10, 63, 64). Interestingly, all these studies used an incremental exercise protocol until exhaustion as intervention. Therefore, the absent increase of the KYN/TRP ratio provoked by EE in the present study could be due to longer exercise duration or rather the time between pre and post exercise measurements. The previously described increases in KYN/TRP ratio after EE may also be caused by the incremental nature of the applied exercise protocols implicating the highest and most exhaustive load immediately before post-measurements. Hence, future studies should examine dose-/time-response relationships of varying EE modalities with consideration of different energy supply systems.

Similar to KYN/TRP ratio, IDO1 expression in PBMCs was unaffected by EE. Since RE resulted in an upregulated KYN/TRP ratio but did not alter IDO1 expression in PBMCs, the underlying mechanisms remain to be elucidated. IDO1 expression may also occur in other cell types or tissues. Furthermore, it is worth mentioning that only one isoform of IDO was investigated in the present study. The physiological roles of IDO2 are by far not as well examined as for IDO1. However, and especially considering the significant increase in KYN/TRP ratio after RE, it cannot be ruled out that elevated IDO2 expression represents an underlying mechanism. Alternatively, the observed increase in KYN/TRP ratio after RE could be driven by the augmented demand of amino acids, as shown by a decrease of serum TRP over time in response to this anabolic exercise stimulus (12, 44).

Kynurenine pathway branch yielding KA

In line with previous studies, KA and KA/KYN ratio increased following EE (41, 50, 60). Here we have shown for the first time, that these increases are only of transient nature, since both outcomes decrease after cessation. In contrast, RE leads to a significant decrease in KA at follow-up. Taken together, EE provokes a more pronounced activation of the KA producing branch of the KYN pathway.

In parallel to the different effects of both exercise interventions on KA and the KA/KYN ratio, KAT4 expression was elevated immediately after exercise only in EE (Figure 4). To our knowledge, it was shown for the first time that KAT4 gene expression is upregulated in PBMCs after an acute bout of EE. To date, increased KAT expression was only shown in longer EE training interventions in muscle tissue in both, humans and rodents (1, 2). It remains to be investigated to what extend acute exercise-induced changes in KA/KYN ratio are driven by PGC-1α transcription coactivator signaling in different tissues (muscle and PBMCs) and how strong these tissues contribute to the conversion from KYN to KA. In greater detail, PGC-1α represents a pan-coactivator of peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcriptional factors and involved in the regulation of different immunomodulatory processes (24). As potential mechanistic insight underlying the observed effects of EE on the KYN pathway yielding KA, the increased KAT4 gene expression in PBMCs may results from a PGC-1α mediated alterations in PPARs. Agudelo et al. emphasized the regulatory role of PPARα and PPARδ together with PGC1-α1 in KAT gene expression in skeletal muscle (1). Indeed, especially PPARγ has been described to be expressed in different immune cells (24). However, a direct link between PGC-1α mediated PPARs signalling and increased KAT gene expression in PBMCs needs to be further investigated.

Kynurenine pathway branch yielding QA

In line with previous studies (41, 60), QA was significantly increased immediately after cessation in EE. In contrast, RE did not show any effects on QA serum concentrations. The different degradation of KYN yielding QA is strongly underlined by the significant interaction effects of the QA/KYN ratio with values in EE being greater than those of RE. So far, studies implicated beneficial effects of exercise for brain health, due to a reduced KYN flux to the CNS (1, 42). These effects have been interpreted by an increased peripheral degradation from KYN to KA, which is not able to cross the BBB. Interestingly, QA which is widely known to have neurotoxic properties, is also not able to pass the BBB. Therefore, an enhanced peripheral clearance of KYN towards QA through exercise might also prevent a pathological accumulation of KYN within the CNS.

Finally, little attention has been focused on another potential reason for commonly observed increases in peripheral QA levels after acute EE (41, 60). QA serves as precursor of de novo NAD^+ synthesis starting with TRP. TRP may not be considered as the most relevant precursor of NAD^+. However, especially EE represents a strong metabolic stimulus, increasing the demand for substrates which are involved in energy supply such as NAD^+. In fact, NAD^+ is known to increase in response to exercise in mammalian cells. The maintenance of consistent or even increased tissue levels of NAD^+ represent a

current research topic in the context of protection against aging and treatment of several diseases (36, 37). Moreover, increased levels of NAD^+ have been associated with enhanced mitochondrial function under metabolic stress (15). Against this backdrop, following exercise trials may also shed light on this important aspect of the KYN pathway. Importantly, exercise-induced increases of peripheral QA concentrations should not be linked to its negative properties within the CNS. Future research should investigate more intermediate metabolites of this branch and should especially focus on the expression of the rate-limiting enzyme KMO, that is potentially sensitive for inflammatory stimuli.

Strengths and Limitations

Strengths of this study comprise the first comparison of two prevalent exercise modalities that were designed as application-orientated sessions on KYN pathway outcomes. Besides commonly assessed pre- and post- exercise measurements, we additionally included a 1h follow-up measurement to gain knowledge on outcome kinetics. Regarding outcome measures, this trial covers a broad range of KYN pathway metabolites, enzymes and mediators as well as their relation to immune cell proportions and numbers. In contrast to previous studies, focusing on KAT expression in muscle, gene expression of IDO1 and KAT4 was assessed in PBMCs for the first time in the context of exercise. Limitations include the constrained transferability of the results to other populations (e. g. females, older or diseased subjects), since a homogenous sample of young, healthy, male adults was investigated. Moreover, future studies may add KMO expression as outcome and include both, gene expression in muscle and PBMCs to determine which tissue/cell type mainly contributes to exercise-induced alterations of the KYN pathway. Finally, different isoforms of IDO and KAT enzyme expression were not measured and remain to be investigated.

Conclusion

In this article, the reviewed literature indicated only restricted evidence for chronic effects of exercise interventions on KYN pathway outcomes. However, long-term intervention trials strongly differ in view of study populations and applied exercise modalities. In contrast, single-bouts of EE consistently provoke acute alterations in KYN pathway outcomes. Here, we directly compared two exercise modalities and revealed that acute resistance and endurance exercise induce different effects on KYN pathway outcomes. Elevated levels after endurance exercise of both KA and QA may lead to neuroprotection by preventing a pathological KYN accumulation within the CNS. Furthermore, the increased level of QA raises the question of an involvement in the compensation of enhanced NAD+ demand as a consequence of augmented energy supply during and/or immediately after endurance exercise. Moreover, the revealed associations between immune cell subsets and KYN pathway outcomes emphasize the hypothesis that repeated acute exercise-induced alterations of the KYN pathway provoke long-term adaptions of the immune system. Especially in view of clinical settings with inflammation-mediated diseases, future studies investigating the interplay between acute and chronic exercise stimuli, the KYN pathway and modulations of the immune system might be of major relevance.

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Supplements

Supplement 1.

Table 1. Mass spectrometric properties of all target analytes: Most intense precursor/product ion pairs were used for quantification (and qualification/identification).

Substance	Precursor ion [m/z]	Product ion [m/z]	Cone voltage [V]	Collision energy [eV]
QA	168	78 (150)	22	20
QA-d3	171	81 (153)	22	20
KA	190	144 (116)	24	18
KA-d5	195	149 (121)	24	18
TRP	205	146 (188)	24	18
KYN	209	94 (146)	20	12
TRP-d5	210	150 (192)	24	18

Table 2. Assay characteristics.

Parameter		TRP	KYN	QA	KA
Calibration curve	Concentration levels	0.5, 2, 5, 20, (50) µg/mL	20, 50, 200, 500, 2000 ng/mL	5, 20, 50, 200, 500, 2000 ng/mL	0.5, 2, 5, 20, 50, 200 ng/mL
	Linear equation	y=1.4756x-0.4224	y=0.0007x+0.0069	y=0.0008x+0.0014	y=0.0086x+0.0072
	R^2	0.9993	0.9999	0.9999	0.9997
Specificity		√	√	√	√
Recovery [%]		70	76	67	73
Imprecision	low:	400 ng/mL: 8%	40 ng/mL: 10%	400 ng/mL: 9%	20 ng/mL: 11%
	medium:	1 µg/mL: 14%	100 ng/mL: 12%	1 µg/mL: 15%	50 ng/mL: 13%
	high:	2.5 µg/mL: 2%	250 ng/mL: 2%	2.5 µg/mL: 2%	125 ng/mL: 3%
LLOQ (S/N > 9:1) [ng/mL]		50	25	10	10

Supplement 2.

Table 1. Documentation of exercise intervention modalities.

	Overall mean exercise intensity (n=24)
Resistance Exercise	
Chest press (% of 1RM ± SD)	68.8 ± 1.7
Lat pull (% of 1RM ± SD)	67.5 ± 5.0
Leg curl (% of 1RM ± SD)	65.3 ± 5.0
Leg extension (% of 1RM ± SD)	70.5 ± 6.0
Back extension (% of 1RM ± SD)	67.5 ± 7.0
Endurance exercise	
Heart rate (% of HR_{max} ± SD)	88.2 ± 5.3
Power output (% of $Watt_{max}$ ± SD)	57.0 ± 5.6

1RM One-repetition maximum (kg); *SD* Standard deviation; HR_{max} Maximal heart rate (bpm); $Watt_{max}$ Peak power output (W)

Elevating body temperature to reduce chronic low-grade inflammation: a welcome strategy for those unable to exercise?

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Abstract

Chronic low-grade inflammation is increasingly recognised in the aetiology of a range of chronic diseases, including type 2 diabetes mellitus and cardiovascular disease, and may therefore serve as a promising target in their prevention or treatment. An acute inflammatory response can be induced by exercise; this is characterised by the acute increase in pro-inflammatory markers that subsequently stimulate the production of anti-inflammatory proteins. This may help explain the reduction in basal concentrations of pro-inflammatory markers following chronic exercise training. For sedentary populations, such as people with a disability, wheelchair users, or the elderly, the prevalence of chronic low-grade inflammation-related disease is further increased above that of individuals with a greater capacity to be physically active. Performing regular exercise with its proposed anti-inflammatory potential may not be feasible for these individuals due to a low physical capacity or other barriers to exercise. Therefore, alternatives to exercise that induce a transient acute inflammatory response may benefit their health. Manipulating body temperature may be such an alternative. Indeed, exercising in the heat results in a larger acute increase in inflammatory markers such as interleukin-6 and heat shock protein 72 when compared with exercising in thermoneutral conditions. Moreover, similar to exercise, passive elevation of body temperature can induce acute increases and chronic reductions in inflammatory markers and positively affect markers of glycaemic control. Here we discuss the potential benefits and mechanisms of active (i.e., exercise) and passive heating methods (e.g., hot water immersion, sauna therapy) to reduce chronic low-grade inflammation and improve metabolic health, with a focus on people who are restricted from being physically active.

Keywords: hyperthermia, passive heating, cytokines, heat shock protein, glucose metabolism

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Chronic low-grade inflammation and chronic disease

Introduction

Type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) are associated with small, yet sustained elevations in circulating levels of pro-inflammatory proteins, a state called chronic low-grade inflammation (11). Causative links between inflammation and the aetiology of those diseases have been suggested (34). For example, in T2DM, chronically elevated plasma concentrations of pro-inflammatory cytokines can impair insulin sensitivity by affecting insulin signalling, potentially through inhibition of C-Jun N-Terminal Kinase (JNK) (72). Indeed, it has been shown that attenuation of JNK activity is associated with protection from insulin resistance in mice (63). For CVD, vascular integrity may be impaired by pro-inflammatory cytokines, facilitating the infiltration of macrophages through the vascular wall to form atherosclerotic plaques (9). Gene knockout studies in mice provide experimental evidence that the actions of tumour necrosis factor α (TNF-α) lead to an increase of plaque formation (19). This aligns with observational epidemiological data that show a positive association between pro-inflammatory markers and future CVD events (147).

These examples indicate that targeting inflammatory pathways might help to prevent and treat chronic inflammatory diseases. The last two decades have seen a host of studies investigating the anti-inflammatory effects of lifestyle interventions, such as exercise (54). Inflammation is multi-faceted in nature. Here we introduce two categories of inflammatory markers that are relatively well studied in the context of exercise, temperature and chronic low-grade inflammation: cytokines and heat shock protein. Referring to literature focused on exercise in addition to passive heating, we then discuss how temperature can affect chronic inflammation, providing rationale for the potential benefits of elevated temperature in chronic disease prevention.

Cytokines

It is recognised that a surplus of body fat is an independent risk factor for T2DM and CVD (151). Confirming the interactions between obesity and inflammation, it has been demonstrated that basal circulating interleukin (IL)-6 concentration in obese individuals is elevated, and that weight loss can reduce the concentration of this risk marker (32, 96). A mechanistic explanation lies in visceral adipose tissue and the residing macrophages within that are a major source of circu-

lating IL-6 at rest (11). Further mechanistic evidence is derived from the associations between obesity, increased intestinal permeability and inflammation (125). Both a high fat diet and increases in body mass increase intestinal bacterial species' DNA in adipose tissue, which correlates with TNF-α mRNA expression in adipose tissue (2). The importance of inflammatory pathways in the aetiology of T2DM is further underlined by data demonstrating that inhibition of pro-inflammatory pathways by knocking out the nuclear factor kappa B (NF-κB) (5) and JNK (65) pathways disrupts the link between obesity and insulin resistance in obese mice. In addition, pro-inflammatory cytokines can prevent tyrosine phosphorylation of IRS-1 upon activation of the insulin receptor and instead promote phosphorylation of IRS-1 on its serine residues, which leads to impaired insulin signalling (72). This might explain why blocking the actions of pro-inflammatory cytokines using anti-TNF-α treatments can improve insulin sensitivity (73).

The cytokine interleukin (IL)-6 has been extensively studied in the context of chronic low-grade inflammation; however, to date, its causative role remains heavily debated (34, 121). Observational studies report a positive association between the chronic elevation of plasma IL-6 concentration and insulin resistance, atherosclerosis, T2DM and CVD (11, 34, 38, 120, 144), linking it with chronic disease. It is important, however, to appreciate the diverse actions of IL-6. For instance, infusion of IL-6 for 2 to 4 h does not alter (133) or even enhances insulin sensitivity (25). There are possible explanations for this discrepancy. Whereas transient increases in plasma IL-6 concentrations can acutely enhance insulin action via AMP-activated protein kinase activation and glucose transporter-4 (GLUT4) translocation (25), chronic increases may impair insulin signalling by inhibiting the phosphorylation of tyrosine residues on IRS-1 (34). In the context of T2DM, it is noteworthy that IL-6 production is upregulated in response to increased concentrations of TNF-α (135). Because the detrimental role of TNF-α in insulin sensitivity and vascular function is relatively well-established (34, 135), it has been suggested that IL-6 may serve as a bystander rather than having a direct impact on health (43). On the other hand, pharmacological blocking of the IL-6 receptor can alleviate symptoms of a range of inflammatory diseases, such as rheumatoid and juvenile idiopathic arthritis (76). However, this also brings about increased concentrations of cholesterol and worsening of insulin sensitivity (117). The exact function of IL-6 in the circulation may also depend on the receptors it binds to following appearance in the circulation. Several cell types (e.g., leukocytes, hepatocytes) express surface IL-6 receptors for *classic* IL-6 signalling, while IL-6 can also bind to soluble IL-6 receptors present in the circulation, after which the resulting complex can bind to cells that do not express the IL-6 receptor (e.g., skeletal muscle cells) in a process called trans-signalling. It is the latter signalling pathway that is suggested to be mainly associated with inflammation (53). Taken together, although the exact underlying mechanisms need further research, chronically elevated plasma IL-6 concentrations are associated with poor metabolic health (11, 34).

Monocytes contribute to the inflammatory profile as they are producers of pro-inflammatory cytokines by signalling through their surface receptors, toll-like receptor (TLR)2, TLR4 and cluster of differentiation (CD) 14 (1, 7). By binding to the TLR-CD14 complex, pathogen-associated molecular patterns can trigger the production of cytokines such as IL-6 and TNF-α through the activation of the NF-κB pathway (7). As a result, the expression of TLRs on the surface of monocytes has been suggested to be a marker for chronic-low grade inflammation (89), particularly TLR2 and TLR4 (47). Indeed, monocytes from individuals with T2DM express more TLR2 and TLR4 when compared with healthy controls (35).

In addition to the surface expression of TLRs, the inflammatory nature of monocytes can be characterised by their expression of CD14 and CD16. Using these two surface markers, monocytes can be divided into three subsets: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$) (154). Interestingly, TLR4 expression is lowest in the classical subsets, as shown in patients with acute myocardial infarction (78) or stable angina pectoris (116). This might explain why a large proportion of intermediate and non-classical monocytes are associated with CVD, T2DM and other chronic diseases (103, 153), suggesting that the distribution of monocyte subsets can be used as a marker for chronic low-grade inflammation. Indeed, in response to an *in-vitro* stimulant (i.e., lipopolysaccharide (LPS)) non-classical and intermediate monocytes produce more of the pro-inflammatory cytokines TNF-α and IL-1β than the classical subset (17, 110).

Heat shock protein

The presence of heat shock protein (HSP) has been confirmed in every eukaryotic cell type and HSP subtypes have been defined based on their molecular mass, ranging from the HSP10 to the HSP110 family. Notably, contrary to what the name might suggest, HSPs are not exclusively induced by heat but are responsive to a range of stressors including hyperthermia, oxidative stress and glycogen depletion (112). With regards to chronic low-grade inflammation, exercise and temperature, the HSP70 family, with its inducible subtype Hsp72, is most widely studied and is the HSP of focus in this review. Note that we use the nomenclature adopted by the Cell Stress Society International, in which HSP refers to the protein family, Hsp refers to the specific protein, and *hsp* refers to the gene and mRNA expression.

Intracellular Hsp72 (iHsp72) functions as a chaperone for protein folding and aids in the maintenance of homeostasis within cells (112). Indeed, the survival rate in mice subjected to heat shock is higher when iHsp72 expression is elevated by a prior non-lethal heat shock compared to control mice (84). When in homeostasis, Hsp72 is bound to heat shock factor-1 (HSF-1) in the cytosol, rendering this complex inactive. In response to physiological stress or inflammation, these molecules are uncoupled, allowing HSF-1 to translocate to the nucleus and activate heat shock elements on the heat shock protein gene. As a result, the transcribed *hsp72* mRNA then leads to an increased Hsp72 protein expression in the cytosol (82). Expression of Hsp72 and its association with metabolic health has been assessed in a variety of cell types, with levels in

leukocytes, adipose tissue and skeletal muscle tissue having gained most attention in the context of chronic low-grade inflammation and metabolic health (63). It is suggested that iHsp72 exerts its anti-inflammatory actions by blocking the activity of the JNK and NF-κB pathways, reducing the production of pro-inflammatory cytokines and enhancing insulin sensitivity (30, 63). Indeed, it has been shown in cell culture experiments that JNK activation is reduced in cells overexpressing Hsp72 (48), and that a high iHsp72 expression prevents the activation of NF-κB and subsequent TNF-α gene transcription (106).

A growing body of evidence supports the importance of HSP in the aetiology of T2DM and CVD (63, 71). In humans, iHsp72 expression in skeletal muscle and adipose tissue is lower in those with T2DM and non-alcoholic fatty liver disease when compared with healthy controls (20, 24, 62). In murine models, Hsp72 knock-out mice develop insulin resistance and obesity (63), and mice in which iHsp72 is overexpressed are protected against the deleterious effects of high fat overfeeding on insulin sensitivity (61). Protection from insulin resistance in mice appears to be associated with the attenuation of JNK. Interestingly, in this particular study JNK attenuation was achieved by heat therapy (63). Moreover, pharmacologically restoring Hsp72 expression induces an 85% increase in glucose clearance rate during intravenous glucose infusion in Hsp72-deficient monkeys (80).

Whereas animal studies have provided compelling evidence of the influence of iHsp72 in skeletal muscle on metabolic health (30, 80), the protective effect of an elevated expression of iHsp72 in immune cells is less clear. Compared with other leukocyte subsets, iHsp72 in monocytes is most responsive to stress and iHsp72 expression shows a dose-response relationship with incubation temperature in isolated cell suspensions (8). Monocytes produce a range of pro-inflammatory cytokines when activated including TNF-α and IL-1β (36); iHsp72 expression in this cell type may therefore directly affect the inflammatory profile of an individual. In addition, and similar to skeletal muscle, monocytes are insulin-sensitive. Their behaviour may therefore serve as surrogate measure for peripheral insulin sensitivity (128). Simar et al. (129), Singh et al. (130) and Njemini et al. (111) found a reduction in resting iHsp72 expression in monocytes as a result of ageing, a process associated with the development of chronic low-grade inflammation (15). Furthermore, increased basal expression of iHsp72 in monocyte-derived macrophages reduces the production of TNF-α and IL-1β in response to *in-vitro* LPS stimulation (36). This finding indicates an anti-inflammatory function of iHsp72 in this cell type.

Hsp72 is also released into the circulation, where its function differs from Hsp72 present within the cell. The tissues that excrete Hsp72 are not fully identified, but there is evidence that the liver, the brain and leukocytes release Hsp72 into the circulation through passive - as well as active - mechanisms (75). In a study using exercise as a stressor, Febbraio et al. (42) showed that skeletal muscle does not contribute to circulating eHsp72 concentrations. In contrast to the anti-inflammatory actions of iHsp72, extracellular Hsp72 (eHsp72) can activate monocytes through the TLR4/CD14 complex, inducing the production of pro-inflammatory cytokines (6). Indeed, elevated basal levels of eHsp72 are linked to impaired insulin sensitivity (28, 35, 85) and the development of atherosclerosis in individuals with hypertension (119). In further support of its potential role in chronic low-grade inflammation, resting eHsp72 concentrations strongly correlate with resting serum TNF-α and CRP concentrations in elderly individuals (111). Thus, by stimulating the production of pro-inflammatory cytokines in circulating immune cells, eHsp72 may exacerbate chronic low-grade inflammation and exert a negative effect on health. As the extracellular, pro-inflammatory, function of Hsp72 appears to differ from the cytoprotective function when present in the cell, it has been suggested that the ratio between extra- and intracellular Hsp72 expression could be a determinant for insulin resistance and T2DM risk (86).

Despite the cross-sectional data suggesting a negative role of eHsp72 on several aspects of health, evidence for its potential to induce pro-inflammatory cytokine release in monocytes and other leukocytes is equivocal (75). It has been suggested that the activation of monocytes following *in-vitro* incubation with eHsp72 can be the consequence of contamination with endotoxins, as opposed to the effect of eHsp72 itself (14, 49). For example, incubating monocyte-derived dendritic cells with endotoxin-free Hsp70 does not induce an acute inflammatory response (14). Moreover, pre-incubation of eHsp72 with polymyxin-B to block the actions of the contaminant LPS abolishes the production of pro-inflammatory cytokines in macrophages (49). Therefore, future *in-vitro* research on the mechanistic actions of eHsp72 should carefully control for possible contamination by endotoxins.

The anti-inflammatory effects of exercise – and the role of temperature

The following evidence derived from the exercise literature helps to understand and partly informs the inflammatory response to hyperthermia. It is heavily summarised; for a broader view on exercise and inflammation, the reader is directed to previous excellent reviews, for example by Gleeson et al. (54) and Petersen and Pedersen (118).

1. **Acute exercise**
   If of sufficient intensity and duration, a bout of exercise induces an acute inflammatory and subsequent anti-inflammatory response, which is thought to be partly responsible for the protective effects of regular exercise (54, 118). IL-6 responds most dramatically to acute exercise and has been suggested to be a main driver of the anti-inflammatory effects of exercise, because the acute post-exercise peak of IL-6 is followed by elevated anti-inflammatory cytokine concentrations (118). Indeed, infusion of recombinant human IL-6 in healthy humans at rest shows that IL-6 independently triggers the production of anti-inflammatory cytokines such as IL-1ra or IL-10, and it increases plasma concentrations of cortisol, a hormone with anti-inflammatory properties (132). Furthermore, acute exercise can increase iHsp72 (108,

112) and eHsp72 concentrations (148), while suppressing TNF-α and IL-1 production (117). The distribution of monocyte subsets within the peripheral circulation is also affected by acute exercise. Most studies report an acute increase in intermediate and non-classical monocytes directly following the cessation of exercise (33, 69, 136), but increases in classical monocytes following exercise have also been reported (94, 103). Potentially reflecting the change in circulating monocyte subsets, reduced monocyte TLR expression in the two-hour recovery period following exercise has been reported (89), which may help explain the mechanisms behind the altered inflammatory profile following acute exercise.

The inflammatory response to exercise is affected by both exercise duration and intensity (117). Importantly, in the context of this review, the increase in body temperature contributes to this relationship. Exercise in the heat results in a greater inflammatory response when compared with exercise in thermoneutral or cold conditions (44, 51, 88, 122, 131). Moreover, clamping core temperature (T_{core}) by cycling in cold water can abolish the acute IL-6 response (122). The amplified acute cytokine response following exercise in the heat may be partly mediated by the increased plasma catecholamine concentrations (122) and carbohydrate utilisation when compared with exercise in thermoneutral or cold conditions (45).

2. **Regular exercise**

Regular exercise is protective against the development of T2DM and CVD (95, 126). Cross-sectional and longitudinal evidence suggests that regular physical activity can reduce chronic low-grade inflammation, as indicated by lower basal circulating concentrations of the inflammatory risk factors IL-6, eHsp72, and numbers of intermediate and non-classical monocytes (16, 54, 59, 152). Possible candidates to explain improvements in the inflammatory profile after exercise training are reductions in visceral adipose tissue (producing pro-inflammatory cytokines at rest) (40), reduced TLR expression on immune cells (47) and changes in the number and phenotype of circulating cells and immune cells residing in tissue (139).

3. **Heat acclimation studies**

Heat acclimation studies provide some insight on the inflammatory effect of exercise training in the heat (reviewed by Amorim et al. (4)). Basal iHsp72 expression has been particularly studied as a mediator for the enhanced heat tolerance after heat acclimation (87): Whereas three days of heat acclimation do not increase basal iHsp72 expression in peripheral blood mononuclear cells (99), ten days of heat acclimation seem sufficient to increase basal iHsp72 expression in peripheral blood mononuclear cells (3, 105). Furthermore, the link between iHsp72 and markers of metabolic health, such as insulin sensitivity (63), suggests that heat acclimation-type exercise training may have wider-reaching effects than on exercise performance alone.

Who benefits from passive heating interventions?

Over the past decade there has been an alarming increase in number of people suffering from T2DM and CVD in the general population (150). The prevalence of these diseases is even higher in those with obesity (12), the elderly (31), or individuals with a physical disability, such as spinal cord injury (SCI) (13). Given the anti-inflammatory benefits of exercise outlined briefly above, it is unsurprising that a common trait of these populations is a reduced physical capacity. Despite this, there is still promise for exercise interventions. Even low-intensity exercise interventions such as regular walking can induce improvements in inflammatory markers in at-risk populations (142). For populations restricted to upper-body exercise modalities (e.g., wheelchair users) it is worth noting that this can induce an acute inflammatory response despite the smaller active muscle mass when compared with lower body exercise (67). Indeed, comparable inflammatory responses have been reported between upper and lower body exercise matched for relative intensity (94). Further, cross-sectional evidence (107), as well as some (10, 124) - but not all (140) - longitudinal upper-body exercise interventions indicate that upper body exercise can reduce inflammatory risk markers in SCI. Metabolic markers such as fasting glucose and insulin in people with SCI are also positively affected by physical activity (23).

Because reductions in physical *capacity* per se do not preclude the inflammatory effects of exercise, it is conceivable that the below-average physical *activity* levels in these at-risk populations (100, 137) contribute to their elevated disease risk. Indeed, environmental, social, and physical barriers to exercise have been identified (97, 143), which may go some way to explain the increased risk for chronic disease. Furthermore, for some populations, exercise of adequate intensity and duration may not be feasible or tolerable. These populations include those with acute injuries (e.g., musculoskeletal injuries, patients recovering from surgery), movement restrictions (e.g., due to obesity, spasticity), secondary complications to chronic disease (e.g., diabetic foot for T2DM, pressure sores for conditions leading to immobility), or cognitive impairments (e.g., dementia). An alternative or addition to exercise may hence represent a welcome strategy for these individuals. Because the acute inflammatory response to exercise is partly mediated by the rise in body temperature (88, 149), it is conceivable that passive heating strategies have the potential to improve the inflammatory profile. Similar to exercise, these strategies have the benefit of being low-cost, non-pharmacological interventions, reducing the financial strain on health-care providers.

The acute inflammatory response to passive heating

There are several ways to increase body temperature passively in humans, of which sauna bathing and hot water immersion (HWI) are the most commonly used. These methods are associated with a range of positive health outcomes, such as weight loss (70), improved sleep quality (37) and vascular

function (21, 27). Nevertheless, the potential of passive heating to reduce chronic low-grade inflammation and improve metabolic health has received relatively scarce attention.

Cytokines

It is suggested that contracting muscle is responsible for the increased circulating concentrations of IL-6 following exercise (134). Animal studies show that the IL-6 production in skeletal muscle increases following passive heat stress as well (146). Welc et al. (145) demonstrated that the upregulation of IL-6 production may be the consequence of HSF-1 activation. Another suggested mechanism for IL-6 release from the muscle in response to hyperthermia is through increased calcium influx after activation of the thermosensitive transient receptor potential 1 (113). Although these studies have provided rationale to study passively elevating body temperature in the context of chronic low-grade inflammation, it should be noted that in animal studies T_{core} is increased to a much larger extent than considered safe in human participants (50, 127, 146). This could make passive heating interventions less potent inducers of an acute inflammatory response in humans. For example, Gupte et al. (57) kept the T_{core} of mice between 41.0° and 41.5°, while in human studies the maximal attained T_{core} during HWI remained between 38° and 39° (41, 66, 114). Furthermore, due to the difference in size between species - and concomitant higher inertia in T_{core} during passive heating in larger species - T_{core} of humans takes considerably longer than that of small animals to increase to a given threshold. This is another important difference between human and animal research to date, in addition to the often reported higher T_{core} investigated in animals.

An overview of studies investigating the acute response of inflammatory markers following passive heating is provided in Table 1. Despite smaller increases in T_{core} during passive heating in human compared with animal studies, 1−2 h HWI induces an acute circulating IL-6 response in humans (41, 66, 88, 93). Consistent with exercise studies (46), this acute IL-6 response appears to be dose-dependent. Laing et al. (88) reported a ~12 fold increase in IL-6 immediately following 2 h HWI in water set at 38.5° while 1 h HWI only results in a ~2−3 fold increase in plasma IL-6 concentration (41, 66, 93).

Heat shock protein

The activation of HSF-1 by passive heating not only results in increased IL-6, but also increased iHsp72 production (146). Raising the temperature of human blood ex-vivo indeed results in an acute increase in iHsp72 expression (68), which is consistent with findings in animal passive heating studies (57, 79). In Vervet monkeys, maintaining T_{core} between 39° and 41° for 30 min using HWI results in a significant increase in *hsp72* expression in skeletal muscle. In addition, maintaining T_{core} of rats around 41.0−41.5° for 20 min using a thermal blanket leads to a ~three-fold increase in iHsp72 protein

Table 1. The acute effect of passive heating on inflammatory markers in humans.

Reference	Design	Population	Main outcomes
Brunt et al., 2018 (22)	1 h HWI up to the shoulder in water set at 40.5°C	Healthy inactive men (N=6) and women (N=4) and sex-matched controls (N=10)	Serum concentration eHsp72 ↔, IL-6 ↑; peripheral blood mononuclear cell iHsp72 ↑
Faulkner et al., 2017 (41)	1 h HWI up to the waist in water set at 40°C	Healthy men (N=14)	Plasma concentration eHsp72 ↑, IL-6 ↑
Hafen et al., 2018 (58)	2 h heating of skeletal muscle using pulsed wave diathermy	Healthy sedentary men (N=10) and women (N=10)	Skeletal muscle iHsp72 ↔
Hashisaki et al., 2018 (60)	1 h in water-perfused suit to achieve 1°C rise in T_{core}	Individuals with spinal cord injury (N=19) and able-bodied controls (N=8)	Serum concentration IL-6 ↑, TNF-α ↔
Hoekstra et al., 2018 (66)	1 h HWI up to the neck in water set at 39°C	Healthy overweight sedentary men (BMI = 31±4 kg/m^2; N=10)	Plasma concentration eHsp72 ↔, IL-6 ↑; monocyte iHsp72 ↔
Iguchi et al., 2012 (74)	30 min in room set at 73°C	Healthy men (N=13) and women (N=12)	Plasma concentration eHsp72 ↑
Laing et al., 2008 (88)	2 h HWI in water set at 38.5°C	Healthy men (N=13)	Serum concentration IL-6 ↑
Leicht et al., 2015 (93)	1 h HWI up to the neck in water set 2°C higher than resting T_{core}	Men with spinal cord injury (N=7) and able-bodied controls (N=8)	Plasma concentration IL-6 ↑, IL-1ra ↑, TNF-α ↔
Morton et al., 2007 (109)	1 h HWI of one leg in water set at 45°C	Healthy men (N=7)	Skeletal muscle iHsp72 ↔
Oehler et al., 2001 (114)	2 h HWI up to the neck in water set at 39.5°C	Healthy men (N=6) and women (N=6)	Monocyte iHsp72 ↑
Whitham et al., 2007 (149)	2 h HWI in water set at 38.5°C; control at 35°C	Healthy men (N=11)	Plasma concentration eHsp72 ↔
Zychowska et al., 2017 (156)	30 min sauna bathing at 98°C	Healthy men (N=18)	leukocytes: *hsp72* mRNA expression ↔, IL-6 ↔, IL-10 ↔

Abbreviations: eHsp72, extracellular heat shock protein 72; HWI, hot water immersion; iHsp72, intracellular heat shock protein 72; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; mRNA, messenger ribonucleic acid

expression in skeletal muscle when compared with control (57). Of note, the acutely increased iHsp72 expression in these two studies was also associated with improved insulin sensitivity.

Despite promising evidence from animal and isolated tissue studies, the iHsp72 response in human whole-body models shows mixed results. Some of the variation may be confounded by the tissue analysed, with relatively few studies investigating skeletal muscle iHsp72 following passive heat stress. This lack of studies may be related to the invasive nature of skeletal muscle sampling. Harvesting monocytes by venepuncture is relatively easy in comparison, and some of the human evidence on passive heating is therefore based on monocyte iHsp72 (66, 114), due to monocyte responsiveness to heat stress (8). Although the acute iHsp72 response to exercise in total leukocytes is similar to the response in skeletal muscle (141), comparing results from studies investigating different cell/tissue types must be done with due caution.

To date, only four studies have investigated the acute iHsp72 response to HWI in humans. These studies found no increase in skeletal muscle (109) or monocytes (66) after 1 h, but an increase in PBMC iHsp72 after 1h (22), and an increase in monocyte iHsp72 expression after 2 h HWI (114). Time is a likely determinant of the iHsp72 response. This is supported by Gibson et al. (52), who demonstrated that T_{core} needs to be maintained above 38.5° for at least 27 min to induce the upregulation of *hsp72* expression following exercise. The extent to which T_{core} is elevated is a likely additional explanatory factor. T_{core} increased by approximately 1.7−2.0ºC in the study showing increases in monocyte iHsp72 expression (22, 114), whereas the increase was ~1.5ºC in the studies showing no change in this parameter (66, 109).

There are limited data about the potential of HWI to induce an acute increase in eHsp72 concentration in humans. Faulkner et al. (41) reported a similar increase in eHsp72 concentration following HWI when compared with exercise matched for heat production. The elevation of muscle temperature was the strongest predictor for the eHsp72 response, explaining 27% of its variance (41). Passive heating by 30 min of sauna bathing, resulting in a 0.8° T_{core} increase, also leads to the elevation of eHsp72 concentrations (74). In contrast, Brunt et al. (22), Hoekstra et al. (66) and Whitham et al. (149) found no significant acute change in eHsp72 following HWI in water set at 38.5−40.5°. Therefore, partly due to the lack of a control condition in some studies and the different designs across studies, the potential of passive heating to elevate eHsp72 concentrations remains equivocal.

Chronic adaptations to passive heating interventions

Acute studies have confirmed the potential of HWI to induce an inflammatory response (41, 66, 88, 93, 114), which has led to the suggestion that chronic HWI treatment may help to reduce chronic low-grade inflammation and improve metabolic health (71, 86, 104, 138). Although there are currently limited human data to support this notion, animal studies provide some insight into the efficacy of chronic passive heat therapy and the few human studies available show promise.

Most animal studies investigating the effect of chronic passive heat therapy on metabolic health and chronic low-grade inflammation have focussed on basal iHsp72 expression and its impact on insulin sensitivity (30, 56, 79, 127). In mice, heat therapy for 16 weeks increased basal iHsp72 expression in skeletal muscle concurrently with improved insulin sensitivity when compared with a sham control condition (30). To further support the importance of iHsp72 for insulin sensitivity, increasing iHsp72 expression by pharmacological means or genetic manipulation resulted in similar improvements in insulin sensitivity (30). A simultaneous increase in basal iHsp72 expression and improvement in insulin sensitivity was also reported in the studies by Gupte et al. (56) and Silverstein et al. (127). Mechanistically, the link between both adaptations following passive heating appears to involve the inhibition of JNK and NF-κB activation (63). Indeed, Chung et al. (30) and Gupte et al. (56) reported reduced activation of these pathways following passive heat therapy. Moreover, in humans, low iHsp72 expression is associated with impaired insulin sensitivity, but also elevated JNK activity (30) (Figure 1).

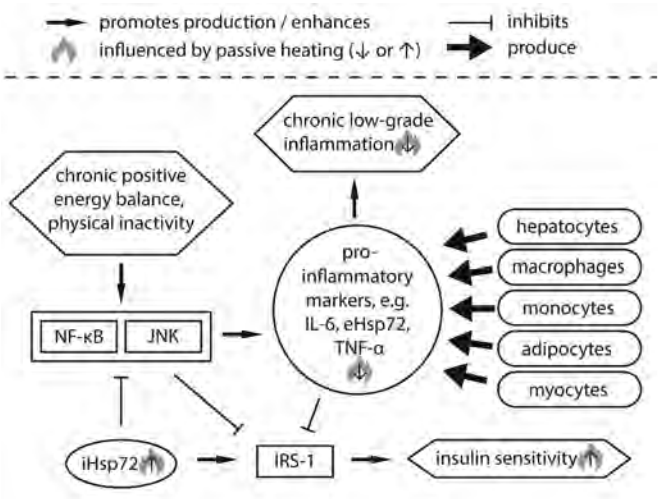

Figure 1 - Chronic impact of passive heating on markers related to inflammation and glycaemic control: overview of evidence to date. A chronic positive energy balance, eventually resulting in obesity, leads to the activation of NF-κB and JNK pathways. This results in an enhanced production of pro-inflammatory proteins and inhibits insulin receptor substrates, attenuating insulin sensitivity. Passive heating has the potential to counteract some of these negative adaptations, reducing inflammation and enhancing insulin sensitivity. eHsp72, extracellular heat shock protein 72; iHsp72, intracellular heat shock protein 72; IL-6, interleukin-6; IRS-1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; TNF-α, tumour necrosis factor α.

In a series of studies, Kavanagh et al. (28, 79, 80) showed that the increase in iHsp72 can also lead to beneficial adaptations in non-human primates, a species genetically closer to humans. In these animals, two HWI sessions per week for five weeks increased basal iHsp72 expression in skeletal muscle, which was strongly associated with fasting blood glucose and the insulin responses during an intravenous glucose tolerance test (79). In keeping with the data derived from mice (30),

pharmacological induction of iHsp72 expression also increased iHsp72 expression and improved insulin sensitivity in non-human primates (80). Together, animal studies have highlighted the potential for chronic passive heating interventions to improve the inflammatory profile and metabolic health, possibly through the elevation of basal iHsp72 expression.

Epidemiological studies indicate that sauna bathing can reduce systemic inflammation (90), as well as all-cause mortality (91). Epidemiological data further show that habitual hot-spa bathing is linked to a lower incidence of hypertension and CVD (98). These findings are supported by controlled passive heating interventions investigating resting inflammatory and metabolic markers in humans, as summarised in Table 2. Hooper (70) showed that three weeks of HWI reduces fasting glucose and glycated haemoglobin concentration in people with T2DM. A reduction in fasting glucose concentration was also observed following two weeks of sauna therapy in patients with congestive heart failure (18), or two weeks of HWI in overweight (but otherwise healthy) individuals (66). Moreover, a two-week HWI intervention reduces plasma IL-6 concentration at rest in people with chronic heart failure (115), and four weeks of sauna therapy reduces IL-6 mRNA expression in leukocytes (156). In contrast, no changes in resting plasma concentration of IL-6 were found in healthy individuals following two- (66) or eight-week (22) HWI interventions, or nine days of passive heat acclimation (77).

The lack of improvements in inflammatory markers following repeated passive heating reported in some of these studies may be linked to the populations that were under investigation, which do not always present with an increased risk for metabolic ill-health *prior* to the chronic intervention (66). For other investigations, the reason could also lie in the relatively modest body temperature increase that was induced in each session, or in the fact that the effect of the acclimation period was assessed on the day after the last passive heating session (77), possibly resulting in the assessment of acute instead of chronic effects. Despite these caveats, the existing evidence suggests that, providing

Table 2. The effect of chronic passive heating interventions on inflammatory and metabolic markers in humans.

Reference	Design	Population	Main outcomes
Brunt et al., 2018 (22)	8 weeks HWI with 36 sessions of 60 min in water set at 40.5°C	Healthy inactive men (N=6) and women (N=4) and sex-matched controls (N=10)	Serum concentration eHsp72 ↔, IL-6 ↔; peripheral blood mononuclear cell iHsp72 ↑
Biro et al., 2003 (18)	2 weeks of daily sauna bathing for 15 min at 60°C + blanket for 30 min	Healthy obese (BMI>30 kg/m^2; N=10)	Fasting glucose concentration ↓
Ely et al., 2019 (39)	8–10 weeks HWI with 30 sessions of 60 min in water set to maintain 38.5–39.0°C of core temperature	Obese women with polycystic ovary syndrome (BMI = 41±1 kg/m^2; N=9 intervention, N=9 resting control)	Fasting glucose concentration ↓, oral glucose tolerance test AUC ↓, adipose tissue insulin signalling ↑
Hafen et al., 2018 (58)	6 days of 2 h heating of skeletal muscle using pulsed wave diathermy	Healthy sedentary men (N=10) and women (N=10)	Skeletal muscle iHsp72 ↑
Hesketh et al., 2019 (64)	6 weeks of passive heating 40–50 min at room air of 40°C	Healthy sedentary (N=10 intervention, N=10 exercising control)	Oral glucose tolerance test AUC ↓
Hoekstra et al., 2018 (66)	2 weeks HWI with 10 sessions of 45–60 min in water set at 39°C	Healthy overweight sedentary men (BMI = 31±4 kg/m^2; N=10 intervention, N=8 resting control)	Fasting glucose ↓, plasma concentration eHsp72 ↓, IL-6 ↔; monocyte iHsp72 ↔,
Hooper, 1999 (70)	3 weeks HWI with 18 sessions of 30 min in water set between 37 and 41°C	Patients with Type 2 Diabetes Mellitus (N=8)	Fasting glucose ↓, glycosylated haemoglobin concentration ↓
Kanikowska et al., 2012 (77)	9 sessions of 10 min HWI in water set at 42°C + blanket for 90 min in 40°C room	Healthy men (N=6)	Plasma concentration IL-6 ↔, TNF-α ↔
Kihara et al., 2002 (83)	2 weeks of daily sauna bathing for 10 min at 60°C + blanket for 30 min	Patients with chronic heart failure (N=20)	TNF-α ↔
Masuda et al., 2004 (102)	2 weeks of daily sauna bathing for 10 min at 60°C + blanket for 30 min	Patients with at least one coronary risk factor (N=14 intervention, N=14 resting control)	Fasting glucose ↔
Oyama et al., 2013 (115)	2 weeks HWI with 14 sessions of 10 min in water set at 40°C	Patients with chronic heart failure (N=16 intervention, N=16 resting control)	Plasma concentration IL-6 ↓, CRP ↓, TNF-α ↓
Zychowska et al., 2018 (155)	4 weeks of sauna bathing, 12 sessions, for 30 min at 98°C	Healthy men (N=22)	Leukocyte: *hsp72* mRNA expression ↓, IL-6 ↓*, IL-10 ↑

Abbreviations: AUC, area under the curve; BMI, body mass index; CRP, C-reactive protein; eHsp72, extracellular heat shock protein 72; HWI, hot water immersion; iHsp72, intracellular heat shock protein 72; IL, interleukin; mRNA, messenger ribonucleic acid; TNF-α, tumour necrosis factor-α. *trend for a decreased resting IL-6 mRNA expression.

sufficient thermal load, improvements in markers for glucose metabolism and chronic low-grade inflammation can be achieved in as little as two weeks. Because animal studies that have induced elevations in basal iHsp72 expression have used longer duration protocols (30, 127), this leaves the question of whether the observed improvements in glucose metabolism reported in humans are orchestrated by the actions of iHsp72. Indeed, no changes in iHsp72 in the presence of fasting glucose reductions have been found following two weeks of HWI in humans (66). Furthermore, as human studies often report more moderate increases in T_{core} than animal studies, it is not uncommon that acutely, iHsp72 remains unaffected (Table 1). It is therefore debatable whether acute increases in iHsp72 are required for a passive heating intervention to be beneficial in context of inflammation and glycaemic control. Other markers of inflammation (e.g., IL-6) do show acute perturbations in the absence of changes in iHsp72, and chronic interventions can improve glycaemic control using protocols of a duration and moderate heat stress too short or not sufficiently intense to acutely increase iHsp72 expression (Table 2).

Future research

The presented evidence supports a potential therapeutic role of passive heating interventions to reduce chronic low-grade inflammation that may particularly benefit clinical populations. However, such interventions bring their own challenges. For example, some populations exhibit impairments of thermoregulatory capacity, including those with T2DM (26) and those with SCI (55). Notwithstanding this, even in populations with normal thermoregulatory control, sweating is largely ineffective for regulating body temperature during HWI interventions due to the inability to decrease body temperature through evaporative heat loss. These clinical populations may therefore not be at a disadvantage *during* HWI. However, impaired thermoregulation affects the return of body temperature to normal *following* HWI (93), which may increase the risk in any emergency situations where a quick return of body temperature is warranted. Furthermore, heat interventions during which skin is exposed to air may induce accelerated elevations of body temperature in these at-risk populations (55). Heat therapy may also be associated with a higher risk for adverse events in the elderly and people with hypertension, T2DM, cardiovascular disease, or allergies (86). Indeed, heart disease is the main natural cause of death during sauna bathing, heat being a contributing factor in half of cases, and the main cause in a quarter of the deaths investigated in one particular study (81). It is further yet to be determined whether the acute elevations in postprandial glucose concentrations following HWI (92) occur in people with T2DM, and whether this might hence influence the feasibility of HWI interventions in this population. Therefore, protocols that have not been adapted and developed for at-risk populations must be carefully evaluated in order to avoid heat illness related events.

Developing protocols that are tolerable and, ideally, enjoyable, should form an important part of future investigations. For example, it could be questioned whether a 120-min HWI session inducing a rise in T_{core} of 2.0ºC as studied by Oehler et al. (114) is realistic to implement in a practical setting, as even a 60-min HWI session inducing a rise in T_{core} of 1.6ºC can be perceived as uncomfortable (66) and physiologically straining (123). Therefore, protocols that induce minimal heat stress stimuli associated with improvements in health markers need to be identified. This may hence improve subjective perceptions of passive heating interventions. Similar to the approach taken in the development of exercise guidelines in a clinical population (SCI) (101), a focus might be put on finding minimal, rather than optimal, heat-doses to induce health benefits. Subjective perceptions may also be improved by targeting specific parts of the body rather than taking a whole-body approach; for example, local heating might reduce whole body heat strain. Such an approach can still result in noteworthy metabolic changes, as shown for targeted heating of one leg, resulting in increased glucose uptake when compared with the contralateral control leg (29). Alternatively, localised cooling might make whole-body heating protocols more tolerable. Finally, different populations (e.g., male/female, young/old, healthy/clinical) may present different characteristics regarding thermoregulation, heat perception and inflammatory profiles. Therefore, specific populations need investigating in detail, because the majority of evidence in controlled human laboratory studies is derived from young, healthy males.

Conclusions

Chronic low-grade inflammation is increasingly recognised in the aetiology of chronic diseases, such as T2DM and CVD. Although exercise can effectively reduce chronic low-grade inflammation, it may not be a feasible intervention to adhere to regularly for populations with reduced physical capacity and/or barriers to exercise. Because the increase in body temperature partly mediates the exercise-induced acute inflammatory response, passive heating strategies may have potential as an alternative or addition to exercise to reduce chronic low-grade inflammation. Indeed, the passive elevation of body temperature acutely influences a range of inflammatory markers that are affected by exercise, which is supported by human, animal and cell culture studies. A small but growing number of chronic passive heating interventions in humans have further explored its effect on inflammatory and metabolic markers. Whereas the literature on improvements of glycaemic control after repeated passive heating in humans is relatively convincing, consistent evidence for improvements of the inflammatory profile has so far been limited to animal studies. This limitation may be related to the reduced thermal load and the relatively short-duration chronic interventions that were investigated in humans. The development of effective and tolerable passive heating protocols to improve the inflammatory profile, alongside glycaemic control, using longer-term chronic interventions in humans should therefore be the aim of further investigations.

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Benefits of exercise and immunotherapy in a murine model of human non–small-cell lung carcinoma

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ABSTRACT

Background: *Lung cancer has the highest incidence and mortality rate in the world. One of the most promising new cancer therapies in recent years is immunotherapy, which is based on the blockade of immune checkpoints such as programmed cell death protein 1 (PD-1). Exercise training is beneficial to maintain and improve the quality of life of cancer patients, and it might also modulate the anti-tumoral efficiency of some chemotherapeutic agents. However, the potential of exercise combined with immunotherapy as a cancer therapy remains to be elucidated. Here, we examined the effects of exercise on tumor growth and its possible adjuvant effects when combined with anti–PD-1 immunotherapy (nivolumab) in a patient-derived xenograft (PDX) model of non–small-cell lung carcinoma (NSCLC).*

Methods: *We generated a PDX model using NOD-SCID gamma mice with subcutaneous grafts from tumor tissue of a patient with NSCLC. Animals were randomly assigned to one of four groups: non-exercise + isotype control (n=5), exercise + isotype control (n=5), non-exercise + nivolumab (n=6) or exercise + nivolumab (n=6). The animals undertook an 8-week moderate-intensity training regimen (treadmill aerobic exercise and strength training). Immunotherapy (nivolumab) or an isotype control was administered 2 days/week, for 6 weeks. Several tumor growth and microenvironment parameters were measured after the intervention.*

Results: *Improvements in aerobic capacity and muscle strength (p=0.027 and p=0.005) were noted in exercised animals. Exercise alone reduced the tumor growth rate with respect to non-exercised mice (p=0.050). The double intervention (exercise + nivolumab) increased tumor necrosis and reduced apoptosis with respect to controls (p=0.026; p=0.030). All interventions achieved a reduction in proliferation compared with the control group (p=0.015, p=0.011, and p=0.011). Exercise alone increased myeloid tumor infiltrates (mostly neutrophils) with respect to the nivolumab only group (p=0.018). Finally, Vegf-a expression was higher in the nivolumab groups (in combination or not with exercise) than in exercise + isotype control group (p=0.045 and p=0.047, respectively). No other significant effects were found.*

Conclusions: *Our results would suggest that aerobic and strength training should be studied as an adjuvant to cancer immunotherapy treatment.*

Keywords: exercise, training, lung cancer, patient-derived xenografts, cancer immunotherapy, immune checkpoints.

INTRODUCTION

Lung cancer is the most common cancer worldwide, and accounts for more than 2.1 million new cases each year and about 1.8 million deaths (10). Non–small-cell lung carcinoma (NSCLC) is a major sub-type of lung cancer with a particularly poor 5-year relative survival rate of 10–13% (20), which is partly attributable to inefficient methods for early detection and lack of curative treatments for advanced disease (10). While surgical resection and chemotherapy remain the cornerstone of treatment in NSCLC (9), checkpoint inhibitor-based immunotherapy is quickly emerging as a possible new treatment modality with the potential to revolutionize cancer care for patients (25).

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On the other hand, the study of the relationship between cancer and exercise continues to evolve. A healthy lifestyle that includes regular exercise and physical activity is epidemiologically associated with a lower risk of cancer – including lung tumors – and cancer mortality (54). Exercise also improves the general physical condition of patients undergoing anti-neoplastic treatment or surgery, and helps mitigate treatment side effects and other complications, and for these reasons people living with and beyond cancer should be advised to be as physically active as possible (56). Regarding lung cancer specifically, growing evidence supports the safety and efficacy of exercise interventions (4). For instance, preoperative exercise considerably reduces the postoperative complication rate (61), with a recent meta-analysis of randomized controlled trials in patients with NSCLC showing improvements in walking endurance, peak exercise capacity, dyspnoea, risk of hospitalization, and postoperative pulmonary complications (53). However, the molecular underpinnings of the potential exercise benefits on lung cancer outcomes remain to be elucidated (4). Some mechanisms have been postulated, such as p53-induced apoptosis in a murine model of lung adenocarcinoma (26), or reduced proliferation and survival of NSCLC cells (41). In addition, the potential anti-tumorigenic effects of exercise on lung cancer –and in fact in most tumors –appear to be related to immunomodulation, as explained below.

The best studied mechanisms are those associated with moderate-intensity aerobic exercise, which involve the improvement of immune function (48). Specifically, innate immunity can be regulated by exercise through reprogramming the tumor microenvironment (39), and by polarizing the relative abundance of different myeloid cell types towards a more anti-tumorigenic phenotype (1). The evidence is particularly strong for natural killer (NK) lymphocyte mobilization and redistribution to the tumor, pointing to a direct anti-tumor effect of exercise, although changes have also been reported in adaptive immunity, with exercise enhancing the mobilization, redistribution and activation of T lymphocytes (principally, cytotoxic $CD8^+$ T cells) (40), increasing their infiltration into the tumor stroma (39, 49). With regard to lung cancer, Pedersen et al. (49) found that wheel running in mice inoculated with Lewis lung cancer cells significantly reduced tumor volume, with an upregulation of the proinflammatory cytokine interleukin (IL)1α, as well as of inducible nitric oxide synthase [iNOS] and markers for NK and T-lymphocyte activity. In patients with NSCLC, tai chi training may promote proliferation of peripheral blood mononuclear cells – with an improvement in their cytotoxicity – and increase circulating NK cell percentage, natural killer T, and dendritic CD11 cells (43), and could also preserve a stable interferon (IFN)γ-producing $CD3^+$ T lymphocytes (T1) to IL4-producing $CD3^+$ T lymphocytes (T2) ratio (70).

Several preclinical models have been developed to study the effects of exercise in cancer (54), with some studies suggesting a greater therapeutic benefit of certain drugs (*e.g.*, low-dose doxorubicin (62)) when used in combination with exercise. The use of the aforementioned models is crucially important for testing the biological plausibility, establishing the therapeutic window and the effective "dose" (intensity, volume and duration of the exercise), and identifying possible predictors of exercise response (33). However, the potential anti-tumor effects of exercise in combination with checkpoint inhibitor-based immunotherapy remain to be elucidated using preclinical cancer models. Although this treatment approach has resulted in unprecedented improvements in survival for patients with lung cancer, not all patients benefit equally and many issues remain unresolved, including the mechanisms of action and the possible effector function of immune cells from non-lymphoid lineages. In this regard, the clinical implementation of antibodies targeting the immune checkpoint inhibitor programmed cell death protein 1 (PD-1), for example nivolumab, can relaunch anti-tumor responses by stimulating the action of infiltrating cytotoxic T lymphocytes in the tumor microenvironment (59, 65). Yet, recent preliminary evidence from our group using a murine patient-derived xenograft (PDX) model of squamous NSCLC devoid of host lymphoid cells provided mechanistic support for an additional immunotherapy mechanism mediated by myeloid cells – specifically neutrophils –, which act as PD-1 inhibitor effector cells responsible for tumor regression by necrotic extension (44). Thus, it would be of interest to use the aforementioned model to investigate whether exercise acts as an adjuvant to anti–PD-1 immunotherapy on malignant tumor cells through mechanisms beyond those related to T lymphocyte involvement.

The aim of the present study was therefore to determine the effect of a physical exercise intervention (specifically, an 8-week combined protocol including aerobic and strength training) during immunotherapy treatment with the anti–PD-1 monoclonal antibody nivolumab in a PDX murine model of squamous cell lung carcinoma – a histologic type of NSCLC. Our main hypothesis was that the exercise intervention would induce significant beneficial effects in terms of physical capacity in PDX mice, and also that the combined effect of physical exercise with nivolumab therapy would enhance the anti-tumor response, with exercise acting as a potential adjuvant treatment.

MATERIALS AND METHODS

Ethical approval for the collection and the use of patient tumor tissue was granted by the Ethics Committee of the *Hospital Universitario Puerta de Hierro* (Madrid, Spain; approval number: PI/144-14). The study was conducted following the Declaration of Helsinki guidelines for the treatment of data and tumor tissue samples from donor patients, collected from May 2015 to October 2018. Eligibility criteria for tissue collection included: patient´s informed consent, a new diagnosis of a primary lung tumor (specifically a NSCLC), not having received previous therapy until surgery and, provision of a sufficient quantity of tumor volume to donate a section for research purposes.

All animal experimental protocols were also approved by the Institutional Ethics Committee of the *Hospital Universitario Puerta de Hierro* (approval number: PROEX 163/14) and were conducted in accordance with European (European Convention ETS 123) and Spanish (32/2007 and R.D. 1201/2005) laws on animal protection in scientific research.

Donor patient

The donor patient was a 79-year-old male who underwent a lobectomy for a nodule in the upper lobe of the right lung.

According to World Health Organization criteria for histological classification and staging (11, 66), the patient´s tumor was a basaloid infiltrating and poorly-differentiated squamous cell lung carcinoma, in clinical stage IIA (pT2a N1 L1 M0).

Animals

Female 8-week-old NOD-SCID gamma mice (NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) purchased from Charles River Breeding Laboratories (Chatillon-sur-Chalaronne, France) were used for the establishment of a human squamous NSCLC PDX model. Mice were housed in the animal facility of *Hospital Universitario Puerta de Hierro* on a 12-hour light/12-hour dark cycle under controlled conditions of temperature (22 ± 1 ˚C) and humidity (45 ± 10%). Mice were accommodated in pairs in ventilated racks under specific pathogen-free conditions, with environmental enrichment *(nestlets)*, and *ad libitum* access to food and water. Mice were handled in laminar airflow cabinets during all the experimental protocols.

Study design

Establishment of a human squamous NSCLC PDX model

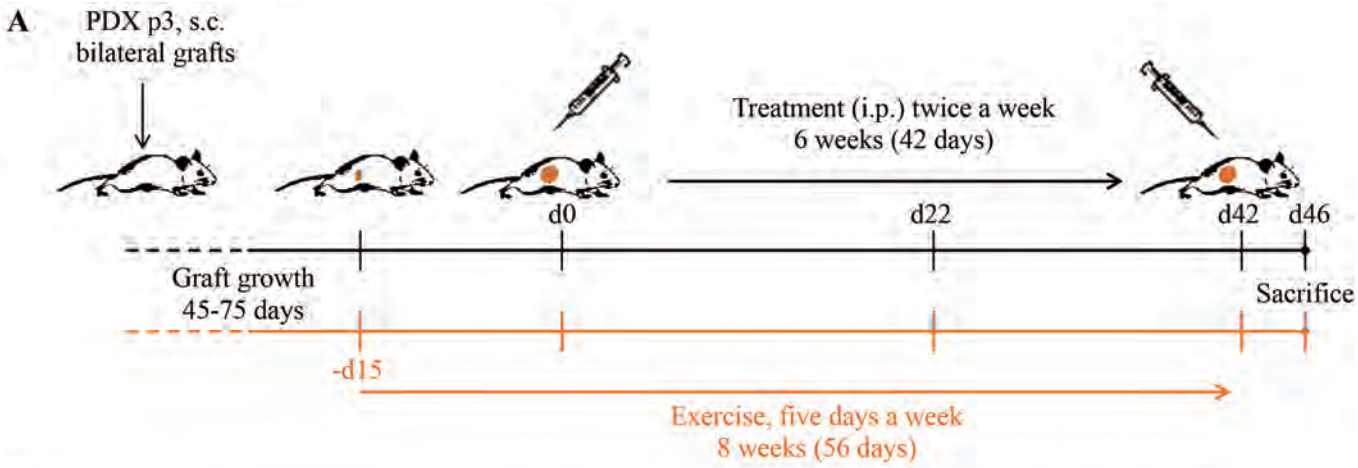
The tumor sample was surgically obtained from the donor patient and sliced into fragments (~3 mm × 2 mm) under sterile conditions. Two specimen pieces were implanted subcutaneously into the bilateral flanks of mice (bilateral grafts) under inhalation anesthesia (sevoflurane in oxygen, Sevorane). This first transplant directly performed from the donor patient to the host mouse was referred to as passage 0 or p0. Whenever palpable, tumor volume was measured with a caliper (AA846R, Aesculap AG, Tuttlingen, Germany) twice weekly using the following formula: $(4\pi/3) \times (w/2)^2 \times (l/2)$, where w = width and l = length. When the volume reached ~1 cm^3, a piece of the tumor was harvested and regrafted into new host mice, in order to maintain the PDX line *in vivo* during subsequent passages; these serial transplants were called p1, p2, and p3, etc. Another portion of the harvested tumor was used for phenotype and molecular analyses to verify that the xenograft model was stable without histopathological changes along passages. When the PDX p2 tumors reached the appropriate size (~100 mm^3), they were regrafted subcutaneously in the bilateral flanks of 22 female mice (p3) (Figure 1A). Finally, transplanted p3 mice were monitored and their bilateral tumor volumes were measured as described above. When the tumors reached ~100 mm^3, the mice were included in the intervention.

Pharmacological and exercise intervention

The pharmacological intervention for the NSCLC PDX model was based on our previous study, which describes an immunotherapy mechanism based on neutrophils (44).

Transplanted p3 mice were paired-matched based on their aerobic capacity (see below), and each pair was randomly assigned to the following experimental groups: non-exercise + isotype control (n=5); exercise + isotype control (n=5); non-exercise + nivolumab (n=6); exercise + nivolumab (n=6). The study design and the experimental groups are shown in Figure 1.

Regarding the pharmacological intervention, isotype control or nivolumab was administrated intraperitoneally (i.p.) twice weekly for six consecutive weeks, beginning at day 0



B

Group	Type	Treatment	Dosage	Duration
Non-exercise + Isotype control	Monotherapy	IgG4κ	150 µg	6 weeks
Exercise + Isotype control	Concomitant	Training	Moderate	8 weeks
		IgG4κ	150 µg	6 weeks
Non-exercise + Nivolumab	Monotherapy	Nivolumab (anti-PD-1)	150 µg	6 weeks
Exercise + Nivolumab	Concomitant	Training	Moderate	8 weeks
		Nivolumab (anti-PD-1)	150 µg	6 weeks

Figure 1. Study design. (A) Schematic representation of the study protocol. Tumor sensitivity to exercise and immunotherapy in a murine PDX model of squamous non–small-cell lung carcinoma (NSCLC). (B) Table showing treatment regimens. Abbreviation: PDX, patient-derived xenograft.

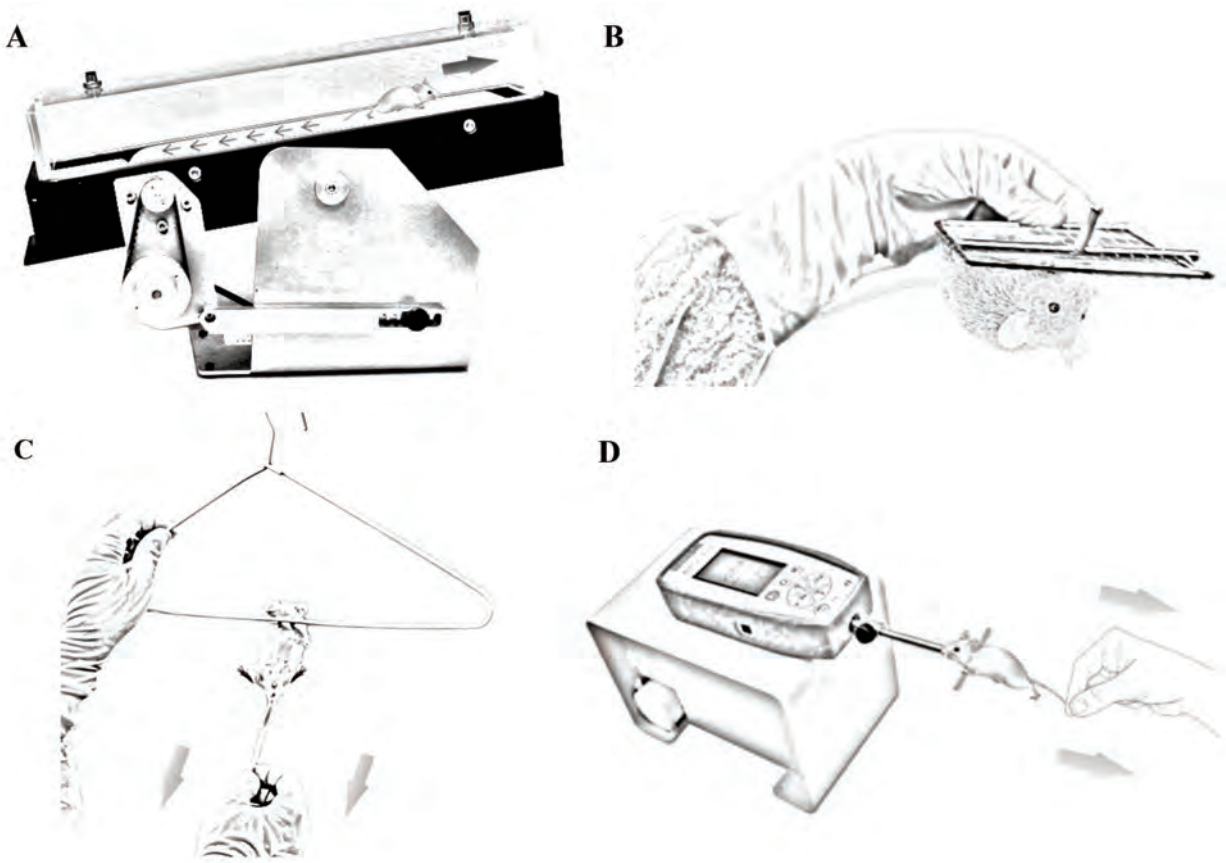


Figure 2. Diagrams of physical exercise equipment. Experimental apparatuses used in the exercise study: (A) Treadmill. (B) Grid. (C) Hanger. (D) Grip strength meter.

(d0) until the end of the experiment (d42). The treatment regimens and doses used in this study are shown in Figure 1B.

The exercise intervention was based on our previous experience (17, 18) and was adapted to the features of the PDX model. The exercise program combined both aerobic and resistance training during five days per week (Monday–Friday; session duration: 40–60 minutes) for a total of eight weeks (from day minus 15 [d-15] to d42). The *aerobic training* regimen included five weekly sessions (session duration: 30–40 minutes) during which the exercise duration, treadmill speed and inclination were gradually increased (Figure 2A). Accordingly, mice started with very low workloads (d-15, 20 minutes at 40% of the maximal velocity obtained during the aerobic performance test that is described below and 0% gradient) and ended with 40 minutes at 80% of maximal velocity and 15% gradient in the last sessions (d42). Electrical stimulation was applied (0.2 mA, 1 Hz) during the first aerobic sessions, but later only gentle tail touching was used as a stimulus to provoke the mouse to move. The *strength training* was performed after the aerobic training, twice weekly (Tuesday and Friday), and included two exercises as recently described (18): horizontal screen exercise (Tuesday; Figure 2B) and hanging exercise with two limbs (Friday; Figure 2C). For the first exercise, the mouse was placed on top of an inverted screen (BIO-GRIPGS model, Bioseb; Chaville, France) and had to climb back over to the top; when this was achieved, the mouse was placed again in the initial position. The number of repetitions was gradually increased (from two to six with a duration of one minute each) with a constant 1-minute rest period between repetitions. For the second exercise, mice were picked up by the tail and placed on a metal cloth hanger taped to a shelf and maintained at 40 cm above a layer of bedding to cushion any fall. Mice were allowed to grasp the wire only using the two forepaws for as long as they could during one set of two-to-six repetitions, with a 2-minute rest period between repetitions. Non-exercise groups could freely move in their cages, but did not perform the exercise intervention.

Sample collection

Peripheral blood samples from the caudal vein were drawn into EDTA tubes at the end of the intervention (d46, 48 hours after the last exercise tests) to study immune cell populations by flow cytometry. Mice were sacrificed after the last blood collection (d46) by cervical dislocation. The bilateral tumors (n=44) were removed and weighed, and their volumes were measured as described earlier. Then, each tumor was divided into several portions for the following analyses: *i)* one piece was fixed in formalin and paraffin-embedded prior to histopathological and immunohistochemical studies; *ii)* a second piece was cryopreserved in OCT (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan) to determine proliferation and apoptosis by immunofluorescence; and, *iii)* a third piece was homogenized in Hank's Balanced Salt Solution (HBSS, Gibco Life Technologies, Gergy-Pontoise, France) to examine for intratumoral immune cell infiltrates using flow cytometry and real-time quantitative polymerase chain reaction (qPCR). To this end, tumors were mechanically homogenized and then digested by gentle agitation in RPMI medium

(Gibco Life Technologies) with D-collagenase (1 mg/ml; Roche Diagnostics, Mannheim, Germany) in a 3:10 proportion for 24 hours at room temperature. When the enzymatic digestion was completed, the suspension was passed through a 40 μm pore size nylon mesh (BD Biosciences, Franklin Lakes, NJ) and centrifuged for 15 minutes at 1500 rpm. After washing twice with HBSS, a part of the resuspended pellet was directly used for leukocyte population identification by flow cytometry and another part was preserved at -80 ºC to analyze immune system-related gene expression.

Outcomes

Aerobic capacity

All mice had two familiarization sessions on a treadmill (Harvard Apparatus; Panlab, Barcelona, Spain; Figure 2A) before the aerobic capacity tests were performed at the beginning and the end of the study. The familiarization sessions consisted of a gradual increase in running duration (5–10 minutes), treadmill speed (0–8 cm/s), and inclination (0–15 degrees), starting with no electrical stimulation and ending with 0.2 mA (1 Hz, 200 ms). Once mice were weighed, they performed a 10-minute warm-up period at 5–10 cm/s (treadmill inclination: 15 degrees) followed by the incremental treadmill test, which started at 5 cm/s with successive speed increments of 2 cm/s every 2 minutes until exhaustion (constant treadmill inclination at 15 degrees), which was defined as the mouse spending more than five continuous seconds on the electric grid and unable to continue running at the next speed. The maximal aerobic capacity achieved was expressed as the total distance run by the mice during each incremental aerobic test considering the weight of the animal [total distance (meters)/ weight (grams)] (adapted from (30, 42)). We also registered maximal velocity achieved in order to determine training intensities for the exercised groups (exercise + isotype control; exercise + nivolumab). To assess aerobic adaptations, we compared the maximal aerobic capacity before and after the training period.

Forelimb grip strength

The maximum forelimb grip strength was measured – as the maximum force (grams) exerted by the mouse before losing grip – using an isometric force transducer (Harvard Apparatus; Panlab, Barcelona, Spain; Figure 2D) on the days following the two aerobic capacity tests. Each mouse took the test three times with a 5-minute rest period between them, and the best reading was recorded as the maximal grip strength in grams. To measure strength adaptations, we compared the maximal grip strength before and after the training period. All animals were weighed at the start and the end of the test.

Tumor volume and tumor growth rate

Tumor volume was measured twice weekly throughout the trial using a caliper, as described above. The response to treatment therapy and/or exercise intervention was quantified in terms of tumor growth rate expressed as a percentage of the total tumor growth with respect to the volume at the beginning of the intervention. The formula (TVdx / TVd0) × 100 was applied, where TVdx refers to the tumor volume measured on a specific day and TVd0 as the tumor volume at the beginning of the pharmacological intervention (set at 100%).

Necrotic index

The percentage of necrotic areas as a measure of tumor regression in response to therapies (pharmacological treatment and/or exercise) was quantitatively analyzed using CaseViewer software (3DHISTECH Ltd., Budapest, Hungary). The average percentage of necrotic areas was evaluated in four paraffin-embedded sections (5 μm, 4 tumors per study group) stained with hematoxylin and eosin. The area for each section was calculated according to the formula: % necrosis area = (Σ necrosis area / total tumoral mass area) × 100.

Cell proliferation

The expression of Ki67 as a proliferation marker (19) was evaluated in 4-μm sections of OCT-embedded tumor tissue by immunofluorescence using a specific proliferative cell antibody (anti-Ki67 human antibody, clone SP6, reference MAD-000310QD, ready to use; Master Diagnostica, Granada, Spain). Antigen unmasking was performed by immersing the slides into a specific buffer (EnVisionTM FLEX Target Retrieval Solution, Low pH, 50×; Agilent Technologies Inc., Santa Clara, CA) during 20 minutes at 97ºC. Tumor samples were then fixed in 100% methanol (-20ºC) for 10 minutes at room temperature followed by incubation with 5% bovine serum albumin for 60 minutes to block the nonspecific protein binding. Samples were stained with undiluted anti-Ki67 mouse antibody, overnight at 4ºC. After three 5-minute washes with phosphate buffered saline (PBS), samples were incubated with the secondary anti-rabbit IgG Alexa 488 antibody diluted 1:200 (Invitrogen Life Technologies, Waltham, MA). After three additional 5-minute washes with PBS, samples were stained with TO-PRO-3 (reference T3605; Invitrogen Life Technologies) diluted 1:500 in PBS for 20 minutes, followed by washing and assembling the samples in PBS/glycerol. Finally, the cell proliferation index was determined by confocal microscopy (20×, numeral aperture of 0.4; TCS-SP5-AOBS-UV; Leica-Microsystems) quantifying the positive nuclear staining (green colour; Arg laser, 500–540 nm) in 8 areas of each slide (~100 cells). The contrast with TO-PRO-3 was displayed in blue colour (He/Ne laser, 633 nm, 645–750 nm). The percentage of Ki67 positive cells (% Ki67) was calculated against the total number of nuclei per field and the average value of the eight images per sample was also calculated.

Apoptosis

End-stage apoptotic cells were investigated in 4-μm sections of OCT-embedded tumor tissue using terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL assay) based on DNA fragment detection (DeadEnd™ Fluorometric TUNEL System; Promega, Madison, WI). To eliminate possible autofluorescence, slides were washed with PBS and incubated with NH_4Cl before the TUNEL assay. Subsequently, samples were incubated with TO-PRO-3 (Invitrogen Life Technologies) diluted 1:1000 in PBS for 20 minutes at room temperature. After three 5-minutes washes with PBS, samples were incubated with the secondary anti-rabbit IgG Alexa 488 antibody diluted 1:200 (Invitrogen Life Technologies), washed and assembled in PBS/glycerol. The fluorescent dye-conjugated dUTP-labeled DNA (displayed in green) and TO-PRO-3 (in blue) were visu-

Table 1. Antibodies per tube and per tumor homogenate sample used for flow cytometry analysis

	FITC	PE	PerCP-Cy7	PE-Cy7	APC	APC-Cy7	PB	AC
peripheral blood Tube 1	hCD33	mCD45.1	hCD4	hCD56	hCD19	hCD8	hCD3	hCD45
Tumor Tube 1	hCD4	mCD45.1	7AAD	hCD56	hCD19	hCD8	hCD3	hCD45
Tumor Tube 2		mLy6G	7AAD	mNK1.1	mCD11B	mCD3		mCD45

Abbreviations: FITC (Fluorescein isothiocyanate); PE (Phycoerythrin); PerCP-Cy7 (Peridinin chlorophyll protein); PE-Cy7 (Phycoerythrin cyanin-7); APC (Allophycocyanin); APC-Cy7 (Allophycocyanin cyanin-7); PB (Pacific blue); AC (AmCyan).

Table 2. List of antibodies used for flow cytometry analysis

Protein	Clone	Brand
hCD33	HIM3-4	BD Pharmigen
hCD4	SK3	BD Pharmigen
hCD56	NCAM16,2	BD Pharmigen
hCD19	HIB19	BD Pharmigen
hCD8	SK1	BioLegend
hCD3	UCHT1	BD Horizon
hCD45	2D1	BD Pharmigen
7AAD	-	BioLegend
mCD45.1	A20	BioLegend
mLy6G	1A8	BioLegend
mNK1.1	PK136	BioLegend
mCD11B	M1/70	BioLegend
mCD3	145-2C11	BioLegend
mCD45	30-F11	BioLegend

alized using a confocal laser scanning microscope (100×, TCS-SP5-AOBS-UV) and images were captured (5 images per preparation; 1024 × 1024 pixels) with Leica Confocal Software (both from Leica-Microsystems). The apoptotic index was determined using the following formula: (number of TUNEL-positive staining cells) / (total number of nuclei) × 100.

Identification of leukocyte populations in tumor stroma (ex vivo)

As described earlier, fresh tumor tissue was homogenized and digested in RPMI media with D-collagenase (1 mg/ml). This was then filtered and prepared for immune cell analysis by flow cytometry (FACSCanto II and FACSDiva software v6.1.2; BD Biosciences) using the specific human and mouse antibodies shown in Table 1 and Table 2.

Identification of gene expression associated with the tumor microenvironment

Total RNA extracted from digested tumor tissues using the RNeasy Plus Mini Kit (Qiagen Inc., Hilden, Germany) was reverse-transcribed into cDNA using SuperScript VILO MasterMix (Invitrogen Life Technologies) in a Mastercycler EP thermocycler (Eppendorf, Hamburg, Germany). All reactions were performed using the same protocol consisting of an incubation step at 25ºC for 10 minutes, followed by one cycle at 42°C for 120 minutes, another cycle at 85°C for 5 minutes, and a cool down cycle at 4°C.

qPCR was carried out using the TaqMan Gene expression and the TaqMan Gex Master Mix (both from Applied Biosystems, Foster City, CA). Each sample was analyzed independently in duplicate for every determination with the 7500 Fast Real-Time PCR System using 7500 software v2.0.6 (Applied Biosystems). Oligonucleotide primers and probes for the amplification reactions were purchased from Applied Biosystems and are shown in Table 3. The expression of the following human and murine genes was studied, all related to immune system and tumor microenvironment: *a)* Human genes: cytotoxic T lymphocyte antigen 4 *(CTLA-4), PD-1,* programmed death ligand 1 *(PD-L1), IL4* and *IFNγ*; and, *b)* Murine genes: arginase-1 *(Arg1)*, vascular endothelial growth factor a *(Vegf-a),* inducible nitric oxide synthase *(iNos), Pd-1* and *Pd-l1* (Table 3). Relative mRNA abundance was normal-

Table 3. List of primers used in real-time quantitative PCR analysis

Species	Gene	Primers
Human	*CTLA-4*	Hs03044418_m1
	PD-1	Hs01550088_m1
	PD-L1	Hs00204257_m1
	IL-4	Hs00174122_m1
	IFNγ	Hs00989291_m1
	GADPH	Hs02758991_g1
Mouse	*Arg1*	Mm00475988_m1
	Pd-l1	Mm00452054_m1
	iNos	Mm00440502_m1
	Vegf-a	Mm01281449_m1
	Pd-1	Mm01285676_m1
	Gadph	Mm99999915_g1

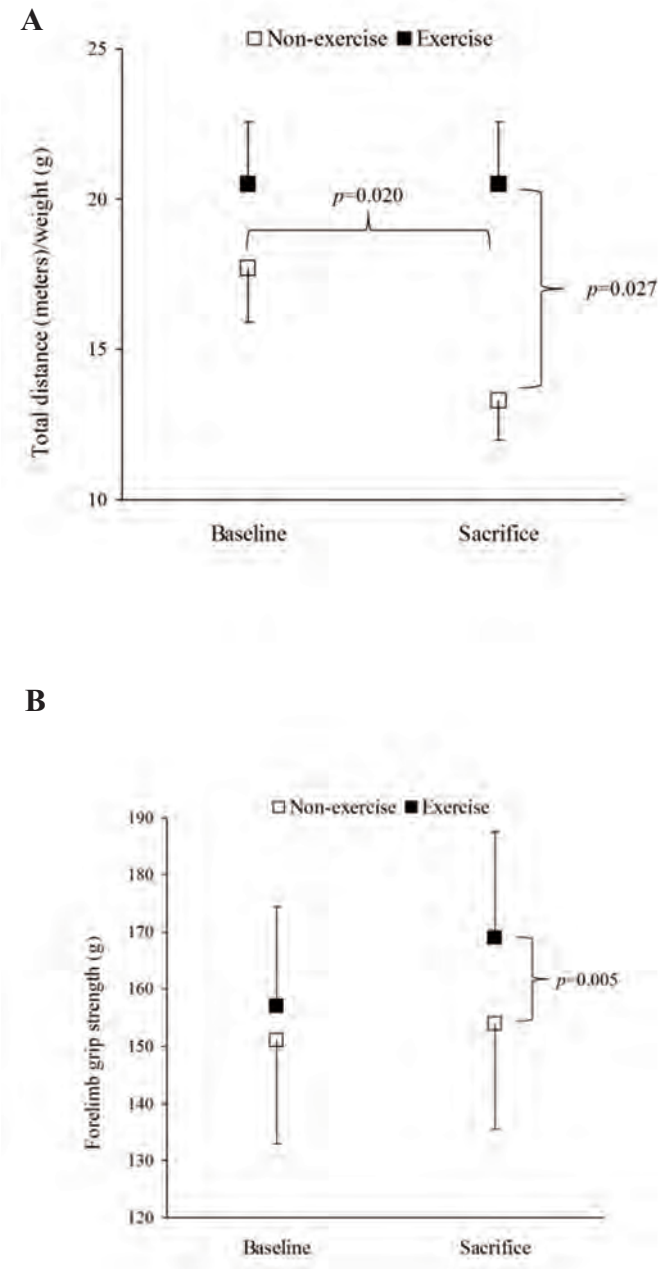


Figure 3. Effect of physical exercise on aerobic capacity. Results consider together the sedentary (non-exercise, white square) and the trained (exercise, black square) groups, before and after the eight weeks of training (to sacrifice). (A) Aerobic performance (distance covered [meters] normalized to weight [g]) before and at the end of the experiment in the control and exercised groups. (B) Muscle strength (forelimb grip strength [g]) before and at the end of the experiment in the control and exercised groups. Values represent mean ± SEM. Data were analyzed using non-parametric tests for within and between-group comparisons (Wilcoxon signed-rank test and Mann-Whitney *U* test, respectively) and significant p-values are shown.

ized to the internal standard, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for human and murine genes, using the relative quantification method. Each sample was analyzed as an independent duplicate for each set of primers and probes. Changes (Δ) in cycle threshold (Ct) values were calculated using the formula: ΔCt = mean Ct for target gene – mean Ct for housekeeping gene (*GAPDH*). Relative gene expression levels were calculated using ΔΔCt analysis, where ΔΔCt = ΔCt of sample – ΔCt of *GAPDH*. The formula 2^(-ΔΔCt) was used to calculate the relative expression ratio in treatment and control groups.

Statistical analysis

Between-group differences were analyzed with the non-parametric Kruskal-Wallis one-way ANOVA test (with post hoc pairwise comparisons done using the Mann Whitney *U* test). The Wilcoxon signed-rank test was used for within-group comparisons over time. Since tumors were bilateral, the sample size per group for all tumor-related variables was the total number of tumors we studied per group. Statistical analyses were performed using IBM SPSS 22.0 package (SPSS, Inc., Chicago, IL) setting the significance level at p=0.05. All graphics were made with GraphPad Prism 6, version 6.01 software (GraphPad Software, San Diego, CA).

RESULTS

Body weight, aerobic capacity and forelimb grip strength

An increase in body weight over time was detected in all mice (1.9 ± 0.4 g on average, p<0.05 for all within-group comparisons). These changes can be attributed, at least in part, to the tumor development process. Between-group analyses showed that the aerobic capacity (p=0.027, Figure 3A) and forelimb grip strength (p=0.005, Figure 3B) (both corrected by body weight) were significantly higher in the exercised mice (exercise + nivolumab or isotype groups) after the 8-week combined training intervention than in their non-exercised peers (non-exercise + nivolumab or isotype groups). Within-group analysis showed that aerobic capacity declined significantly in the non-exercised groups at the end of the study (p=0.020), but was maintained in the exercised groups (p>0.05).

Tumor volume and tumor growth rate

When we compared tumor growth rates between groups, we found a delay in tumor growth in the exercise + isotype control group compared with the non-exercise + isotype control group (p=0.050) (Figure 4A), and also a smaller tumor volume at sacrifice (Figure 4B). By contrast, no differences in tumor growth were observed between the exercise + nivolumab group and the non-exercise + nivolumab group. Macroscopically, we observed that the final tumor volume in the double intervention (exercise + nivolumab) group tended to be larger than that in the other study groups, but this difference was not statistical significant (p>0.05).

Necrotic index

Tumor regression was analyzed by evaluating the percentage of necrotic areas as an index of the tumor response to the different interventions at the study end. Between-group comparisons showed that the necrotic index was significantly higher in the double intervention group than in the non-exercise + isotype control group (p=0.026) (Figure 5).

Cell proliferation

Cell proliferation was assessed by immunostaining for Ki67. As shown in Figure 6A and B, the non-exercise + isotype control group had the highest cell proliferation between the four study groups (40% of Ki67-positive proliferating cells). Significant lower Ki67 immunostaining was found in the exercise + isotype control, non-exercise + nivolumab and exercise + nivolumab groups when compared with the non-exercise + isotype control group (p=0.015, p=0.011 and p=0.011, respectively; Figure 6B).

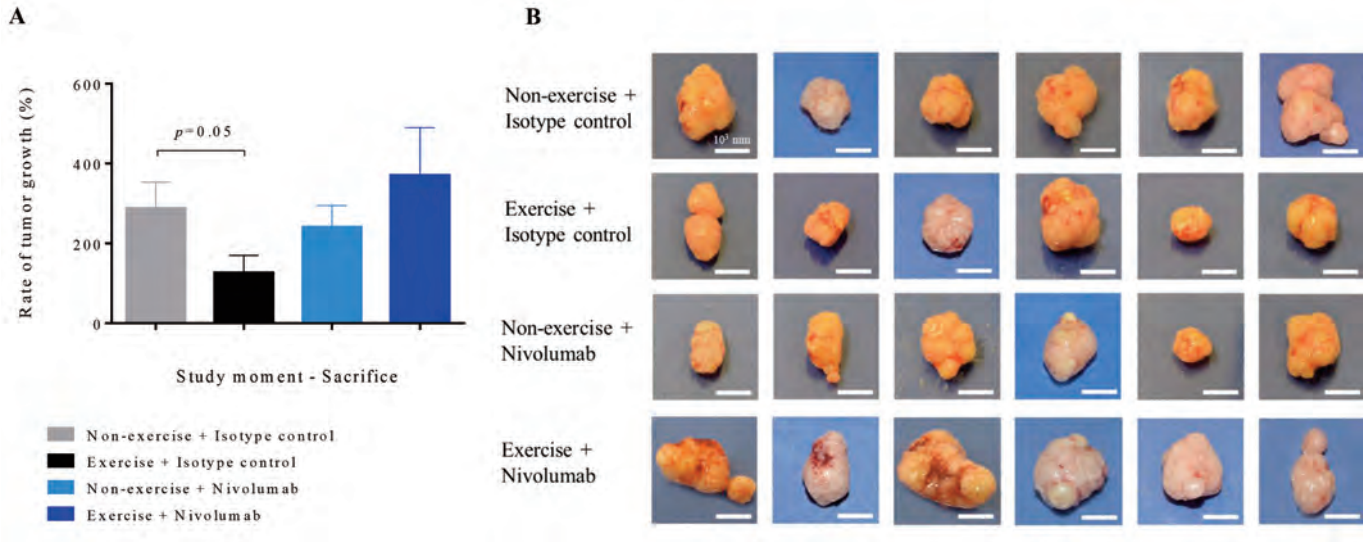


Figure 4. Tumor response to exercise and anti–PD-1 immunotherapy. (A) Tumor growth rate at sacrifice of the different groups. Tumor growth rate was expressed as a percentage of the change of tumor volume with respect to initial tumor volume, which was considered 100%. Data are expressed as mean ± SEM and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.173). (B) Panel with representative ex vivo images of the tumor sizes at the end of the experiment (at sacrifice moment). Scale bars, 10 mm.

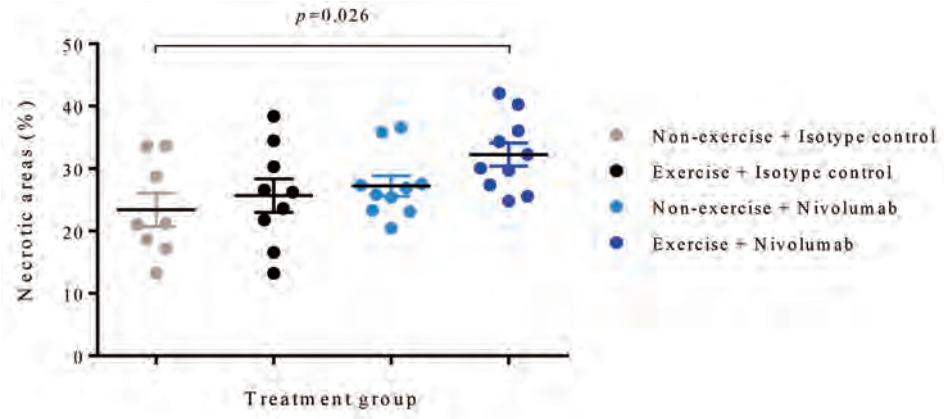


Figure 5. Analysis of tumor necrosis in the experimental groups. The necrosis index is expressed as a percentage of necrotic areas. Values represent the percentage (mean ± SEM) and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.074).

Apoptosis

We noted very little apparent apoptosis in any of the four groups of mice examined, and TUNEL-positive cells were not strictly located to the necrotic areas (Figure 7A). The non-exercise + isotype control group contained the largest number of TUNEL-positive cells (1.65 ± 0.59%), which was significantly greater than in the exercise + nivolumab group (p=0.030) (Figure 7B).

Identification of leukocyte populations in peripheral blood and in tumor stroma

We used flow cytometry to analyze several human and mouse immune cell populations in peripheral blood and in tumors in the four study groups (see Tables 1 and 2). We failed to detect human leukocytes (hCD45$^+$) in both blood and tumor samples, and the immune component was 100% murine (mCD45.1$^+$). Similarly, human T and B lymphocytes and NK cells were also absent, so we concluded that the lineage present in the samples was myeloid, from the murine host. Identical results were found for all study groups.

Focusing on tumor homogenates, we found a significantly higher tumor infiltration of leukocytes in the exercise + isotype control group than in the non-exercise + nivolumab group (p=0.018; Figure 8A). The percentage of neutrophils, monocytes and eosinophils were very similar in all study groups (Figure 8B), although there was a quasi-significant trend for an increase in tumor-infiltrating neutrophils in the exercise + nivolumab group when compared with the non-exercise + isotype control group (p=0.060).

Gene expression associated with the tumor microenvironment

We next used qPCR to test for the expression of human genes related to the immune system and the tumor microenvironment in RNA extracted from tumor tissues (*i.e.*, *CTLA-4, PD-1, PD-L1, IL-4* and *IFNγ*), and found that their expression was undetectable in all the study mice (results not shown). In an analysis of murine genes related to the tumor microenvironment, we found that *Vegf-a* expression was higher in the nivolumab groups (in combination or not with exercise) than in exercise + isotype control group (p=0.045 and p=0.047, respectively) (Figure 9). We also noted a trend for lower *Arg1* expression when exercise was applied as the only intervention compared with the exercise + nivolumab group (p=0.068). The remaining genes *(iNos, Pd-1* and *Pd-1l*) showed similar expression levels in all experimental groups.

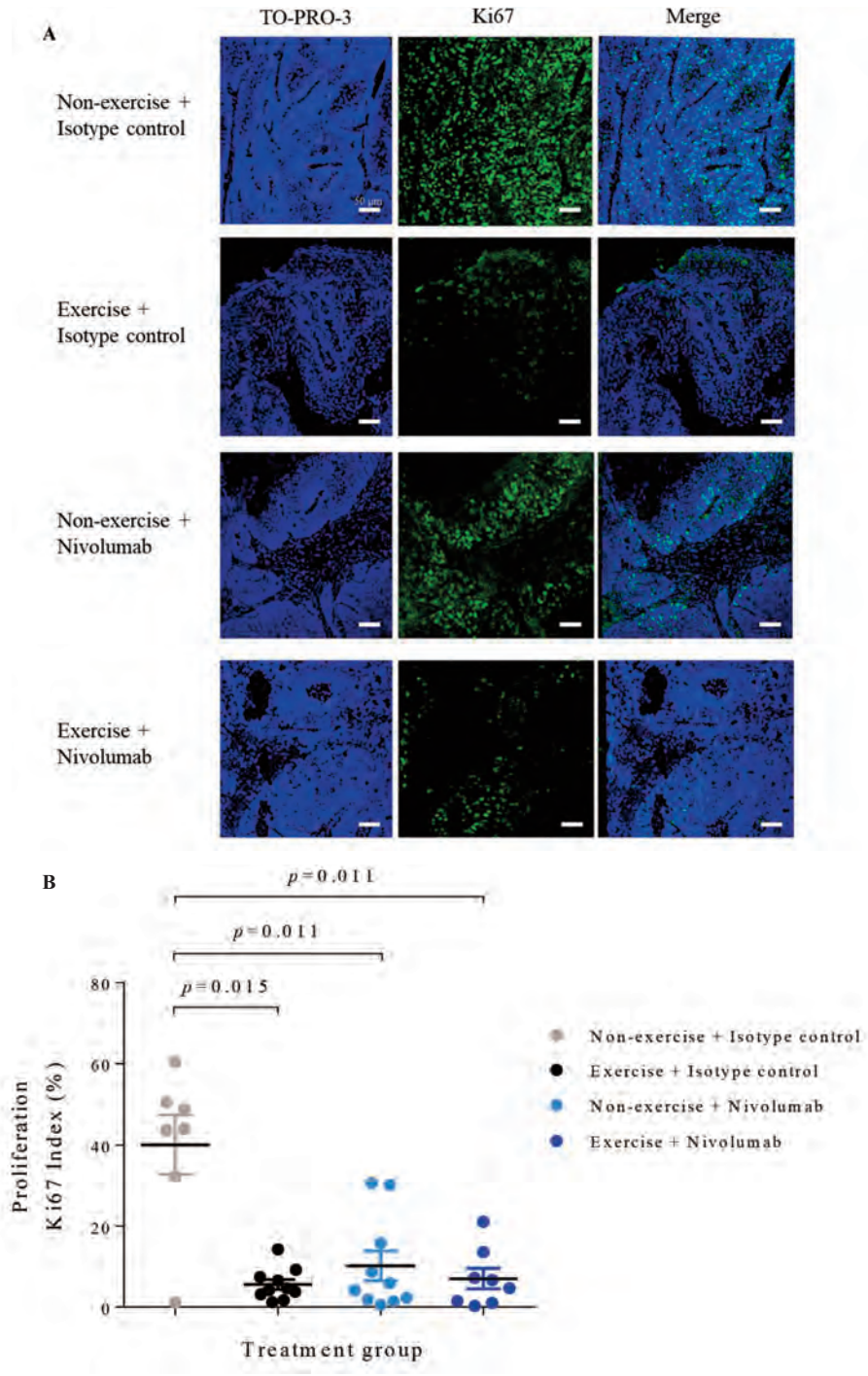


Figure 6. Effect of exercise and anti–PD-1 treatment on cell proliferation. (A) Panel of representative images of Ki67 immunofluorescence staining (green). Nuclei were stained with TO-PRO-3 (blue). Right panels show merged images. Scale bars, 50 μm. (B) Scatter plot, with the individual percentages of Ki67-positive cells in tumors in each group. The lines in the dot plots represents the mean ± SEM of each group and significant pairwise differences are shown (Kruskal-Wallis p-value for group effect = 0.027).

DISCUSSION

Our main findings were that: *(i)* exercise *per se* – and also in combination with nivolumab – reduced cell proliferation in tumors compared to non-exercise and exercise alone also increased myeloid tumor infiltrates (mostly neutrophils) with respect to the nivolumab only group; and *(ii)* the combined intervention (exercise + nivolumab) increased tumor necrosis with respect to the non-intervention control group. To the best of our knowledge, this is the first study to analyze the effect of a physical exercise intervention (applying an 8-week combined protocol including aerobic and strength training) as a potential adjuvant therapy for anti–PD-1 treatment using a murine PDX model of NSCLC. The fact that this model is devoid of host lymphoid cells allows to gain mechanistic insight into the effects of exercise – combined or not with immunotherapy – on tumor development, particularly with regard to the role played by neutrophils, which have been much less studied than lymphocytes.

On the other hand, we found that an exercise intervention combining aerobic and resistance training, and based on public health recommendations for adults (24), conferred significant benefits on aerobic capacity and forelimb grip strength in mice irrespective of immunotherapy treatment. These results are in line with previous studies performed in patients with NSCLC (53) and in preclinical murine models of lung cancer (50) and other tumors (*e.g.*, slowing prevention of muscle wasting (6, 29, 52)).

Studies on physical exercise as an adjuvant therapy in patients with cancer generally focus on increasing their general health status and tolerance to treatments, which might contribute to improve their prognosis and survival. Although immunological adaptations to exercise in cancer have being studied previously (see 54 for a review), there is no *direct* biological evidence in humans for the potential beneficial effects of exercise per se, on tumor regression or on delaying tumor progression. However, previous studies have demonstrated how exercise can reduce the tumor growth rate in preclinical models (e.g., 26, 49, 51, 73), but because of the broad heterogeneity with respect to type of cancer, murine models and exercise interventions used in these studies, it is difficult to draw clear conclusions. In our murine PDX model, physical exercise by itself was found to delay tumor growth and to result in a smaller tumor volume at the end of the study compared with non-exercised mice, irrespective of immunotherapy. However, the final tumor volume as a result of the double intervention (exercise + nivolumab) tended to be larger than that observed in the remainder of the study groups. In this regard, we recently showed in the current mouse PDX model that anti–PD-1 therapy, alone or sequentially combined with cis-

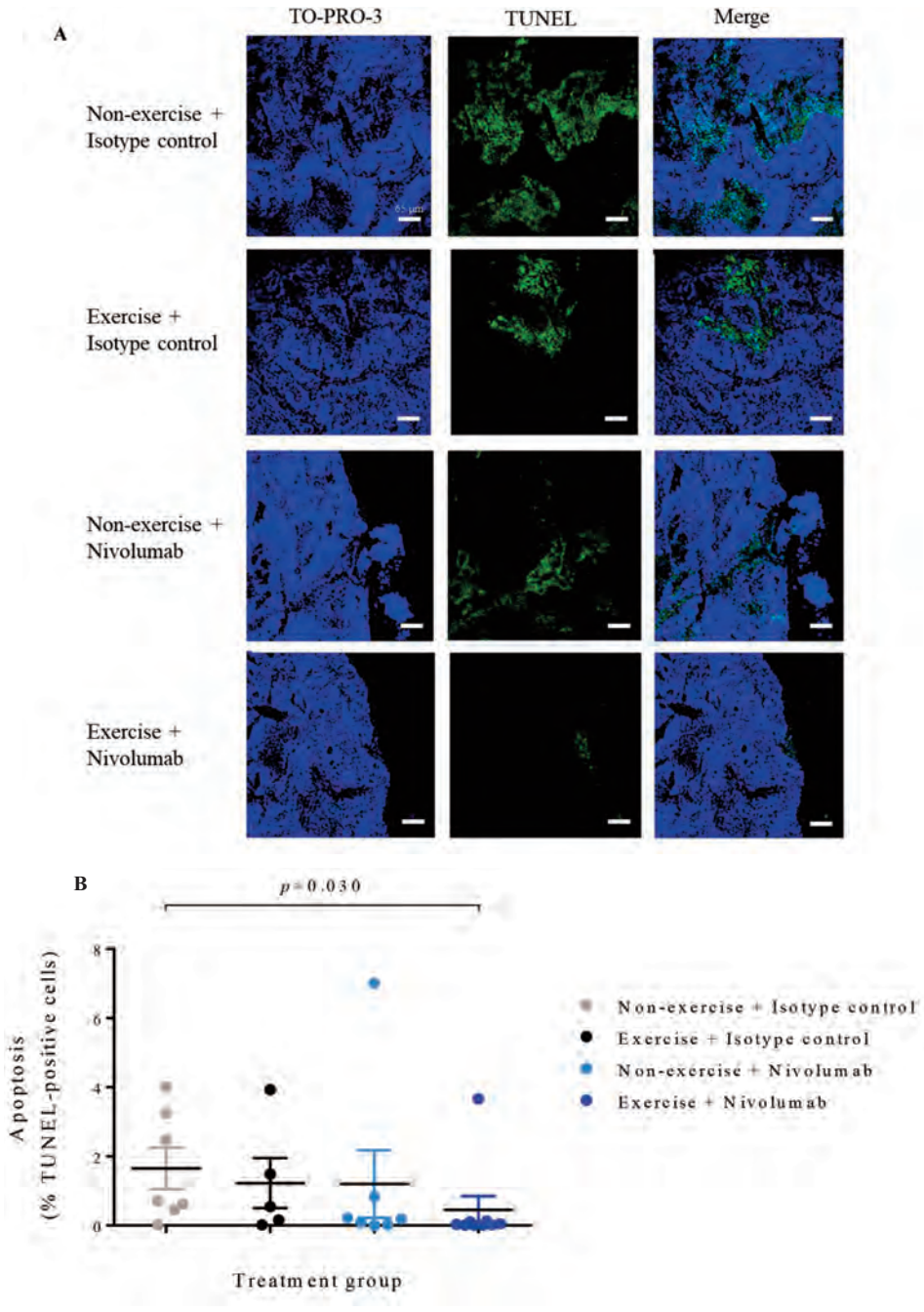


Figure 7. Effect of exercise and anti–PD-1 treatment on tumor apoptosis evaluated by TUNEL assay. (A) Panel of representative images of TUNEL immunofluorescence staining (green). Nuclei were stained with TO-PRO-3 (blue). Right panels show merged images. Scale bars, 65 µm. (B) Scatter plot, with the individual percentages of TUNEL-positive cells (apoptotic) of the tumors in each group. The lines in the dot plots represents the mean ± SEM of each group and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.089).

platin, led to a paradoxical response, that is, to an increase in tumor growth rate (in the anti–PD-1 phase) with large and friable tumors in some cases, which were associated with exudates containing inflammatory polymorphonuclear neutrophils (PMNs) from areas of reactive necrosis (44). This phenomenon is reminiscent of unconventional responses of checkpoint inhibitor-based immunotherapy, such as pseudoprogression, which can be observed in patients´ solid tumors (including NSCLC) treated with this type of immunotherapy (12, 13, 37). These 'paradoxical' or 'unconventional' responses are associated both with immune cell – PMNs and lymphocytes – recruitment and with the intratumoral inflammatory environment triggered by those cells (12).

In murine cancer models, aerobic exercise training at moderate intensity has been shown to induce apoptosis in tumors, essentially through caspase activation and a reduction in *Bcl-2* expression (3, 7, 26, 27, 73). By contrast, our results suggest that apoptosis is probably not the mechanism of cell death that results in tumor regression in response to nivolumab, exercise or their combination, in this model. Indeed, the exercise + nivolumab combination produced the lowest levels of apoptosis, although the percentage of positive TUNEL staining was low (<2% on average) in all study groups. Regarding the results of cell proliferation in our study (measured as Ki67-positive cells), exercise and nivolumab (alone or in combination) significantly reduced cell proliferation with respect to the non-exercise + isotype control group (i.e., 6–10% versus 40% in the control group), but no differences were observed between the treatment groups. Thus, we consider that exercise per se could be responsible for the decrease in proliferation found in the tumors from this group. In this regard, exercise can inhibit cell proliferation by a variety of mechanisms. These include decreases in circulating growth factors such as insulin-like growth factor 1 (54), activation of AMP-activated protein kinase (AMPK), and down-regulation of protein kinase B, which collectively down-regulate the activity of the mammalian target of rapamycin (mTOR) (74). Direct and indirect (through Akt inhibition) downregulation of mTOR, as well as of mTOR downstream signalling (ribosomal protein S6 kinase beta-1 [p70S6K]), has also been shown in NSCLC cells exposed to post-exercise serum, together with stimulation of proliferation and survival through inhibition of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (41). However, some controversy exists because other authors associate exercise with an increase in tumor cell proliferation (71).

Exercise modulates the tumor microenvironment by acting on the innate and adaptive immune systems (28, 39), increasing the peripheral blood levels of T lymphocytes and NK cells, their mobilization to the tumor stroma (31, 49), or their cytotoxicity against tumor cells (28, 39). However, in the PDX model used in this study, flow cytometry analysis revealed the absence of T lymphocytes and NK cells in the tumor grafts and also in peripheral blood, which is common in

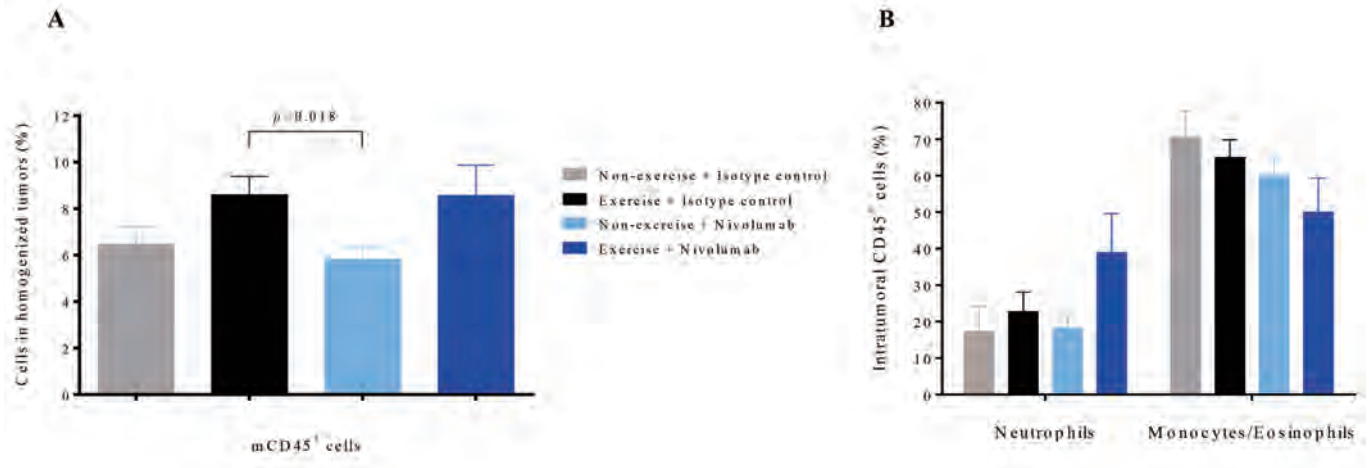


Figure 8. Leukocyte populations in PDX tumor homogenates. (A) Analysis of murine leukocytes in homogenized tumors. Results represent the percentage of mCD45.1 positive cells with respect to the total amount of cells that are detected as events in the flow cytometer. (B) Analysis of populations of murine myeloid cells in homogenized tumors. Populations of neutrophils and monocytes/eosinophils are shown, with respect to the total numbers of cells that are detected as events in the flow cytometer. The data (A and B) are expressed as the mean ± SEM and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.056 for mCD45.1$^+$ leukocytes, 0.310 for neutrophils, and 0.293 for monocytes/eosinophils). Neutrophil phenotype: mCD45.1$^+$ mCD11b$^+$ mLy6G$^+$; monocyte/eosinophil phenotype: mCD45.1$^+$ mCD11b$^-$ mLy6G $^-$.

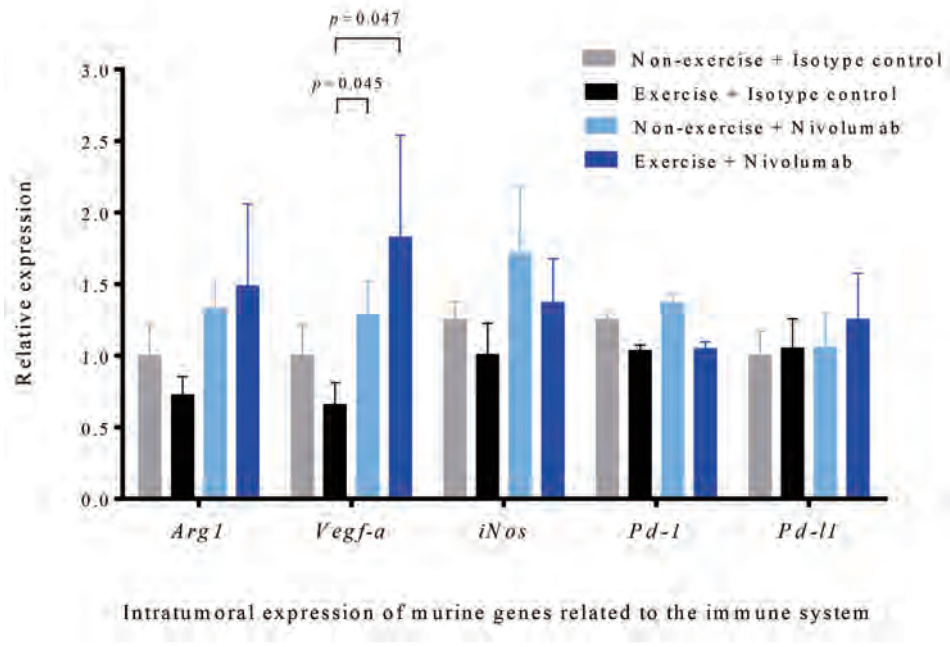


Figure 9. Analysis of murine gene expression related to immune system and microenvironment. Relative expression of arginase-1 *(Arg1)*, vascular endothelial growth factor-a *(Vegf-a)*, inducible nitric oxide synthase *(iNos)*, programmed cell death protein 1 *(Pd-1)* and programmed death ligand 1 *(Pd-l1)* by qPCR in homogenized tumors. Represented are the mRNA intratumoral relative levels, which are expressed as mean ± SEM. Significant pairwise differences are shown (p=0.161 for Kruskal-Wallis group effect in Vegf-a.)

this type of model (60). Animal and human studies show that exercise can also impact the innate immune component by increasing the levels of myeloid cells such as macrophages, monocytes and neutrophils, both in peripheral blood and in tissue infiltrates (64, 69). In addition, exercise can regulate the reprogramming of the tumor microenvironment, by promoting the polarization of myeloid cells towards a more anti-tumorigenic phenotype (1, 22, 23). As expected for PDX models of solid tumors (72), including NSCLC (46), we found that the leukocyte-infiltrated tumor component was completely replaced by a murine infiltrate. We cannot confirm whether some human cells were preserved in host mice, but they were below the limit of detection in tumor homogenates. Accordingly, in the absence of murine NK cells and lymphocytes, myeloid cells were considered as the infiltrated cells present in the stroma. We found an increase in the number of total leukocytes in tumor infiltrates from exercised mice treated with the isotype control, which is in line with previous research on exercise-induced changes to myeloid cell abundance (64, 69). We also found a trend towards an increase (p=0.06) in the proportion of neutrophils in the tumor stroma of the exercise + nivolumab group (38%) versus the non-exercise + isotype control group (17%), which is an important finding as neutrophils dominate the immune cell composition in NSCLC (35). Moreover, there was a decrease in the number of monocytes/eosinophils in the tumors of exercised mice that received nivolumab, which was concomitant with an increase in neutrophils. In turn, leukocyte populations in tumor stroma appeared to be balanced in the pharmacological (nivolumab) and non-pharmacological (physical exercise) groups, which might create an environment that can lead to potential beneficial effects of both therapies, since the tumor microenvironment is characterized by a loss of homeostasis in immune cell populations (47, 63).

Immunotherapy treatment can modify the cellular component of the tumor microenvironment and, consequently, the gene expression profile related to the immune system of the

intratumor environment (21, 57). Physical exercise also produces this effect, as it is known to mobilize myokines (mainly proteins and small peptides [*i.e.*, cytokines]) and immune cells at the systemic level (1, 39, 54). Although the expression of all murine genes evaluated was detected in the tumors of all experimental groups, a significant increase in *Vegf-a* expression was observed 8 weeks after nivolumab administration (combined or not with exercise) compared with the exercise + isotype control group, which showed the lowest expression of this gene. Vegf-a is involved in angiogenesis in the tumor microenvironment (5). The effect of exercise on Vegf-a is controversial because both a decrease (in the vicinity of Dalton's lymphoma (68) or in breast tumor tissue from female mice (58)) and an increase in its expression has been reported (67). Tsai et al. showed an increase in serum Vegf-a levels in tumor-bearing mice inoculated with Lewis lung carcinoma (LLC) compared with baseline, but without significant differences in terms of survival rate or tumor growth compared with the control group [67]. In turn, Alves et al. observed 2.5-fold higher Vegf-a mRNA levels in an LLC mice model undergoing daily high-intensity interval training after tumor cell injection compared with non-exercised mice, with a significant reduction of tumor mass and an increase in survival (2). On the other hand, the finding that exercise might result in enhanced tumor perfusion (through increases in Vegf-a) had led some authors to hypothesize that exercise may enhance the delivery of drugs (45) and thus exercise emerges as a potential co-adjuvant intervention when combined with drug therapy, improving the efficacy of the latter (8). Thus, the fact that Vegf-a expression was increased with nivolumab treatment, especially when combined with exercise, does not necessarily reflect a deleterious effect. On the contrary, this finding can be interpreted as a mechanism by which anti–PD-1 immunotherapy promotes PDX tumor perfusion, implying vascular remodeling, better drug distribution and myeloid cell – mainly neutrophils – mobilization with the potential to exert an anti-tumor (cytotoxic or 'necrotizing') effect. In effect, the treatment that tended to yield the highest necrotic index (Figure 5) as well as the highest Vefg-a tumoral levels was the double intervention exercise + nivolumab group. Morover, neutrophils participate actively in the remodeling and (neo)angiogenesis processes of the tumor and its extracellular matrix as a result of VEGF-α production and activation (14, 15).

Our study has several limitations and strengths. Firstly, the sample size was low and the study was done in only one immunotherapy-responder line of PDX mice. On the other hand, the NOD-SCID gamma mice used as the experimental model lack T cells, B cells and mature NK cells, so it was not possible to study the interaction of those immune cells with both the tumor microenvironment and the interventions applied in this study. However, the fact that we studied PDX mice devoid of host lymphoid cells might be actually viewed as a potential strength as they represent a unique animal model to study the effects of exercise on tumor development through mechanisms other than the classically advocated T lymphocyte or NK cell involvement. Therefore, we were able to study the effects of the anti–PD-1 (nivolumab) immunotherapy, the exercise training program, and their combination by mechanisms independent of the adaptive immune system, specifically through myeloid cells such as neutrophils. One of the strengths of this study was the application of a training modality combining aerobic and resistance exercise, with the latter been applied here for the first time in the field of exercise in preclinical cancer models, and with the knowledge on the potential benefits of exercise against cancer been confined up to date to aerobic exercise only. Interestingly, preliminary evidence suggests that chemotherapy-treated patients with lung cancer who joined exercise sessions using resistance bands managed to maintain white blood cell levels during treatment compared with a control group (36). Our study is also the first to combine exercise with immunotherapy, specifically an immune checkpoint inhibitor that is receiving growing attention in oncology for its potential to improve treatment responses compared to traditional treatments. In addition, the study was focused on the analysis of the tumor response to exercise and/or to the anti–PD-1 therapy, assessing several molecular markers of growth, progression and cell death and also the anti-tumoral transcriptome, with particular emphasis on the infiltrated innate immune system and the production of *Vegf-a.*

CONCLUSION

The combination of an aerobic and strength exercise training program in a murine PDX model of NSCLC improved aerobic capacity and maximal strength in mice. Exercise per se was also able to reduce cell proliferation in tumors from these mice, as reflected in a reduction in Ki67 staining and in tumor volume. When exercise was combined with nivolumab, both therapies influenced tumor regression through an increase in necrosis and a decrease in the proliferative index. An increment in *Vegf-a* expression was also found in the exercise + nivolumab group, although future research should examine the biological relevance of this result. In conclusion, we consider that despite the need for a deeper analysis of the effect of exercise in clinical and pre-clinical studies, its application should be taken into consideration as a co-adjuvant therapy to pharmacological treatment. Furthermore, PDX models as the one we used here might allow other researchers to gain deeper insight into the role of non-lymphoid immune cells, particularly neutrophils, against tumor development, with these cells been scarcely studied to date compared with lymphocytes.

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Mobilizing serum factors and immune cells through exercise to counteract age-related changes in cancer risk

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ABSTRACT

An increasing body of evidence suggests that age-related immune changes and chronic inflammation contribute to cancer development. Recognizing that exercise has protective effects against cancer, promotes immune function, and beneficially modulates inflammation with ageing, this review outlines the current evidence indicating an emerging role for exercise immunology in preventing and treating cancer in older adults. A specific focus is on data suggesting that muscle-derived cytokines (myokines) mediate anti-cancer effects through promoting immunosurveillance against tumourigenesis or inhibiting cancer cell viability. Previous studies suggested that the exercise-induced release of myokines and other endocrine factors into the blood increases the capacity of blood serum to inhibit cancer cell growth in vitro. However, little is known about whether this effect is influenced by ageing. Prostate cancer is the second most common cancer in men. We therefore examined the effects of serum collected before and after exercise from healthy young and older men on the metabolic activity of androgen-responsive LNCaP and androgen-unresponsive PC3 prostate cancer cells. Exercise-conditioned serum collected from the young group did not alter cell metabolic activity, whereas post-exercise serum (compared with pre-exercise serum) from the older men inhibited the metabolic activity of LNCaP cancer cells. Serum levels of candidate cancer-inhibitory myokines oncostatin M and osteonectin increased in both age groups following exercise. Serum testosterone increased only in the younger men post-exercise, potentially attenuating inhibitory effects of myokines on the LNCaP cell viability. The data from our study and the evidence in this review suggest that mobilizing serum factors and immune cells may be a key mechanism of how exercise counteracts cancer in the older population.

Keywords: ageing, cancer development, exercise, immunosurveillance, cancer growth-inhibitory molecular factors, immune-regulatory myokines

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INTRODUCTION

The global increase in the elderly population is associated with growing challenges both for individuals and health care systems (5, 25, 108). The incidence and prevalence of most cancers increase with ageing (9, 64, 70), and this has major health implications. It is therefore important to improve the understanding of the efficacy of lifestyle-based strategies for preventing and treating cancer with advancing age. Epidemiological and observational evidence shows that regular physical activity and exercise[1] protect against the development of some of the most common cancers (such as breast, colon and endometrial cancer), and reduce the risk of disease recurrence and mortality (1, 64, 70, 74). The protective effects of exercise against cancer are proposed to revolve around changes in the endocrine and immune systems (56, 58, 73). Notably, age-related alterations in the immune system may contribute to the increased cancer risk in the older population (43). Recognizing the increasing body of evidence suggesting immune benefits of exercise especially in older adults (34, 116), this notion has important implications for the role of exercise immunology in the protection against cancer with ageing.

Recent experimental data have also shown that blood serum collected after exercise from mice (57), healthy younger adults (105), and cancer patients and survivors (32, 33), inhibits cancer cell growth *in vitro.* These effects of exercise-conditioned serum are likely due to the exercise-induced secretion of endocrine factors by skeletal muscle – termed myokines – and/or other tissues into the blood (32, 33, 57, 105). Muscle-derived endocrine factors that may mediate cancer-inhibitory effects of exercise through controlling cancer cell metabolism and growth kinetics include, for example, irisin, osteonectin (also known as secreted protein acidic and rich in cysteine, or SPARC), and oncostatin M (OSM) (57, 93). Furthermore, certain myokines, including interleukin (IL)-6, IL-7 and IL-15, have immune regulatory effects (56, 91, 93), which might have important consequences for counteracting carcinogenesis. For example, IL-6 regulates the exercise-induced redistribution of cytotoxic natural killer (NK) cells from the blood circulation into tumours, thereby decreasing tumour growth (95). The data suggesting a myokine-mediated 'crosstalk' of skeletal muscle with immune and cancer cells, provide a novel conceptual framework that links active skeletal muscle and immune function with exercise-associated protection against cancer (34, 58). However, so far, only very little information is available on how ageing influences these muscle-immune- and muscle-cancer cell-interactions.

Prostate cancer is the second most common cancer in males, and, in particular, a type of cancer that becomes more common as men age (1). The epidemiological evidence for a preventive effect of physical activity specifically against prostate cancer is limited (1, 64, 70, 74). However, epidemiological data suggest that physical activity is associated with a 38% reduction in the relative risk of cancer-specific mortality in individuals diagnosed with prostate cancer (74). Observational studies have also shown an exercise-dependent reduction in the risk of disease recurrence for prostate cancer (58, 63). Moreover, a study by Rundqvist et al. has demonstrated that serum collected from 10 healthy men (aged between 18 and 37 years) after one hour of cycling inhibited the growth of prostate cancer cells (LNCaP) *in vitro*, as compared with serum obtained in resting conditions (105). Nevertheless, there are no data available on whether ageing influences the exercise-induced changes in the capacity of serum to inhibit the viability of cancer cells in general, and prostate cancer cells in particular. Expanding upon the work by Rundqvist et al. (105), the primary aim of the current study was to compare how serum collected pre- and post-exercise from younger and older men affects prostate cancer cell growth. We hypothesised that acute exercise would improve the capacity of serum to inhibit prostate cancer cell growth in younger and older men, as compared with serum collected in resting conditions. As another extension to the study by Rundqvist et al. (105), we used two different prostate cancer cell lines, androgen-responsive LNCaP and androgen-unresponsive PC3 cells, for incubation with the serum. Androgen signalling plays an important role in normal male physiology and prostate cancer (29, 106). Considering that systemic levels of androgens, such as testosterone, change in response to exercise (79, 125) and with ageing (53, 109), we used these two cell lines to gain insights into potential androgen-related mechanisms. In addition, we assessed whether there are age-related differences in the exercise-induced changes in the serum concentrations of testosterone and candidate immune-regulatory or cancer-inhibitory cytokines including myokines IL-6, IL-15, irisin, osteonectin, and OSM.

In addition to our original contribution, we have outlined the current evidence on the role of exercise-induced changes in serum myokine/cytokine concentrations and the immune system in counteracting cancer in the older population. Several reviews on the impact of exercise on immune function in the context with either ageing (34, 116) or cancer (56) are available. A recent review by Hojman et al. has provided an updated status of research related to the molecular mechanisms underlying the link between exercise and cancer prevention and treatment (58). To present a contemporary view, this review combines and integrates available data on how myokines, cytokines and the cellular immune system mediate protective exercise effects against cancer specifically with advancing age, with the major focus on human studies.

METHODS

Ethical approval

The study was approved by the Queensland University of Technology Human Research Ethics Committee (ethics no. 1500000881). A total of 22 healthy males volunteered to participate and all participants provided written, informed consent before their inclusion in the study.

Study participants

Twelve healthy males aged 20 to 33 years (young age group) and ten healthy males aged 60 to 73 years (old age group) were recruited for this project. Before the enrollment into the study, all participants were assessed by a medical physician and were classified as clinically healthy. This medical entrance examination included a standard medical history questionnaire, height, weight, body mass index (BMI), and blood pressure measurements. The participants also completed a physical activity questionnaire. Both age groups had a

normal BMI of 18.5–25 kg/m^2. Exclusion criteria included any evidence of acute or chronic diseases such as cancer, or heart, lung, nerve or muscle disease and diabetes. Further exclusion criteria included training for and participation in any competitive sports events, smoking and the use of blood-thinning medication, and other drugs such as anti-inflammatory medicines or statins. All participants performed an incremental exercise test on a cycle ergometer (LODE Excalibur Sport, LODE BV, Groningen, The Netherlands) to determine their maximum oxygen uptake ($\dot{V}O_{2\,max}$). After a warm-up of 5 minutes, the test commenced at a power output of 25 W, with increments of 25 W/min until cadence dropped below 60 rpm, or voluntary exhaustion occurred. Gas analysis during the test was performed using a TrueOne2400 metabolic cart (ParvoMedics, East Sandy, UT, USA), while heart rate was monitored by telemetry (Vantage NV, Polar, Finland). The participants' anthropometric and physiological characteristics are summarized in Table 1.

Table 1. *Anthropometric and physiological characteristics of the study participants*

	Total group (n=22)	Young age group (n=12)	Old age group (n=10)	P value
Anthropometric characteristics				
Age (years)	44.05 ± 18.46	28.2 ± 2.6	63.1 ± 6.9	0.000*
Height (m)	1.80 ± 0.06	1.80 ± 0.08	1.80 ± 0.03	0.923
Weight (kg)	76.9 ± 6.5	74.1 ± 6.2	80.15 ± 5.6	0.025*
BMI (kg/m^2)	23.8 ± 2.2	22.8 ± 2.1	24.9 ± 1.92	0.024*
Physical activity/exercise level				
Duration (min/week)		243 ± 193	492 ± 432	0.047*
Physiological characteristics				
$\dot{V}O_{2\,max}$ (L/min)	3.20 ± 0.6	3.27 ± 0.61	3.12 ± 0.69	0.602
$\dot{V}O_{2\,max}$ (mL/kg/min)	41.7 ± 8.1	44.1 ± 7.3	38.9 ± 8.4	0.148
METs at $\dot{V}O_{2\,max}$	12.0 ± 2.4	12.6 ± 2.1	11.1 ± 2.6	0.180

Data are presented as mean ± SD. BMI, body mass index; $\dot{V}O_{2\,max}$, maximum oxygen uptake; METs; metabolic equivalents. * Significantly different between age groups.

Exercise protocol

On the morning of the exercise trial, the participants were provided with a standardized breakfast. This standardized breakfast consisted of four high-fiber low-sugar cereal biscuits (983 kJ, 0.9 g fat, 44.2 g carbohydrates, 8.2 g protein) and 200 mL milk (538 kJ, 6.8 g fat, 10.2 g carbohydrates, 6.6 g protein). The participants were asked to have their breakfast 2 to 2.5 h before the exercise trial. Furthermore, the participants were required not to exercise in the 24 h preceding the exercise trial and to refrain from any caffeine and alcohol consumption during this time frame. As in the study by Rundqvist et al. (105), the exercise trial consisted of 20 min of cycling at a work rate corresponding to 50% of their $\dot{V}O_{2\,max}$, before the work rate was increased to a work rate corresponding to 65% of their $\dot{V}O_{2\,max}$ for an additional 40 min. Immediately before and immediately after exercise, approximately 25 mL of blood was collected through a 21-gauge butterfly needle inserted into an antecubital vein. Blood was collected into vacutainers (Becton Dickinson Biosciences, San Jose, CA, USA), containing either ethylenediamine tetraacetic acid (EDTA; for hematological analysis) or a clot activator (CAT; for serum analysis and serum incubation with the cell lines). For serum analysis and serum incubation experiments, blood samples were centrifuged at 1500 × g for 10 min at 22 °C, within 20 minutes of collection. Serum was aliquoted and stored at –80 °C until analysis or use for cell culture experiments.

Hematological analysis

Pre- and post-exercise whole blood collected in vacutainer tubes with EDTA was used for assessment of the hematological profile (including concentrations of total leukocytes and leukocyte subpopulations) using an automated hematology analyzer (Ac T diff 2, Beckman Coulter, Brea, CA, USA). Before data acquisition, the instrument accuracy and precision were checked using the 4C-ES Cell Control (Beckman Coulter). The collected blood samples were gently mixed before placing the tube into the analyzer.

Cell culture

Two commonly used prostate cancer cell lines from the American Type Culture Collection (ATCC), PC3 (ATCC® CRL-1435™) and LNCaP (ATCC® CRL-1740™), were used in this study. Both cell lines differ in their biological characteristics, with PC3 cells demonstrating stronger invasiveness compared to LNCaP cells *in vitro*. LNCaP cells are androgen-responsive, unlike PC3 cells, which are not responsive to androgens (72). Both cell lines were cultured in 75-cm^2 flasks using phenol red-free Roswell Park Memorial Institute medium-1640 (RPMI-1640) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1 mg/mL streptomycin, 100 units/mL penicillin.

Cellular metabolic activity assay

The Alamar blue assay® (ThermoFisher, Waltham, MA, USA) was used to assess the effect of pre- and post-acute exercise serum on the metabolic activity, an indirect measure of the cellular viability, of the prostate cancer cells. This assay is a widely used to evaluate the metabolic function and cellular health of cultured cells (102). Specifically, it incorporates

an oxidation-reduction indicator that fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As the cultured cells grow, their metabolic activity results in a chemical reduction of this indicator. Continued growth maintains a reduced environment, whereas inhibition of growth maintains an oxidized environment. In 400 µL of growth medium, LNCaP and PC3 cells were seeded (at cell densities of 5.82 x 10^3 cells/cm^2 and 2.91x 10^3 cells/cm^2 for the LNCaP and PC3 cells, respectively) into individual wells of 48-well plates and incubated at 37 °C with 5% CO_2 for 24 h. On the following day, the cells were serum-starved for 3 h. After serum starving, 200 µL of RPMI 1640 medium supplemented with 5% FBS and 5% each participants' rest or exercise serum were added to each well plate in triplicate. Cells were returned to the incubator at 37 °C with 5% CO_2 for another 96 h, with the medium refreshed at the 48 h-time point. At the 96 h-endpoint, Alamar blue was added to each well at a final concentration of 8% (v/v). The plates were incubated at 37 °C for another 4 h and from each well, 100 µL of medium were transferred to a 96-well black plate in duplicate. Fluorescence intensity (excitation 544 nm, emission 590 nm) was detected using the POLARstar OPTIMA plate reader (BMG, Labtech, Ortenberg, Germany). After the Alamar blue assay, the cell monolayers were washed twice with PBS to remove residual Alamar blue reagent, and then stored at –80 °C for at least 48 h before the PicoGreen assay was performed.

DNA content assay for determining cell number

In addition to assessing the metabolic activities of prostate cancer cells, the cell number and cellular proliferation were measured indirectly by using a DNA quantification assay. This was achieved by using the PicoGreen reagent (ThermoFisher, Waltham, MA, USA) in conjunction with a DNA standard curve according to the manufacturer's instruction. After thawing the frozen plates, 0.5 mg/mL proteinase K (Invitrogen™) in phosphate-buffered EDTA (PBE) was added into each well and the plates were incubated at 37 °C with 5% CO_2 overnight. The detached cells were thoroughly resuspended and incubated at 56 °C for another 8 h. Subsequently, the cells were centrifuged (Microfuge 18, Beckman Coulter) at 2000 rpm for 5 min, and the supernatant containing DNA was diluted (1:50) in PBE. The DNA standard curve was prepared with lambda DNA standard using a dilution series, consisting of 1000, 500, 250,125, 62.5, 31.25, 15.625 ng/mL). The standards and samples were plated in triplicates of 100 µL into black 96-well plates. PicoGreen working solution (100 µL) was added to each sample or standard, and the plates were incubated for 5 to 10 min at room temperature and protected from light. The fluorescent signals were detected using a POLARstar OPTIMA fluorescence plate reader (excitation 485 nm emission 520 nm).

Serum testosterone analysis

Serum concentrations of total testosterone were assessed by electrochemiluminescence using an automated immunoassay analyser (Cobas E411, Roche diagnostics, Indianapolis, IN, USA) and native reagents (Elecsys Testosterone II, Roche diagnostics, Indianapolis, IN, USA). The manufacturer-reported measuring range is 2.5–1500 ng/dL. Analysis was performed with technical duplication, resulting in a coefficient of variation of 1.3 %. Due to difficulty to collect sufficient blood volumes from some of the participants, it was not possible to analyze serum testosterone from the whole study population. Samples from 10 young participants and seven old participants were included in the testosterone analysis.

Serum cytokine/myokine analysis

A Milliplex Myokine immunoassay kit (Millipore Corp., Billerica, MD, USA) was used to simultaneously measure serum levels of five known myokines, including IL-6, IL-15, irisin, osteonectin (also known as SPARC), and OSM (catalogue number: HMYOMAG-56K), as previously described (45). The assay was performed according to the manufacturer's instructions and all samples were run in duplicate. Briefly, 25µL of the provided standards, controls or blanks were added to the appropriate wells. In addition, 25 µL of each thawed serum aliquot was diluted 1:2 in assay buffer and then added to the sample wells prior to the addition of another 25 µL of assay buffer. A 25 µL aliquot of the provided serum matrix was added to wells containing standards, controls and blanks. A final 25 µL volume of working solution containing multiple microbeads, labelled with specific antibodies against each of the aforementioned factors were then added into each well and allowed to incubate overnight on a plate shaker at 4 °C. The plate was then washed twice with 200µL Milliplex wash buffer and the beads were incubated for 1 h at room temperature in 25 µL of detection antibodies. A 25 µL aliquot of Streptavidin-Phycoerythrin was then added to each well and allowed to incubate for 30 min at room temperature with agitation. The plate was then washed twice more, 150 µL of drive fluid was added to each well and the plates were then incubated for 5 min on a plate shaker. The plate was read on Millipore Magpix System (Millipore), and data was analysed using Milliplex Analyst software (V5.1, Millipore). Mean inter-assay coefficient of variation for the five factors analyzed were as follows: IL-6: 2.4 %; IL-15: 1.9 %; irisin: 2.3 %; osteonectin: 2.9%; OSM: 2.1%. Minimum detectable concentrations (minDC) and sensitivity (i.e., minDC+2SD; indicated in brackets) were as follows: IL-6: 0.53 pg/mL (0.9 pg/mL); IL-15: 0.73 pg/mL (5 pg/mL); irisin: 224.42 pg/mL (281 pg/mL); osteonectin: 3.62 ng/mL (7.5 ng/mL); OSM: 0.96 pg/mL (6 pg/mL).

Statistical analysis

Statistical analyses were conducted using SPSS version 25 (IBM, New York, USA) and GraphPad Prism version 7 (GraphPad Software, Inc., San Diego, CA). To compare the means between two unpaired groups, normality was tested using the Shapiro-Wilk test. If the data followed a Gaussian distribution, a Student T-test was performed; otherwise, the non-parametric Mann-Whitney test was performed. To compare among the two cell lines treated with pre- and post-exercise serum, a two-way repeated-measures ANOVA was performed, in conjunction with Sidak's multiple comparisons test. A paired T-test was used to compare the blood leukocyte, serum testosterone concentrations and cytokine/myokine concentrations pre- and post-exercise. Furthermore, a two-way repeated measures ANOVA was conducted to determine interaction effects of exercise and age on serum concentrations of testosterone and cytokines/myokines. Statistical significance was set at a $P<0.05$.

RESULTS

Exercise-induced changes in total leukocyte counts and leukocyte subpopulations in the blood circulation

Total circulating leukocyte counts, granulocyte and lymphocyte counts increased significantly (P<0.05) in both age groups after the exercise trial. No differences were detected in the magnitude of these changes between the two age groups. All changes in leukocyte subpopulations are summarized in Table 2.

Effects of exercise on the metabolic activity of prostate cancer cells

For the young age group, there was no significant difference in the metabolic activity of LNCaP and PC3 cells treated with pre- versus post-exercise serum (Figure 1). For the old age group, there was a significant reduction in LNCaP cell metabolic activity when cells were treated with post-exercise serum compared with pre-exercise serum (P<0.01). For the PC3 cell line, there was no significant difference in the metabolic activity when cells were treated with pre- versus post-exercise serum from both the young and older group.

Effects of exercise on prostate cancer cell numbers

As a proxy indicator of cell growth/proliferation, there was no difference in the total amount of DNA for LNCaP cells and PC3 cells treated with pre- versus post-exercise serum (Figure 2). There were no exercise age interaction effects for the total amount of DNA.

Table 2. *Blood concentrations of leukocytes and subpopulations before and after the exercise trial in both age groups*

	Young age group (n=12)			Old age group (n=10)		
	Pre-exercise	Post-exercise	P value	Pre-exercise	Post-exercise	P value
WBC (x10^9/L)	5.45 ± 1.24	7.42 ± 1.31*	<0.001	6.47 ± 1.7	8.44 ± 2.77*	0.004
LY (x10^9/L)	1.69 ± 0.41	2.35 ± 0.83*	0.004	1.86 ± 0.31	2.32 ± 0.56*	0.019
MO (x10^9/L)	0.39 ± 0.18	0.48 ± 0.12	0.098	0.38 ± 0.18	0.40 ± 0.17	0.629
GR (x10^9/L)	3.36 ± 0.98	4.60 ± 1.22*	0.002	4.25 ± 1.51	5.70 ± 2.44*	0.022

Data are presented as mean ± SD. WBC, white blood cells; LY, lymphocytes; MO, monocytes; GR, granulocytes.
* Significantly different from pre-exercise.

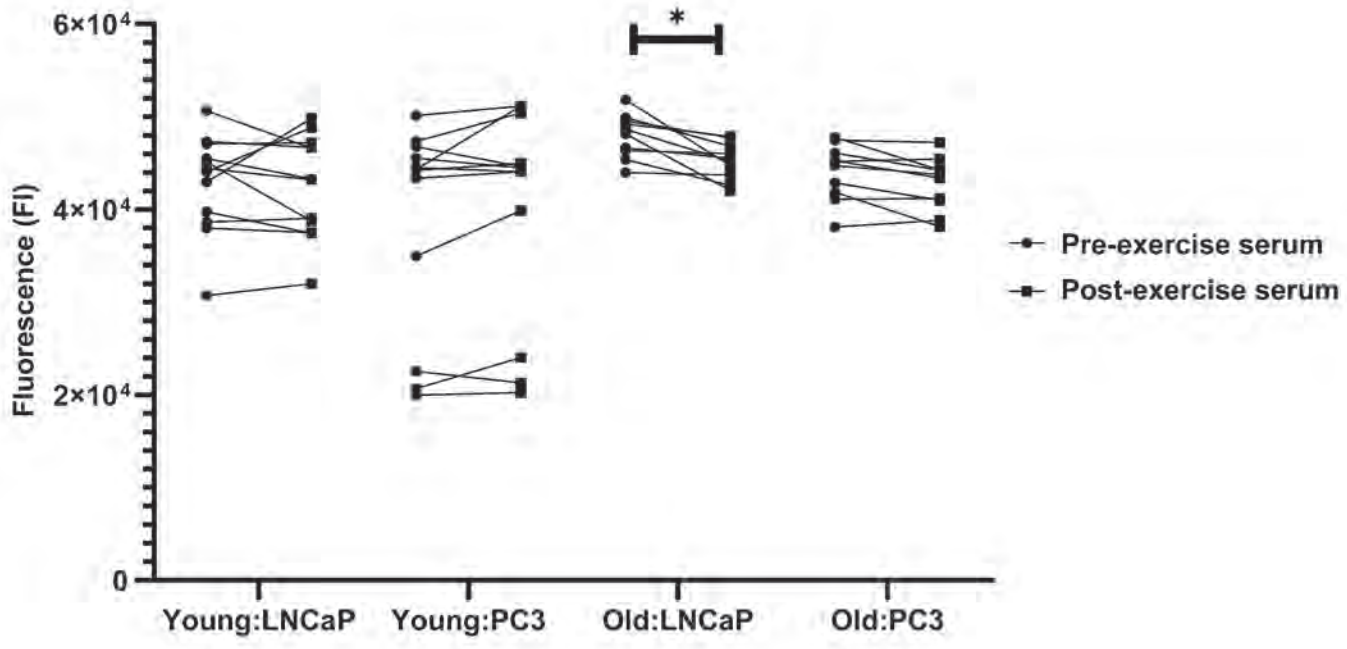

Fig. 1. Effects of exercise on the metabolic activity of LNCaP and PC3 prostate cancer cells after incubation with serum collected from the young and older men, as assessed by using the Alamar blue assay.

Metabolic activity results measured with Alamar blue reagent for LNCaP and PC3 cells treated with pre- or post-exercise human serum, separated based on the age group (young n=12, old n=10) and cell types (LNCaP, PC3). Values shown are the averages of the technical replicates for cells treated with each participant's pre- (circle) or post- (square) exercise serum. Each connected set of dots represent a different individual. A two-way repeated measure ANOVA with Sidak's multiple comparisons test was used to compare between the pre- and post-exercise response for each cell line within young or old age groups. *, P<0.05

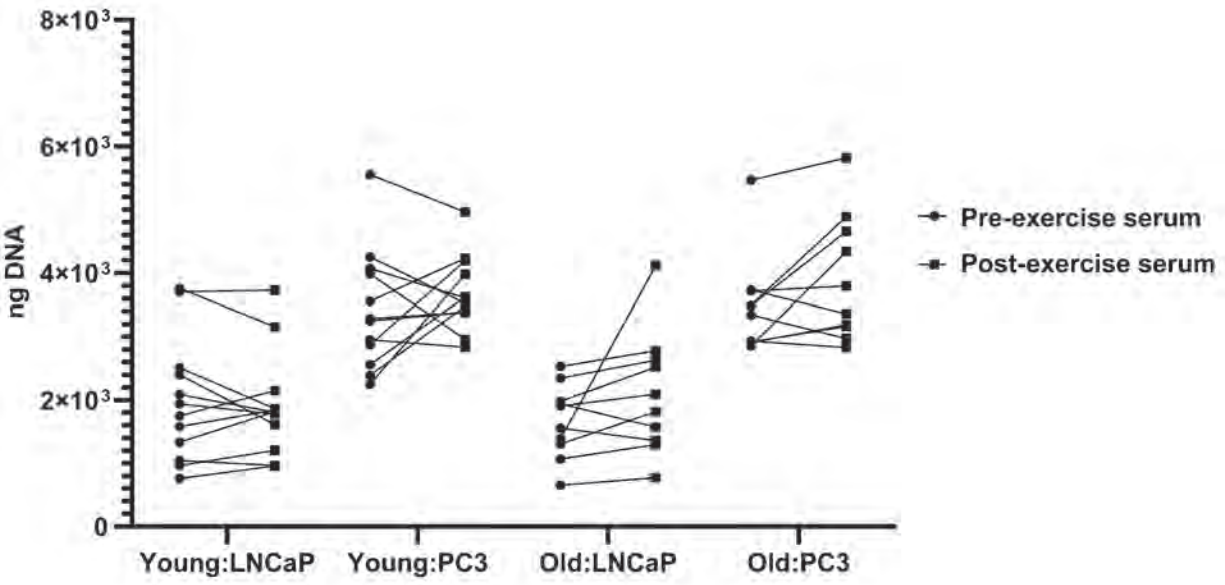

Fig. 2. Effects of exercise on numbers of LNCaP and PC3 prostate cancer cells after incubation with serum collected from the young and older men, as assessed by using the PicoGreen assay.

Combined cellular DNA results using the Alamar blue reagent for LNCaP and PC3 cells treated with pre- or post-exercise human serum, separated based on the age group (young n=12, old n=10) and cell types (LNCaP, PC3). Values shown are the averages of the technical replicates for cells treated with pre- (circle) or post- (square) exercise serum. Each connected set of dots represent an individual. A two-way repeated measure ANOVA with Sidak's multiple comparisons test was used to compare between the pre- and post-exercise response for each cell line within young or old age groups.

Exercise-induced changes in serum testosterone concentrations

Serum testosterone concentration increased from pre- to post-exercise in the young group (P<0.01), but not in the old group (shown in Figure 3). Results of the two-way repeated measures ANOVA on the serum testosterone data showed that there was neither an age effect nor an interaction effect of exercise and age.

Exercise-induced changes in serum concentrations of candidate cytokines/myokines

Serum concentrations of osteonectin and OSM increased in both young and older men from pre- to post-exercise (*P*<0.05). Significant effects of exercise for the total study group (P<0.001) as well as for the young (P<0.05) and the older group (P<0.01) were evident for both osteonectin and

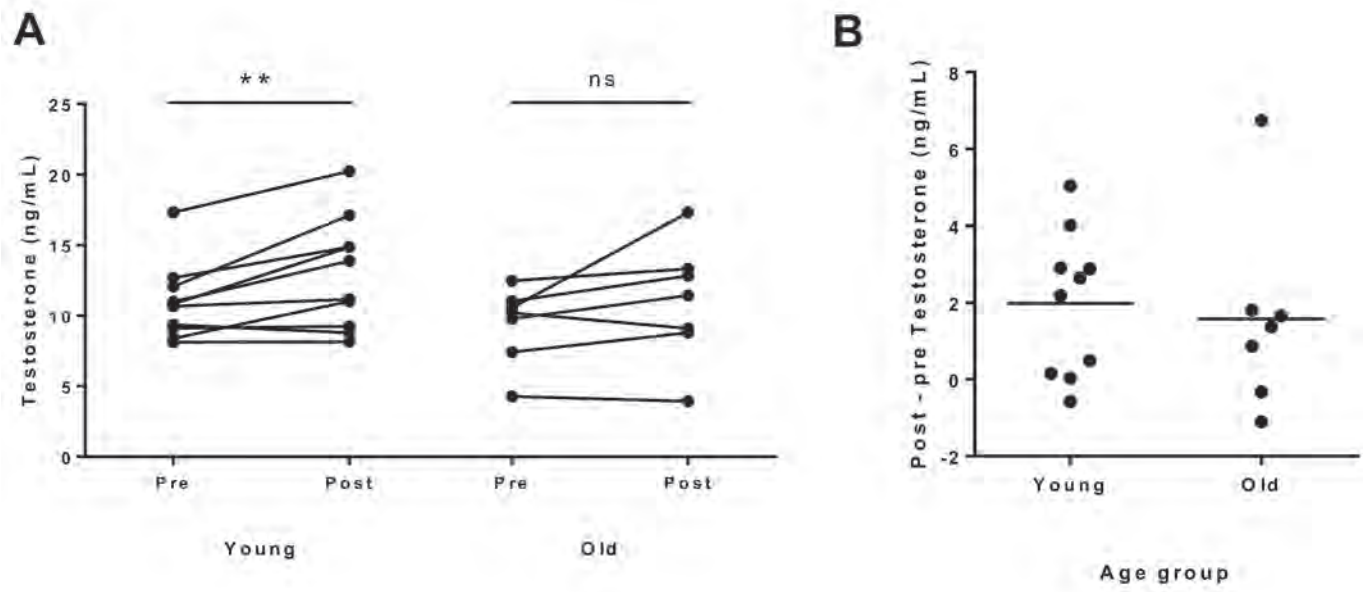

Fig. 3. Changes in the serum concentrations of testosterone from pre- to post-exercise in the young and older adults.

Univariate scatterplots of the level of testosterone (A) in pre- or post-exercise serum samples from each participant grouped on their age group (young n = 10, old = 7). Each connected set of dots represent a different individual. Fig. 3B shows the change in the testosterone levels post-exercise for each individual. A two-way repeated measures ANOVA was used to compare between the pre- and post-exercise response for each age group and interaction effects of exercise and age. A paired T-test showed that testosterone increased in the young group. ns = non-significant (*P*>0.05); **, *P*<0.01.

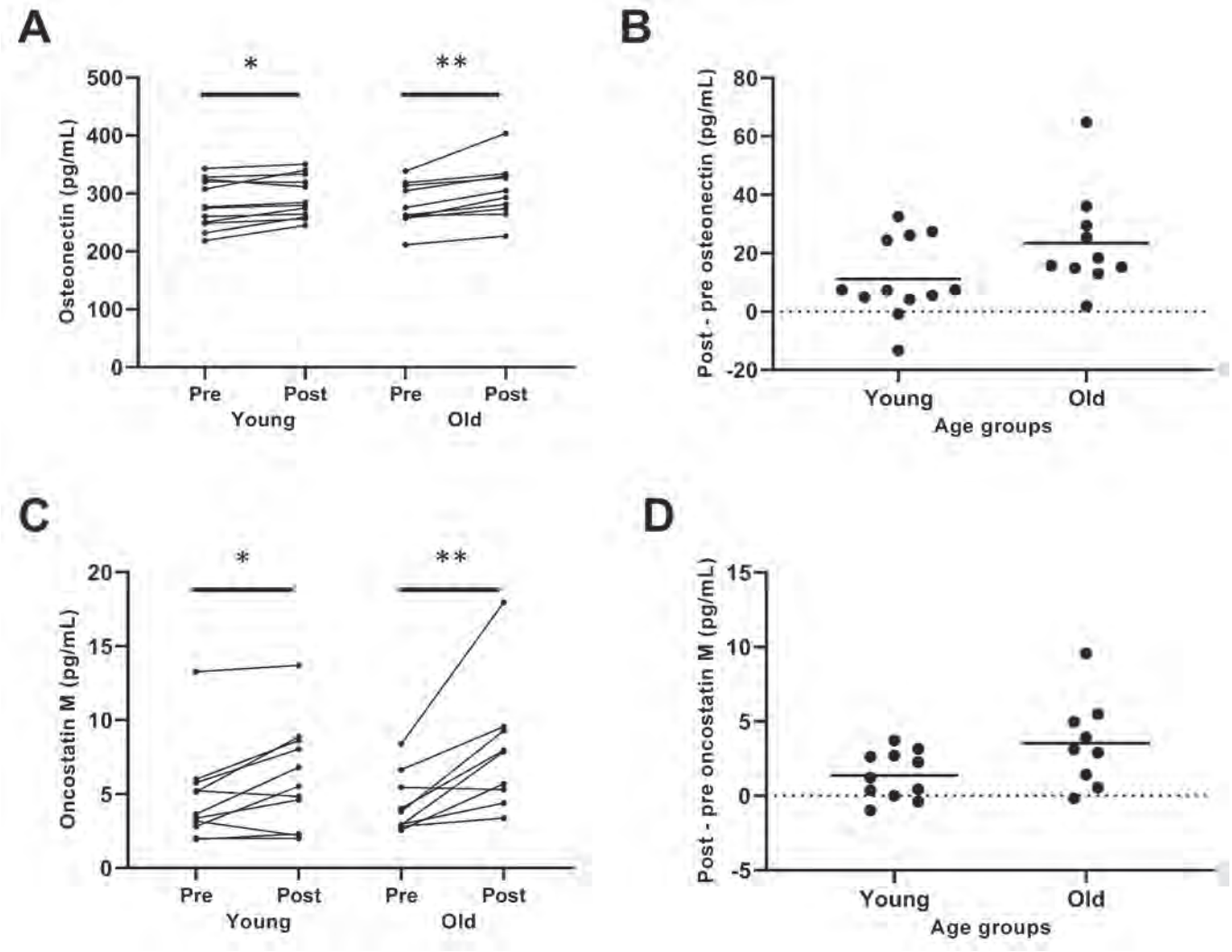

Fig. 4. Changes in the serum concentrations of osteonectin (SPARC) and oncostatin M (OSM) from pre- to post-exercise in the young and older adults.

Univariate scatterplots of the level of osteonectin (A) and OSM (C) in pre- or post-exercise serum samples from each participant grouped on their age group (young n=12, old n=10). Each connected set of dots represent a different individual. The change in the osteonectin and OSM levels post-exercise for each individual are represented (B, D), with the mean depicted as a horizontal line. A two-way repeated measures ANOVA was used to compare between the pre- and post-exercise response for each age group and interaction effects of exercise and age. *; $P<0.05$; **, $P<0.01$.

OSM. There was no effect of age or interaction effect of exercise and age. The pre- to post-exercise changes in the serum osteonectin and OSM concentrations in both age-groups are shown in Figure 4. Serum levels IL-6, IL-15 and irisin were not consistently detectable in all samples or did not change after exercise (data not shown).

DISCUSSION

The most important finding of this study was that, compared with pre-exercise serum, serum collected from older men after a single bout of exercise reduced the metabolic activity of (androgen-responsive) LNCaP prostate cancer cells *in vitro*. By contrast, no such effect was observed after treatment of the cancer cells with pre- versus post-exercise serum collected from young men. Furthermore, there was no difference between the effects of pre- versus post-exercise serum on the total amount of DNA of LNCaP and PC3 prostate cancer cells. Exercise elicited an increase in the serum levels of osteonectin (also known as SPARC) and oncostatin (OSM) in both age groups, while serum testosterone concentrations increased only in the young adults following the exercise trial.

Prostate cancer is the second most common cancer in men and the incidence of the disease increases with advancing age (1). For examining whether acute exercise influences the capacity of serum from young versus older men to modify prostate cancer cell viability *in vitro*, we used two commonly used prostate cancer cell lines, LNCaP and PC3 cells. A major difference between these prostate cancer cells lines is that unlike the PC3 cell line, the LNCaP cell line is responsive to androgens, including testosterone and testosterone metabolites (28). The androgen signalling-axis plays a central role in both prostate physiology and in the pathogenesis of prostate cancer (29, 106). Androgens, particularly testosterone, likely contribute to prostate cancer growth during advanced stages of the disease (20, 100). However, contrary to the more traditional concept that high serum testosterone levels could be a risk fac-

tor for prostate cancer, more recent evidence suggests that high levels or variations in serum concentrations of androgens (including testosterone) within a 'normal' physiological range are not associated with an increased risk of developing prostate cancer (37, 78, 100, 101, 136). Experimental and epidemiological data indicate a complex relationship between systemic concentrations of androgens and the growth versus the differentiation of a prostate tumour (100, 101). A variety of mechanisms, including pre- and post-receptor regulation and intra-tumoural androgen synthesis, contribute to the complexity of dysregulated androgen signalling in prostate tumourigenesis (29). Furthermore, various cytokines and growth factors, such as IL-6, IL-8, epidermal growth factor receptor and insulin-like growth factor 1 (IGF-1), have been implicated in the crosstalk with androgen receptors in prostate cancer (29). Blood testosterone concentrations in young, healthy men have been reported to either increase or decrease after acute endurance exercise, dependent on the exercise duration and intensity (79, 125). Data also suggest that regular endurance training can help to counteract the age-related decline in basal systemic testosterone concentrations in middle-aged and older men (53, 109). In our study, serum testosterone concentrations increased in response to the exercise trial only in the young participants, but not in the older adults. The lack of an acute response of testosterone to exercise in the older men might reflect a reduced capacity to produce or secrete testosterone with age, as was suggested by a previous study (3).

With regards to the serum effects on prostate cancer cells, in our study, post-exercise serum (compared with pre-exercise serum) from the older men inhibited the metabolic activity of androgen-responsive LNCaP (but not that of androgen-unresponsive PC3) prostate cancer cells. Contrary to previous findings by Rundqvist et al. (105), exercise did not influence the capacity of serum from younger men to affect the viability or metabolism of either of the prostate cancer cells. Various biological mechanisms might have played a role in the discrepancy between the findings of these studies. For example, age-dependent interactive effects of androgens with cytokines and growth factors on the LNCaP prostate cancer cells following exercise may be a potential mechanism contributing to the differences in the effects of post-exercise serum from young versus older men. As a possible explanation, the exercise-induced increase in serum testosterone in the young adults might have influenced the cell metabolic activity and 'masked' inhibitory effects of certain cytokines or myokines. Different assays used to assess prostate cancer cell growth as well as different incubation conditions might also account in part for the disparity between the studies.

Another important observation of our study was that serum levels of two candidate cancer-inhibitory myokines, OSM and osteonectin, increased in both younger and older men following exercise. The outcome of a previous study has suggested that OSM might suppress cancer cell growth (57). By incubating a human mammary cancer cell line (MCF-7) with serum collected from mice after exercise, Hojman et al. showed that the exercise-conditioned serum inhibited the proliferation and increased caspase activity of the cancer cells (57). Additional mechanistic investigations indicated that incubating these cells with recombinant OSM inhibited cell proliferation and induced apoptosis, whereas adding anti-OSM-antibodies to the cell media reduced the induction of caspase activity (57). Moreover, OSM was upregulated in the mouse muscle and increased in the serum after exercise, suggesting that OSM is a myokine (57). Although OSM displays contrasting roles in cancer (dependent, at least partly, on the investigated tissue compartment) (60), the findings by Hojman et al. suggest that OSM is a possible candidate myokine mediating an inhibitory effect on mammary cancer cell growth (57). Our data tend to support the concept that OSM is a candidate factor that inhibits the viability or metabolism of cancer cells. However, considering that the metabolic activity of LNCaP prostate cancer cells only decreased after incubation with post-exercise serum from the older group, the present study points toward a context-dependent nature of such effects. Such context-dependent effects might relate to the differential serum testosterone responses between the two age groups, interactions between OSM and testosterone or other androgens, and the different physiology (e.g., androgen responsiveness) of the two different prostate cancer cell lines.

Notably, our study appears to be the first showing an exercise-induced increase in blood serum levels of osteonectin in both young and older humans. A previous study by Aoi et al. has identified this matricellular protein as a myokine secreted by skeletal muscle contractions into the blood circulation in mice and healthy young men (6). Using a colon cancer mouse model, it was shown that regular exercise inhibited colon tumourigenesis in wild-type mice but not in osteonectin-null mice, suggesting an anti-tumourigenic effect of osteonectin (6). Similar as for OSM, the data from the present study do not reveal any apparent effects of the serum osteonectin response to exercise and the viability and metabolic activity of the prostate cancer cells after treatment with pre- to post-exercise serum. Osteonectin is a multifunctional protein that regulates cell-cell and cell-matrix interactions in a cell type- and context-dependent manner (123). Future research may, therefore, focus on factors that influence the effects of osteonectin, for example, on different (cancer) cell types and in different tissue environments.

In both age groups, the exercise trial induced changes in blood leukocyte concentrations, i.e., increases in total leukocytes, lymphocytes and granulocytes (including neutrophils). These changes in the cellular immune system are characteristic for moderate to more intense endurance exercise (90, 129). On the contrary, serum levels IL-6, IL-15 and irisin were not detectable or did not change immediately after exercise. As one possible explanation, the assay used might have lacked the sensitivity to detect potentially minimal serum changes in these myokines. For example, available human data on changes in the plasma/serum concentrations of IL-15 following acute exercise suggest that increased IL-15 levels are small (88, 122). Furthermore, while there has originally been some controversy about the specificity and methods used to measure irisin (4, 22), irisin has meanwhile been identified in human plasma by mass spectrometry (59). However, the reported increases in circulating irisin levels after exercise training have also been relatively small (59). Considering that these myokines only increase to such small extents, we might have missed peak concentrations, in case IL-15 and irisin increased in a slightly delayed manner after exercise. With regards to IL-6, the lack of a detectable increase in the serum after exercise was somewhat surprising. Most (but not all) studies that have assessed systemic IL-6 responses to acute exercise have shown increases in IL-6 plasma or serum concen-

trations, dependent on the combination of mode, intensity and duration of exercise (as discussed below) (22, 88, 92). The few studies that did not find an increase in plasma or serum IL-6 levels mostly involved cycling at a more moderate intensity (22, 69). It remains possible that the combination of mode (i.e., cycling), intensity and duration (20 min at 50% $\dot{V}O_{2\ max}$ followed by 40 min at 65% $\dot{V}O_{2\ max}$) in this study was not sufficient to elicit a detectable systemic IL-6 response.

It is also worth mentioning that, taking into account the age-associated decline of $\dot{V}O_{2\ max}$ (50), the fitness level of the older study participants based on their $\dot{V}O_{2\ max}$ was relatively high (i.e., 38.95 ± 8.4 mL/kg/min; mean ± SD), as compared with the younger participants (44.1 ± 7.3 mL/kg/min). This somewhat higher training status of the older individuals, relative to the young group, may have contributed to the observed effect of post-exercise serum on the LNCaP cells. Higher physical activity levels and regular exercise training might influence serum concentrations of cytokines, myokines and growth factors both at rest and following acute exercise (22). Dependent on what serum factors are considered, training might induce a greater or a smaller acute response to exercise. For example, as discussed below, there is some evidence suggesting that long-term training might attenuate the pro-inflammatory cytokine response to a single bout of exercise in older animals and humans (75, 85).

A limitation of the current study was the lack of data on whether the treatment with pre- and post-exercise serum also affects the metabolism and viability of normal prostate cells. Originally, we planned to compare the effects of human serum on prostate cancer cells versus non-tumourigenic human prostatic epithelial cells (i.e., the RWPE-1 cell line; ATCC® CRL-11609™). However, due to technical problems with the culturing and serum treatment of this normal prostate cell line, we were not able to provide evidence on effects on these cells. The findings by Rundqvist et al. suggested that the growth-inhibitory effect of exercise-conditioned serum from healthy men was specific for prostate cancer cells, but did not influence the growth of fibroblasts (105).

Together, our findings contribute important new information on the effect of a single bout of exercise on the capacity of serum, collected from young versus older men, to modify the metabolic activity of androgen-responsive prostate cancer cells. These data provide tentative support of the notion that acute exercise elicits beneficial effects against the viability of prostate cancer cells specifically in older men. Additional investigations are required to substantiate the impact of ageing on these effects, and to further investigate mechanisms contributing to the differential responses of the androgen-responsive versus the androgen-unresponsive prostate cancer cells. Future research may also focus on examining potential interactive effects of androgens with key candidate cancer-inhibitory cytokines/myokines such as OSM and osteonectin. Furthermore, as discussed in our review below, another emerging aspect that warrants further investigation is the exercise-dependent interplay of serum factors with the cellular immune system.

Mechanisms linking age-related changes of the immune system to cancer development

Ageing, immune function, and cancer development

The incidence and prevalence of most cancers, in particular breast, colon and prostate cancer, increase with ageing (9, 64, 70). Various age-associated molecular, cellular and physiological changes have been proposed to contribute to the increased cancer risk with advancing age (9). At a cellular level, for example, underlying ageing processes such as increased oxidative stress, macromolecular damage, genomic instability, cellular senescence, and dysregulated cellular growth and differentiation might affect the development and growth of cancer (9, 18, 41, 49). Over the past years, there has been increasing research interest in the putative role that age-related alterations in the immune system play in the increased cancer incidence and prevalence in older adults (34, 43, 68). Advanced age is associated with remodelling and dysregulation of the immune system, which is commonly referred to as 'immunosenescence' (34, 68, 87). The age-associated decreased immune competence likely results from lifelong exposure to antigens and pathogens (particularly latent cytomegalovirus or CMV infection), intrinsic changes in immune cells and genetic predisposition (40, 68). Bone marrow ageing and thymic atrophy also contribute to the age-related impairment in the development and function of immune cells, such as T- and B-cells (7, 67). Longitudinal studies in octo- and nonagenarians have shown that age-related changes in various robust immune variables are associated with clinical outcomes, including frailty and mortality (2, 119, 130, 131). Based on the results of these studies, a cluster of immunological variables that are predictive of mortality and accepted as 'hallmarks' of human immune ageing has been established (68, 87). Referred to as the 'Immune Risk Profile' (IRP) (68, 87), this cluster includes variables indicative of T-cell senescence, such as an inversion of the peripheral blood $CD4^+$ to $CD8^+$ T-cell ratio (as discussed below). A causal relationship between the decline in the normal functioning of the immune system with ageing and cancer has not been established (43, 44). However, various age-related immune changes may contribute to cancer development and progression in older adults (43, 44).

The potential link of 'immunosenescence' with cancer development

The phenotypic and functional changes in both the innate and the adaptive immune systems with ageing and the physiological mechanisms underlying these changes have been comprehensively covered elsewhere (7, 43, 67, 68, 111). This review focuses instead on some of the age-associated alterations in the immune system that have been suggested to play important roles for cancer development. An increasing body of evidence from clinical epidemiology and experimental studies in animal models support the notion that a fully functioning immune system is central for counteracting carcinogenesis (49, 73). In a process that is termed immunosurveillance, both the innate and adaptive cellular arms of the immune system track, recognize and eliminate antigens and abnormal cells (43, 49). Effective immunosurveillance recognizes and eliminates premalignant lesions and cancer cells before the formation of a clinically recognizable tumour (43). Among the key components of immunosurveillance against cancer development are cytotoxic immune cells, specifically natural killer (NK) cells and CD8+ T-cells (44). Natural killer cells are cytotoxic effector cells of the innate immune system that, under normal physiological conditions, can recognize and eradicate tumour cells without prior antigen exposure (116). Age-related changes in the phe-

notypic and functional characteristics of these cells are therefore likely to contribute to the survival of cancer cells and increased cancer risk with ageing (43, 44, 68). With regards to NK cells, ageing is associated with a phenotypic redistribution of NK cell subsets characterized by decreased numbers of more immature and more cytotoxic $CD56^{hi}$ NK cell subtypes, coupled with increased numbers of more secretory $CD56^{low}$ NK cell subtypes (19, 44, 111). Furthermore, ageing is accompanied by an impairment in the NK cell cytotoxicity at the single cell level (54). These alterations may impair the efficacy of NK cells to control transformed cells with advancing age (44, 68). In addition to an inverted $CD4^+$ to $CD8^+$ T-cell ratio, key features of T-lymphocyte senescence include: decreased proportions of naïve T-cells (34); increased proportions of T-cells expressing surface markers associated with a late differentiated, exhausted and senescent phenotype (34, 68, 116, 118); increased secretion of pro-inflammatory cytokines by T-cells (7, 34); an imbalance in helper T cell phenotypes, with a shift toward type 2 (Th2) > type 1 (Th1) cells (43, 116); decreased T-cell proliferative responses (7, 34, 68, 116); and decreased $CD8^+$ T-cell cytotoxicity (34, 44). Together, these age-related alterations are indicative of a restricted repertoire and functional capacity of T-cells (43, 116). All of these changes might contribute to an impaired immune response against cancer development, but the decreased killing functions of $CD8^+$ T-cells is specifically a central aspect in this context (44).

Ageing is also associated with impairments in Toll-like receptor (TLR) signalling (103, 111, 127). As a critical component of cellular innate immune function, TLRs recognize pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). They also link innate and adaptive immune responses (24, 62, 111). By sensing of danger and damage signals originating for example from pre-malignant lesions, TLRs are important for an effective immunosurveillance against carcinogenesis (43, 133). Age-associated decreases in TLR functions may therefore not only contribute to the impairment in immune responses to infectious diseases and vaccination, but also to a decreased capacity to recognize and eliminate abnormal and transformed cells in the elderly (127).

Other aspects of immune ageing that might contribute to a reduced immunosurveillance against cancer development include impaired dendritic cell functions (potentially contributing to a decreased presentation of tumour antigens to T-cells) (21, 44, 68); an increase in immunosuppressive immune cells such as regulatory T cells and myeloid-derived suppressor cells (which might suppress anti-tumour T-cell responses) (43, 44, 48); and decreased effector functions of neutrophils and monocytes/macrophages (43, 55, 111). Regarding the age-dependent dysregulation of innate immunity, a more general characteristic is that innate immune cells are already activated in the basal state, whereas their responses to additional stimulation are impaired (44, 111). In accordance with the presence of a stronger inflammatory milieu (as discussed below), ageing is associated a shift in monocyte subpopulations towards more pro-inflammatory phenotypes, and an increased production of pro-inflammatory cytokines by monocytes (8, 30, 44, 111).

'Inflammageing' and cancer

Another central feature of ageing that is, in part, affected by immunosenescence is chronic low-level inflammation, also termed 'inflammageing' (34, 40). Inflammation is an integral component of the innate immune response to infectious pathogens and tissue damage, but the resolution of inflammation is central for restoring homeostasis (16, 89, 129). Inflammageing describes a state of systemic inflammation that is unresolved and associated with a two- to four-fold increase in the circulating levels of inflammatory mediators such as cytokines (e.g., tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6) and acute-phase proteins (e.g., C-reactive protein (CRP)) in older individuals (15, 16, 40). The exact aetiology underlying this age-associated chronic inflammation is incompletely understood (40). Potential mechanisms that contribute to unresolved inflammation with ageing include accumulating macromolecular and cell damage, dysfunctional mitochondria, cellular senescence, an imbalance in the gut microbiota, and the accumulation of visceral fat (16, 40, 91). Each of these sources activates a network of inflammatory pathways. In addition, as described above, the dysregulation of components of the innate and adaptive immune systems, such as increases in basal immune cell activation and pro-inflammatory cytokine production, play an important role in chronic inflammation with advancing age (16, 40, 68, 111). Although a causal relationship has not been established, there is evidence suggesting that chronic low-grade inflammation is a major contributor to the pathology of several age-related conditions and chronic diseases, including cancer (16, 40, 91, 131). Chronic inflammation may contribute to cancer development by enhancing oxidative stress and by altering the transcriptional regulation of cytokines, oncogenes and tumour suppressor genes (40, 44). Unresolved inflammation also supplies or activates various bioactive molecules within the tumour microenvironment that promote tumour growth (49). These molecules include growth factors that enhance proliferative signalling; anti-apoptotic factors that suppress (cancer) cell death; extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis; and signalling factors that activate the epithelial-mesenchymal transition (a cellular program that broadly regulates invasion and metastasis) (49).

Exercise training improves or preserves the normal functioning of various components of the immune system in older adults (116). Furthermore, acute exercise produces an anti-inflammatory environment in the body, and exercise training mediates additional anti-inflammatory effects in the long-term (91, 129). In the following sections, we discuss available evidence whereby exercise may contribute to counteracting cancer development through modulating immune function and inflammation in general, and in particular in the ageing population.

The emerging role of exercise immunology in cancer prevention and survival

Epidemiological evidence linking physical activity and exercise with a decreased cancer risk

There is strong evidence from epidemiological research showing that physical activity reduces the risk and improves the survival for several cancers, including some of the most common cancers (1, 64, 70, 74). A recent systematic review of 45 reports comprising hundreds of epidemiologic studies with several million study participants has indicated that physical activity is associated with an approximate 10 to 20 per cent

reduction in the relative risk for cancers of the breast, colon, endometrium, bladder, stomach, oesophagus (adenocarcinoma) and kidney (74). Notably, the levels of physical activity that were associated with this risk reduction corresponded with the amount of physical activity recommended in the US 2018 Physical Activity Guidelines Advisory Committee Scientific Report (i.e., at least 150 to 300 minutes of moderate-intensity aerobic activity plus muscle-strengthening activity at least two days each week (110)) (74). Furthermore, a dose-response relationship between physical activity and specific cancer risk was evident for several cancers, with the strongest evidence for a dose-dependent reduction in the risk for breast and colon cancers (74). Data from randomized clinical trials also support the beneficial effects of physical activity for cancer primary prevention and the reduction in the risk of disease recurrence (42, 63). Potential mechanisms underlying the benefits of physical activity or exercise for protection against cancer include exercise-dependent reductions in cancer risk factors such as sex hormones, metabolic hormones, and pro-inflammatory factors, as well as improved immune function (73). Over the past few years, there has been increasing evidence in support of the concept that the exercise-dependent regulation of the immune system and inflammation plays a central role in counteracting cancer (56, 58). Recent experimental studies have also provided important mechanistic information in agreement with this concept (95).

Exercise-induced effects on the cellular immune system that might counteract cancer

Changes in the number and composition of blood leukocytes are one of the most prominent effects of acute exercise bouts on the immune system, most likely as a result of hemodynamic shear stress and in response to catecholamines, glucocorticoids and cytokines (90, 94, 129). Being an integral part of the physiological stress response to exercise, these changes in the cellular immune system likely reflect a redeployment of specific immune cell subtypes, for example, out of the blood to peripheral tissues that require enhanced immunosurveillance following physical stress (90). Importantly, acute exercise preferentially mobilizes immune cells with potent effector functions, including NK cells and $CD8^+$ T-cells, both of which are highly cytotoxic against tumours (as discussed above) (90, 115). A recent preclinical study by Pedersen et al. demonstrated how important the exercise-induced mobilisation and redistribution of NK cells are for counteracting tumour incidence and progression (95). Using a model of voluntary wheel running in mice, this study showed that six weeks of exercise training reduced tumour onset and growth across different tumour models by at least 60 per cent, as compared with non-exercising control conditions (95). Additional mechanistic experiments within this study suggested that these protective effects resulted from the exercise-dependent mobilization of NK cells, their infiltration into the tumours and the subsequent destruction of tumour cells (95). Exercise also appears to "prime" effector functions of immune cells, thereby enhancing immunosurveillance (90, 115). As shown by a study in healthy cyclists, NK cells present in the blood during exercise recovery, as compared with resting conditions, are more efficient killers of various cancer cell lines *in vitro* (12). However, it is poorly understood how exercise mediates tumour-killing NK cell functions.

Furthermore, the mobilisation, redistribution, transcriptional reprogramming and functional changes of neutrophils following acute bouts of exercise are among the most pronounced exercise-induced effects on the cellular immune system (80, 90, 129). Neutrophils and their heterogeneity are suggested to play an important role in cancer (81). Additional research may, therefore, focus on whether the beneficial effects of exercise against cancer are mediated through regulation of neutrophils (56).

Experimental evidence linking myokines with cancer protection

The concept that skeletal muscle is an endocrine organ provides another important mechanistic basis for linking exercise immunology with cancer protection (34, 56, 91). Contracting muscle is capable of producing and releasing several hundred cytokines and other peptides with autocrine, paracrine and endocrine effects, referred to as myokines (88, 91). Several myokines have immune regulatory functions, including, for example, IL-6, IL-7 and IL-15 (56, 91, 93). Other muscle-derived endocrine factors, such as OSM, irisin, and osteonectin (also known as secreted protein acidic and rich in cysteine (SPARC), may mediate cancer-inhibitory effects of exercise more directly by controlling growth kinetics of cancer cells (57, 93) (discussed below).

The first identified and most studied myokine is IL-6 (92). The magnitude of the exercise-induced increase in plasma IL-6 is related to exercise duration, intensity, the muscle mass involved during exercise, and muscle glycogen levels (92). In addition to its potent metabolic effects, IL-6 likely also mediates some of the anti-inflammatory and immune-regulatory effects of exercise (93, 120, 121, 129). IL-6 is commonly used as a marker of inflammatory status and associated with various age-related pathologies that have a strong chronic inflammatory component (40, 131). However, the transient response of IL-6 and other cytokines to exercise is markedly different from the cytokine cascade induced in pathological situations (92, 93, 129). For example, in contrast to inflammatory responses to sepsis or chronic low-grade inflammation, the IL-6 production by contracting muscles does not involve the activation of the pro-inflammatory nuclear factor- pathway. Nor does muscle-derived IL-6 stimulate the production of the pro-inflammatory cytokine TNF-α (92, 97). Under physiological conditions such as during exercise, muscle-derived IL-6 has metabolic and anti-inflammatory effects (92, 97). The acute increase in plasma IL-6 in response to exercise is followed by increases in the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1ra) and IL-10 (79, 80, 97). Experimental data support a direct role of IL-6 in these anti-inflammatory effects. For example, it has been shown that infusion of IL-6 at concentrations corresponding to levels obtained after exercise enhances plasma levels of IL-1ra and IL-10 and inhibits TNF-α production in healthy humans (120, 121). Muscle-derived IL-6 also contributes to the exercise-induced redeployment of leukocytes (121). The study by Pedersen et al. (described above) showed that exercise-induced increases in IL-6 played a central role in regulating NK cell trafficking and NK cell-dependent tumour control (43).

Interleukin-7 has also been identified as a myokine (51). It is also produced in the thymus and is essential for the development and survival of T-cells and NK cells (107, 113, 128).

Another myokine with immune-regulatory properties is IL-15 (91). In addition to its metabolic effects, IL-15 is an important proliferative and activating factor for T-cells and NK cells (107, 128). In a clinical trial, IL-15 administration positively affected lymphocyte homeostasis and immune responses in cancer patients (26). However, at present there is only little information available on whether IL-7 and IL-15 responses to exercise have clinical implications (35).

In addition to these observed effects of myokines on immune cell activity, some experimental studies suggest that specific myokines affect cancer cell viability and tumour growth kinetics in a more direct manner (58). In a preclinical study by Hojman et al. (57), post-exercise blood serum collected from mice inhibited mammary cancer cell proliferation and induced apoptosis of these cells *in vitro* (29). Further mechanistic experiments suggested that OSM might be a key candidate myokine mediating the observed inhibitory effects on cancer cell growth (57).

Moreover, osteonectin has been identified as another novel myokine (6, 23). It was shown that osteonectin inhibits colon cancer cell growth *in vitro* and reduces colon tumourigenesis in exercising mice (6). Both the transcription and translation of osteonectin was downregulated in skeletal muscle of old sedentary mice as compared with young sedentary mice, suggesting an impact of ageing on the expression of osteonectin (6). In an experimental model, enhanced growth of pancreatic tumours has been observed in osteonectin-null mice (14). Osteonectin is involved in both physiological processes such as development and tissue remodelling, and pathological conditions such as cancer (123). Considering that osteonectin is also expressed by other tissues (including neoplastic tissues) and that it might also favour tumourigenesis (123), additional investigations examining factors that influence the function of osteonectin (e.g., different microenvironments) might help to better explain its contrasting roles in cancer.

In general, several human studies have shown that blood serum collected after acute exercise from healthy men (105), breast cancer patients (32), and colorectal cancer survivors (33) inhibits the growth of prostate cancer cells (105), breast cancer cells (32), and colon cancer cells (33). These data support the conceptual framework that exercise-induced changes in the blood, likely through the secretion of molecular factors by skeletal muscle and/or other tissues, contribute to the protective effects of exercise against cancer development (58). Although these findings are promising, to date, only a limited number of potential cancer-inhibitory myokines and other 'exercise factors' have been identified. In addition to identifying these factors, future research may also examine whether myokines play a role in the exercise-dependent enhancement of certain functions of the cellular immune system, such as the cytotoxicity of NK cells against tumours.

Multiple health benefits of exercise are mediated through long-term adaptation of physiological systems, such as skeletal muscle or the cardiovascular system, following regular physical training (39, 52). Notably, it has been suggested that the transient, but repetitive release of 'anti-oncogenic' molecular factors into the blood following single bouts of exercise may be at least as important for tumour control as chronic changes in resting blood concentrations of 'risk factors' such as pro-inflammatory cytokines (32, 33, 56, 58). This notion agrees with the data from some of the aforementioned *in vitro* serum incubation studies. Serum collected after acute exercise inhibited tumour growth kinetics, while serum obtained in resting conditions following longer-term exercise training did not (32, 33).

Exercise-dependent regulation of inflammation and anti-inflammatory effects in the context with cancer protection

An increasing body of evidence suggests that the anti-inflammatory effects of exercise are of major importance for health (39, 91, 129). Considering the association of cancer with low-grade chronic inflammation (as described above), it is therefore likely that exercise-associated anti-inflammatory effects also play an important role for counteracting cancer (56, 91, 129). It is widely recognized that regular exercise exerts its anti-inflammatory effects by producing an anti-inflammatory environment with each bout of exercise, and through a reduction of visceral fat (the latter of which is pro-inflammatory in nature, e.g., by secreting pro-inflammatory cytokines and adipokines) (91, 93, 97, 129). The anti-inflammatory effects of single exercise bouts are, at least partially, mediated by the release of myokines, such as IL-6, into the blood circulation (92, 93, 97). As described above, the IL-6 release from contracting skeletal muscle has anti-inflammatory effects, e.g., by stimulating the production of IL-1ra and IL-10 by blood mononuclear (immune) cells (92, 97, 121).

Numerous studies have investigated the systemic responses of cytokine and other inflammatory mediators to exercise (88, 129). Dependent on various factors, including exercise duration and intensity and the extent of exercise-induced muscle damage, exercise induces a transient increase in cytokines/myokines (e.g., IL-1ra, IL-6, IL-8, IL-10) and acute-phase proteins (e.g., CRP) in the circulation (79, 80, 88, 129). While skeletal muscle cells within contracting muscles are the main contributors to the exercise-induced increase in circulating IL-6, other potential cellular sources of circulating cytokines include cells within the microvasculature, fibroblasts and leukocytes (88). The changes of cytokines and acute-phase proteins in the circulation following exercise likely are an important part of the body's response to the exercise-associated physiological demands (88). Under non-pathophysiological conditions, even a more pronounced immune-endocrine, cytokine and stress response to a single bout of strenuous exercise is a tightly coordinated and dynamic process that is followed by counter-regulatory and anti-inflammatory mechanisms (90, 129). Furthermore, regular exercise training and a high degree of physical fitness appear to attenuate the systemic inflammatory response to acute exercise (65), which might reflect adaptive mechanisms for counteracting 'overshooting' inflammation. In the context with cancer, such robust, but rapidly resolved systemic inflammatory responses to repeated bouts of exercise might enhance the clearance of transformed cells and nascent tumours (56). Moreover, chronic exercise training might 'shape' acute inflammatory responses in a manner that facilitates the protection from carcinogenesis (56). In a rodent model, the acute inflammatory response to a liver carcinogen was attenuated and more rapidly resolved after voluntary wheel running for six weeks, leading to a faster and more effective clearance of damaged liver cells as compared with less active mice (11).

In contrast to the transient response of inflammatory mediators to acute exercise, data from cross-sectional and interven-

tion studies suggest that regular physical training is associated with lowered systemic levels of inflammation markers in resting conditions (42, 47, 84, 91, 97). For example, a randomized controlled trial that involved 400 previously inactive, healthy women aged 50 to 74 years, showed reduced basal blood concentrations of CRP and IL-6 after a year-long exercise training (42). Moreover, a stronger reduction of CRP and IL-6 was observed with increased weekly exercise time (42).

Collectively, there is some evidence suggesting that the cumulative effect of repeated responses of cytokines/myokines to acute exercise along with the regulation of inflammation by regular exercise are important mechanisms that contribute to counteracting tumour growth and progression (32, 33, 56, 58). Long-term exercise training likely also contributes to a reduced risk of cancer development by suppressing chronic inflammation (58, 91).

Beneficial effects of exercise on the ageing immune system and the potential role of these effects in counteracting cancer with advancing age

Immune-protective and anti-inflammatory effects of exercise in older adults

Accumulating evidence suggests that regular exercise or physical activity improves immune function in the older population (34, 116). A causative link between exercise-associated benefits for immune health and the risk of chronic disease, especially cancer, has not been established to date (34, 126). Nevertheless, cross-sectional data show that several of the immune biomarkers that are key components of the IRP cluster (e.g., T-cell responsiveness to mitogens, naïve/memory T-cell ratio) are positively displayed in physically active compared with sedentary elderly individuals (83, 114, 116, 118). In agreement with the concept that exercise has immune benefits in older adults, prospective population-level studies have shown that regular physical training reduces the risk of infection, as compared with sedentary behaviour (34, 86). Data from clinical trials indicating that increased physical activity enhances the efficacy of vaccinations in aging humans also support this concept (135). Furthermore, as discussed above, data from both cross-sectional and intervention studies suggest that regular exercise is associated with lowered systemic levels of inflammation markers in healthy individuals and in populations at-risk of diseases associated with low-grade inflammation, such as the elderly (42, 47, 84, 91, 97). In a recent animal study, Nilsson et al. observed that lifelong aerobic exercise alleviated systemic inflammation, including key drivers of the cytokine cascade and tumour progression (TNF-α, IL-6, and IL-1β), and protected against several different types of cancer in naturally-aged mice (85). The extent to which these immune-protective and anti-inflammatory effects of exercise contribute to a decreased cancer risk in older humans is unknown (34, 126). However, various components of the immune system that improve with exercise in older adults, such as increased cytotoxic functions of NK cells and $CD8^+$ T cells, play important roles for immunosurveillance against cancer (34, 44).

Exercise-induced effects on the cellular immune system in older adults

Some, but not all, studies investigating the effects of regular physical training on NK cell number and function in healthy, older individuals reported benefits of exercise (17, 83, 116, 134). Compared with their sedentary, less fit counterparts, elderly women with a high aerobic capacity were shown to have superior NK cell cytolytic activity, despite similar NK cell numbers in the blood (83). *In vitro* NK cell cytotoxicity against a target cell line (human leukemic K562 cells) was higher in a group of sedentary men and women (aged 650.8 years) after six months of aerobic exercise training (134), but not in postmenopausal women (aged 50–75 years) after a 12-month intervention involving aerobic exercise training (17). Furthermore, 12 weeks of moderate aerobic exercise training did not improve NK cell function in previously sedentary women aged 67–85 years (83). Only a few studies have investigated the effects of exercise on NK cells in cancer patients. For example, 15 weeks of aerobic exercise training increased NK cell cytotoxic activity in postmenopausal breast cancer survivors (aged 596 years) (38), while eight weeks of combined aerobic and resistance exercise did not alter NK cell cytotoxicity against K562 cells in breast cancer patients (aged 35–72 years) (82). Among other influencing factors, the mixed results among these studies are likely due to differences in exercise doses and modes, the duration of the training interventions, and the diversity of the initial health and fitness status of the participants. Of note, comparable reductions in tumour volume with exercise were observed in adult and old mice in the aforementioned experimental study by Pedersen et al. (95), suggesting a similar capacity of exercise to enhance tumour-killing NK cell functions in both age groups. More research is needed to examine under which conditions (particularly concerning the individuals' health status) exercise training is most efficient for augmenting cytotoxicity of NK cells against cancer cells. Additional translational research is also required to verify the interplay of exercise and ageing on these 'anticancer' functions of NK cells in humans. Further benefits of exercise training on the innate immune system in older adults include lower monocyte subpopulations displaying a more pro-inflammatory phenotype ($CD14^+CD16^+$) (124), and improved neutrophil migratory dynamics (10). In general, however, relatively little is known about the influence of exercise training on innate immune cells, especially neutrophils, with ageing (116).

With regards to T-cells, cross-sectional data are relatively consistent in showing greater T-cell proliferative responses and lower proportions of senescent T-cells in physically active, as compared to sedentary older adults (76, 83, 116, 118). A cross-sectional study involving 102 healthy males aged 18–61 years has demonstrated that aerobic fitness ($\dot{V}O_{2\,max}$ was associated with a lower age-related accumulation of senescent $CD8^+$ T-cells (118). Detailed analysis of the data from this study suggests that the relative fitness status of individuals might have a more pronounced effect on phenotypic shifts in blood T-cells than ageing *per se* (118). A study by Duggal et al. assessed phenotypic characteristics of immune cells in 125 older cyclists (55–79 years) who had maintained a high level of physical activity for much of their adult lives, as compared with 75 age-matched, healthy individuals who were not involved in regular exercise (35). An accumulation of senescent T-cells was observed in both the sedentary and the active older adults, yet the masters cyclists displayed a higher proportion of naïve T-cells and recent thymic emigrants (indicative of a better-preserved thymus output), higher serum levels of IL-7

and lower serum levels of IL-6 under resting conditions (35). Contrary to findings in cross-sectional studies, results from longitudinal studies examining T-cell mediated-immunity in older individuals are less consistent (17, 83, 116, 134). Features of T-cell senescence were either unchanged (17, 83, 134) or improved in previously sedentary healthy elderly individuals following training interventions (134). In postmenopausal breast cancer survivors, T-cell proliferation responses were increased after a 15-week aerobic training intervention (38). Furthermore, a recent study in pre-diabetic adults (aged 57.0 ± 5.2 years) showed that 3 weeks of regular endurance exercise training stimulated the production and mobilization of naïve T-cells, while terminally differentiated effector memory cells might have disappeared by apoptosis (98). These findings agree with the concept that regular exercise mobilizes 'older' functionally exhausted/senescent lymphocytes to undergo apoptosis and create 'vacant space' for the expansion of newly functional T-cells (77, 114). Together, these findings suggest that the benefits of exercise training might be greatest in individuals who already display more profound impairments within the peripheral T-cell compartment (e.g., cancer survivors) at the beginning of an exercise intervention (116).

Effects of ageing on systemic cytokine responses to exercise

Relatively little is known about the interplay of ageing and regular physical training on the systemic changes of cytokines and myokines in response to acute exercise. In a naturally ageing mouse model, acute exercise elicited a lower pro- and anti-inflammatory cytokine response in young and old aerobically trained mice, compared with sedentary old mice (85). This might reflect a protective adaptation associated with long-term exercise training to prevent excessive inflammation (85). Available data in older humans are varied. In an earlier human study that investigated the relation of ageing and muscle-derived IL-6, healthy older men released the same amount of IL-6 from skeletal muscle as healthy young men at the same relative exercise intensity (96). This suggests that ageing skeletal muscle maintains its capacity to produce and release IL-6 into the circulation in response to exercise. In a recent study, differential systemic cytokine changes were reported in young and middle-aged non-athletes and masters athletes (aged 53.1±8.8 years) following an incremental test to exhaustion (75). Blood IL-4 and IL-6 concentration increased only in the young adults, while an increase in IL-1 concentration was observed only in the masters athletes (75). Moreover, although the concentrations of all other assessed cytokines did not change in response to exercise in any of the three groups, the TNF-α/IL-10 ratio (used as a proxy marker for the overall pro- and inflammatory cytokine balance) increased only in untrained middle-aged adults (75). Another study observed no measurable circulatory IL-6 and IL-10 response to either moderate- and higher-intensity exercise in healthy older adults with either lower or higher levels of physical fitness (132). Collectively, while there is some evidence suggesting that long-term exercise training may attenuate the pro-inflammatory response to exercise with advancing age, available data do not enable us to make more definitive conclusions about the impact of ageing on the systemic cytokine response to exercise. More research is warranted comparing these responses between older and younger adults and between older trained and untrained adults.

Methodological considerations of *in vitro* cancer cell culture assays

There are important methodological considerations for *in vitro* cell culture studies. Traditional studies in which cancer cells are cultured as two-dimensional (2D) monolayers on rigid, plastic surfaces cannot fully represent the complexity of the tumour microenvironment (71). *In vivo*, cancer cells interact not only with neighboring cancer cells, but also other cell and tissue types such as fibroblasts, adipocytes, the surrounding vasculature, as well as cells from the immune system (13). Three-dimensional (3D) cell cultures, such as tumour spheroids, are better able to recapitulate *in vivo* cellular physiology including cell-cell and cell-matrix interactions, nutrient transport properties, and oxygen and nutrient diffusion gradients (36, 99). Gene and protein expression profiles of 3D cultures are better able to recapitulate *in vivo* profiles than 2D cultures (61). Furthermore, within a 3D tumour spheroid configuration cancer cells are more reflective of the *in vivo* situation. The cancer cells form spheroids which then develop a necrotic core and an outer proliferative zone (104) and respond to stimuli differently in comparison to 2D culture systems. For example, the cancer cell response to chemotherapy, growth factors and interaction with the extracellular matrix are more physiologically relevant in 3D as compared to 2D models (36, 99). With these points in mind, future studies investigating the response of cancer cells to exercise may consider utilizing 3D cell culture systems for a more physiologically relevant *in vitro* model.

Evidence from *in vitro* or *in vivo* studies demonstrates that exercise does not completely eliminate cancer (31, 95, 105). Rather, pre-incubating cancer cells with serum collected from individuals post-exercise decreases the ability of those cancer cells to form tumours *in vivo* (i.e., the tumour take rate) or colony-forming ability in *in vitro* studies. Furthermore, pre-incubation of cancer cells with post-exercise serum slows tumour growth in *in vivo* cancer models. Culturing cancer cells in the presence of post-exercise serum decreased cancer cell growth in *in vitro* models of several cancers (31, 33, 57, 95, 105, 117, 126). As discussed above, previous studies have suggested a link between myokines and modulation of the tumour microenvironment. However, *in vitro* serum incubation studies generally only represent the effects of a single 'snapshot' in time, such as a specific time point following exercise. As such, they cannot replicate the cumulative effect of repeated acute exercise responses that may lead to tumour growth control *in vivo* (58).

Hojman et al. postulated that exercise may have a more potent effect on cancer metastatic potential, as opposed to direct control of cancer cell viability (58). In addition, exercise is reported to normalize tumour vasculature, alter the systemic immunological profile and enhance tissue-immune cell surveillance (66). Future research may therefore also focus on the effects of exercise on the tumour microenvironment, rather than only on the malignant cells themselves.

Exercise can influence the tumour microenvironment by affecting tumour metabolism, nutrient and growth factor signaling, autophagy, lactate levels, angiogenesis and the localized immune response (66). Generally, cancerous cells escape detection by suppressing the immune response within the tumour microenvironment. Recognizing the beneficial effects of exercise on immunosurveillance (as discussed above),

future investigations may, therefore, focus on this aspect by using 3D models. The immunosurveillance effects of activated NK and T cells have already been modelled in 3D spheroid culture systems, but not in the context of exercise. Co-culture studies of allogenic $CD8^+$ T and NK cells, stimulated with IL-15, and patient-derived colorectal cancer spheroids demonstrated that these immune cells can infiltrate into the cancer spheroids and concomitantly increase their expression of activation markers (CD25 and CD107a), leading to increased cancer cell apoptosis (27). Sherman et al. used a 3D lung carcinoma spheroid model to demonstrate that NK cells could migrate towards a chemotactic gradient, infiltrate the tumour spheroids and cause cancer cell destruction (112). Moreover, serum from participants engaged in acute exercise activated NK cells, leading to increased NK cell cytotoxic activity directed against lymphoma and multiple myeloma cells *in vitro* (12). Together, this suggests that exercise serum could be used to study immune cell infiltration and activity directed against cancer cells using 3D spheroid culture models.

CONCLUSION AND FUTURE DIRECTIONS

Emerging data suggest that the immune system and the 'crosstalk' of contracting skeletal muscle with immune and cancer cells through myokines mediate some of the protective effects of exercise against the development and progression of cancer. Recognizing the increased cancer risk with advancing age, more research is needed for verifying these effects in experimental and clinical settings in older humans.

In particular, more well-controlled, long-term studies measuring molecular and cellular markers combined with clinical outcomes are required to determine the immune-protective and anti-carcinogenic effects of regular exercise training in the older population. Another aspect that warrants further investigation is whether there are 'dose-dependent' exercise effects in this context. As discussed above, some evidence suggests dose-response relationships between physical activity levels and the risk for certain cancers (i.e., a decreased cancer risk with increased exercise 'doses') (74). Data from a previous meta-analysis showed that even former elite athletes, including elite endurance athletes (i.e., a population group with exceptionally high exercise loads), have a lower risk of cancer mortality than the general population (46). There is also strong epidemiological and clinical evidence that supports the widely recognized notion that the numerous health benefits of regular exercise outweigh potential negative effects of even very strenuous exercise training (such as a transient dysfunction of certain immune components (90, 129)). Future research may focus on determining whether there is an optimal range of training loads that confers the most benefits for immune function, health, and cancer protection, and whether hypothetical dose-response benefits are age-specific and change over the lifespan. Additional investigations are also required for identifying which exercise modes (e.g., endurance- or resistance exercise, or a combination of both) are most efficient in enhancing anti-carcinogenic and anti-tumorigenic effects of the cellular immune system and cytokines/myokines especially in older adults.

Future research could also take advantage of 'omics' technologies (e.g., blood plasma proteomics) and advanced bioinformatics methods (e.g., network-driven methods) to identify 'anti-cancer' candidate factors as well as linkages of such factors with functional and clinical outcomes. Furthermore, using 3D cell culture platforms will be a key extension upon previous investigations that used 2D cell culture models for examining how blood serum collected before and after exercise interventions influences the viability of cancer cells *in vitro*. Such 3D cell culture models more realistically mimic the *in vivo* tumour microenvironment, as compared with 2D structures. This is especially important with regards to the interactions of cancer cells with exercise-induced cytokines, myokines and other serum factors. Another emerging aspect that warrants further investigation is the exercise-modulated interplay between serum factors and the cellular immune system. In the light of data suggesting that exercise enhances anti-tumourigenic functions of immune cells, such as cytotoxic NK cells (12, 115), investigating whether such tumour-killing immune cell functions are affected by exercise-induced changes in the serum, will provide important mechanistic information in this regard.

The evidence in this review suggests that mobilizing cancer-inhibitory and immune-regulatory serum factors and immune cells may be a key mechanism of how regular exercise mediates its protective effects against cancer in the older population. Collectively, this is a promising and important area of research within the domain of exercise immunology. It is likely that advancing the understanding of the role of immune-endocrine responses to exercise for counteracting carcinogenesis will expand a variety of potential targets for preventing and treating cancer, and help to define more efficient exercise prescriptions especially for older adults.

AUTHOR CONTRIBUTIONS

O.N., J.M.P., J.M.H., T.J.P., and E.W.T. conceived and designed this research project; J.H.H., J.McG., G.M.M., P.D.-G., and L.R. performed experiments and contributed to the acquisition of the research data; J.H.H., J.McG., J.M.P. and O.N. analysed and interpreted the research data; J.H.H. and J.M.P. prepared figures; O.N., J.H.H., and J.McG. drafted this manuscript; O.N., J.McG., G.M.M., J.M.H., T.J.P., E.W.T., and J.M.P. edited and critically revised this manuscript. All authors approved the final manuscript version.

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Macrophage immunophenotype but not anti-inflammatory profile is modulated by peroxisome proliferator-activated receptor gamma (PPARγ) in exercised obese mice

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ABSTRACT

Moderate aerobic training may be therapeutic for chronic low-grade inflammatory diseases due to the associated anti-inflammatory response that is mediated by immune cells. The peroxisome proliferator-activated receptor gamma (PPARγ) regulates the M1 (pro-inflammatory) and M2 (anti-inflammatory) polarization, as well as the immunometabolic response of macrophages. Against this background, the present study seeks to clarify whether the conditional deletion of PPARγ in macrophages would have any effect on the anti-inflammatory role of moderate aerobic training. To test this hypothesis, two mice strains were used: PPARγ LyzCre+/+ (KO) and littermates control animals (WT). Each genotype was divided into 1) sedentary high-fat diet (HF) and 2) high-fat diet and moderate aerobic training (HFT) (n = 5–8 per group). The experimental protocol lasted for 12 weeks, comprising 4 weeks of HF diet only and 8 weeks of HF diet and aerobic training (5 times/week, 50–60 minutes/day at 60% of maximum speed). Metabolic analyses were carried out on the serum glucose homeostase, adipose tissue morphology and cytokine content, and macrophage cytokine production. Immunophenotyping and gene expression were also performed. KO male mice were more prone to hypertrophy in the subcutaneous adipose tissue, though only the IL-1β ($p = 0.0049$) was higher compared to the values observed in WT animals. Peritoneal macrophages from KO animals exhibited a marked inflammatory environment with an increase in TNF-α ($p = 0.0008$), IL-1β ($p = 0.0017$), and IL-6 ($p < 0.0001$) after lipopolysaccharide stimulation. The moderate aerobic training protected both genotypes from weight gain and reduced the caloric intake in the KO animals. Despite the attenuation of the M2 marker CD206 ($p < 0.001$) in the absence of PPAR-γ, the aerobic training modulated cytokine production in LPS stimulated peritoneal macrophages from both genotypes, reducing proinflammatory cytokines such as TNF-α ($p = 0.0002$) and IL-6 ($p < 0.0001$). Overall, our findings demonstrate the essential role of PPARγ in macrophage immunophenotypes. However, the deletion of PPARγ did not inhibit the exercise-mediated anti-inflammatory effect, underscoring the important role of exercise in modulating inflammation.

Keywords: adipose tissue, cytokines, immune system, moderate aerobic training, transcriptional factor

INTRODUCTION

Obesity is a global public health concern with a rising prevalence since the industrial revolution due to the increase in caloric intake (1). The obese adipose tissue is characterized by hypertrophy and subsequent higher lipolysis, which can cause ectopic lipid accumulations and impairments of insulin sensitivity (2). Together with the accumulation of fat depots and an increased risk of cardiovascular diseases, obesity may be a trigger to the inflammatory response characterized by a low-grade but chronic (3-4).

The long-term stimulation of innate immune cells exacerbates the release of cytokines, adipokines, and acute phase proteins (3, 5). One of the first studies describing obesity as a trigger of low-grade inflammation was conducted by Hotamisligil (6), in which the tumour necrosis factor alpha (TNF-α), a potent proinflammatory cytokine from visceral adipose tissue, was considered the link between inflammation and insulin resistance.

Although macrophages are considered the central players in local inflammation within adipose tissues, other immune cells play relevant roles in the origin and maintenance of low-grade inflammation. These include different subsets of T and B lymphocytes, eosinophils, and NK cells (7). However, of all the cells from the innate immune system, macrophages are the majority in the adipose tissue stromal vascular fraction (8). Classically activated macrophages (M1) exhibit a more glycolitic metabolism and a higher secretion of TNF-α, IL-6, and IL-1β from obese adipose tissues (9). In contrast, lean adipose

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tissues not only recruit immune cells with anti-inflammatory characteristics, but also release cytokines such as adiponectin, which induces the polarization of alternatively activated macrophages (M2) (8).

Macrophages recognize pathogens and some fatty acids by a transmembrane protein called Toll like receptors (TLRs). TLR-4 recognizes lipopolysaccharides (LPS) (10) and triggers a pathway that leads to the dissociation of an inhibitory subunit from the nuclear factor kappa B (NF-κB) complex and its subsequent translocation to the nucleus, leading to the transcription of pro-inflammatory genes (11). Diet-derived fatty acids modulate monocytes/macrophages In addition to TLRs expression patterns, saturated fatty acids are believed to activate TLR4-mediated signalling pathways (12). In contrast, some studies have shown that polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid have anti-inflammatory properties due to the ability to activate the peroxisome proliferator activated receptors (PPARs) (13).

PPARs are a family of transcriptional factors that regulate several genes related to lipid metabolism and lipogenesis. Three members from the PPARs family have been identified (α, β/δ, and γ), with diversified tissue distribution (14). PPARα is considerably expressed in tissues with high metabolic rates, such as liver, heart, muscle, brown adipose tissue, and some immune cells. Its main role is the regulation of fatty acid metabolism (15). PPAR β/δ is involved in the transcription of genes responsible for fatty acid uptake, beta-oxidation, and energy decoupling. This fosters the reduction of lipotoxicity and the improvement of insulin sensitivity (16). PPARγ is considered the main regulator of adipogenesis. It has metabolic functions such as adipocyte differentiation, lipid storage, and glucose metabolism, as well as anti-inflammatory functions that are expressed essentially via macrophages. Some of the PPARγ target genes are lipoprotein lipase, CD36, and adiponectin (2). Two anti-inflammatory mechanisms of PPARγ have been previously identified. One is the suppression of proinflammatory transcription factors, including STAT, NF-κB, and AP-1, via competition for the same PPAR responsive element (PPRE) (17). The other mechanism is the non-removal of corepressor complexes from inflammatory genes, keeping them in a suppressed state (18).

For PPARγ binding to specific DNA sequences to occur, heterodimerization with a second member of the nuclear receptor family, the retinoic X receptor (RXR), is required. In the absence of ligands, PPARγ silencing occurs by transcriptional corepressor complexes. Alternatively, binding of agonists ligands induces conformational changes in PPARγ structure. This leads to the trapping of transcriptional coativators (19). Previous human studies have shown that PPARγ activation leads monocytes into the anti-inflammatory profile by alternative M2 macrophage polarization (20). Additionally, pre-treatment with proinflammatory cytokines down-regulates PPARγ gene expression in bone marrow-derived macrophages. In addition, the pre-treatment of adipocytes with arachidonic acid hinders PPARγ gene and protein expression (21).

In 2010, Kawanishi et al. showed that moderate aerobic training (16 weeks on the treadmill) was able to increase M2 (CD163) mRNA expression of surface markers and reduce M1 (CD11c) in obese mice with a concomitant reduction of TLR-4 mRNA. Besides this, exercise has been considered an important PPARγ activator by different mechanisms such as gene expression (22), DNA-binding (23), or ligands generation (24). Our group has previously showed that PPARα plays an important anti-inflammatory role in acute exercise (25). PPARγ also plays an essential role in the control plasma lipids (26) and in the alternative activation of monocytes in human (27) and animal models (28) in moderate-intensity aerobic training.

Due to the need for therapeutic strategies for chronic inflammatory diseases, such as diabetes, we chose not to use a therapeutic target already well-established in the literature, PPARγ. Rather, we chose to apply an acknowledged treatment, physical exercise, based on the hypothesis that the absence of PPARγ would impair the well-known anti-inflammatory effect of exercise. As such, in the context of obesity the over-activation of inflammatory TLR4 pathway, we seek to investigate whether macrophages PPARγ influences the exercise-mediated anti-inflammatory effect. To evaluate this, we analyzed alterations in the metabolic and inflammatory parameters of PPARγ CreLox mice induced to obesity by a high-fat diet and subsequently submitted to an 8-week moderate-intensity aerobic training.

MATERIALS AND METHODS

Animals and diet

The Animal Care Committee of the Institute of Biomedical Sciences approved all the experimental protocols (University of São Paulo, Brazil, Protocol 112/13CEUA). Mice with a selective deletion of PPARγ in the myeloid lineage (PPARγ Cre+) were produced by crossing PPARy Lox/P mice (The Jackson Laboratory, stock no. 004584) with Lys Mcre mice (The Jackson Laboratory, stock no. 004781). The animals were selected by genotyping using primers and protocols indicated by the manufacturer Figure S1. The mice in the study were male 8–10-week-old mice maintained at 22 ± 2°C in a 12-hour light/dark cycle.

The mice were fed with a high-fat diet (HF, 59% of calories from fat, 15% from proteins, and 26% from carbohydrate) consisting of a modified AIN-93 diet with an increased lipid content and a reduced carbohydrate content (Table S1) (29). The basic composition of the high-fat diet were: hydrogenated vegetable fat, sucrose, casein, dextrinized corn starch, corn starch, microfine cellulose, sunflower oil [omega 6], mineral mix, vitamin mix, L-cystine, and choline bitartrate. Two or three mice were kept in each cage and both water and the diet were supplied *ad libitum*. The food consumption pattern was recorded weekly and the caloric intake was calculated (Figure 1C).

Experimental groups

The mice were subdivided into four groups (8–10 mice each): wild type sedentary (WT HF) [21.6 ± 1.12g], knockout sedentary (KO HF) [21.41 ± 1.33g], wild type trained (WT HFT) [20.51 ± 1.54g], and knockout trained (KO HFT) [19.65 ± 1.15g]. All the animals were adapted to a treadmill for 1 week (10 min—10 m/min) and performed a maximum test speed in three moments (week 1/8, week 4/8, and week 8/8). Only trained groups were submitted to the exercise training protocol (5 days/week, 1 hr/day 55–65% of maximum speed

test) (30-31). After 72 hours of resting from the last maximum speed test, the animals were euthanized (6hr fasting).

Analysis of serum lipid concentrations

The mice were euthanized and blood was collected from the same site of decaptation, and the serum was obtained by centrifugation at 3000rpm for 15 minutes at 4°C. The non-esterified fatty acids (NEFA; HR Series NEFA HR; Wako Pure Chemical Industries, Ltd., Richmond, VA), glucose, triacylglycerol (TAG), and total cholesterol (TC) were analyzed in the serum by a colorimetric method (Labtest, Lagoa Santa, Brazil). All the assays were performed in duplicates.

Glucose (GTT) and insulin (ITT) tolerance tests

The mice received an intraperitoneal injection of D-glucose (2 $g.kg^{-1}$ body weight) or insulin (0.375 $U.kg^{-1}$ body weight) after a 4-hour fast. For the glucose tolerance test (GTT), blood drops were collected from the tail vein before and at 15, 30, 60, and 90 minutes after the glucose injection. The glucose concentrations at each time point were used to calculate the area under the curve (AUC) (32). For the insulin tolerance test (ITT), blood samples were collected from the tail vein before and at 5,10,15,20, 25, and 30 minutes after insulin injection. The constant for serum glucose disappearance (KITT) was calculated by linear regression of glycemic levels measured between 5 and 15 minutes after insulin injection according to Bonora (1989) (33). An interval of 72 hours between the two tests was observed. Accu-Chek Performa (Roche, São Paulo, SP, Brazil) was used to assess the levels of glucose.

Citrate Synthase

The activity of citrate synthase was determined using 5. 5'-dithiobis- (2-nitrobenzoic acid DTNB) using muscle (soleus) homogenates in 100 mM Tris, pH 8.0, 0.1 mM acetyl-CoA, 0.1 mM 5,5'-dithiobis- (2-nitrobenzoic acid), 0.1% triton X-100. 2 µg of supernatant protein were used for the reaction that was started with 0.2 mM oxaloacetate and monitored at 412 nm for 3 minutes at 25 ° C.

Enzyme-linked immunosorbent assay (ELISA)

Subcutaneous (inguinal) adipose tissue (80–100 mg) and adipocytes extracted from the same depot were homogenized in radioimmunoprecipitation assay buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid [EDTA] at pH 7.4) containing 10$\mu g.ml^{-1}$ of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation, the supernatant was used to determine the protein concentration by the Bradford assay (Bio-Rad, Hercules, CA). Insulin from the serum and cytokines from the supernatant of cultured macrophages and from the adipose tissue were assessed in duplicates by ELISA (DuoSet ELISA; R&D Systems, Minneapolis, MN).

Peritoneal macrophage isolation and culture

Macrophages were obtained by washing the peritoneal cavity with Roswell Park Memorial Institute (RPMI) culture medium (Sigma-Aldrich), enriched with 2% fetal bovine serum (FBS), and antibiotics (1%). The medium collected was centrifuged, the supernatant aspirated, and the cell pellet resuspended in 1 ml of complete medium The cells were counted in a haemocytometer using trypan blue (0.4%) exclusion and 5x105 viable macrophages were cultured in medium (RPMI) supplement with fetal bovine serum (10%), and antibiotics solutions in standard conditions (37°C in humidified 95% air; 5% CO2). After 2 hours, nonadherent cells were removed by washing with RPMI. Adherent cells were then collected using cold phosphate-buffered saline (PBS) 2% simulated body fluid for further flow cytometry analysis or incubated with 2.5µg.ml−1 of lipopolysaccharide (LPS; Escherichia coli, serotype 0111:B4; Sigma Aldrich) for 24 hours (34). The medium was collected for the determination of interleukin-6 (IL-6), IL-10, IL-1β, monocyte chemoattractant protein-1 (MCP-1), IL-1ra, and TNF-α by ELISA (R&D System) according to the manufacturer's instructions.

Adipose tissue-macrophages and peritoneal macrophages flow cytometry

Adipose tissue was digested with collagenase type II (2 $mg.ml^{-1}$; Sigma- Aldrich) in PBS with EDTA (2 mM; Sigma-Aldrich) and 0.5% FBS for 60 minutes at 37°C in a shaker. The suspension was filtered using a cell strainer 100 µm and subsequently centrifuged at 400 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was resuspended in 3 ml lysis buffer (17 mM Tris–HCl + 0.144M de cloreto de amônia, pH 7.2) for 2 min for purification of leukocytes. After centrifugation at 3,000 rpm for 10 minutes at 4°C, the cells were washed with PBS, and stained with the following antibody panel: Anti-CD11b, F4/80, CD11c, CD206, diluted 1:100 (BioLegend, San Diego, CA). M1 macrophages were characterized by the expression of CD11c in adipose tissue-resident macrophages and CD86 in peritoneal macrophages concomitant with F4/80 and CD11b markers and M2 macrophages by the expression of CD206 concomitant with F4/80 and CD11b markers (Table S2). The characterization of subpopulations of leukocytes was performed on the FACS CANTOII machine (Beckton Dickson, Franklin Lakes, NJ) and data analysis was performed with FlowJo 9.5.3 software Tree Star (Ashland, OR).

Real-time reverse transcription PCR

Macrophage total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY), following the method described by Chomczynski and Sacchi (35). Reverse transcription to complementary DNA (cDNA) was performed using the high capacity cDNA kit (Applied Biosystems, Foster, CA). Gene expression was evaluated by real time PCR according to Higuchi et al. (36), using SYBR Green Master Mix (Applied Biosystems) as a fluorescent dye. Primer sequences are shown in Table S3. Quantification of gene expression was carried out using the RPLP0 gene as an internal control, as previously described by Liu and Saint (37).

Histological sections

Tissues were removed, fixed in 4% formaldehyde (phosphate buffer) at pH 7.4, for a period of approximately 24 hours at room temperature. After fixation, the tissues were dehydrated in ethanol, clarified in xylol and embedded in paraffin at 60°C. Subsequently, the paraffin blocks containing the tissues were cut into a microtome (Leica®, Germany),

resulting in cuts of 5 μm in thickness. The sections were stained with hematoxylin and eosin (H & E). For histological investigation. An image analysis system consisting of a digital camera (Evolution, Media Cybernetics, Inc., Bethesda, MD) attached to a light microscope (Eclipse 400, Nikon) was used. High quality images (2048 × 1536 pixels) were captured with ImagePro Plus 4.5.1 (Media Cybernetics) and quantified areas of adipocyte perimeters and diameters by Fiji Software (ImageJ) Madison, Wisconsin, USA - Adiposoft (38).

Statistical analysis

All the groups were first tested for the distribution normality (Shapiro Wilk test) and subsequently compared in terms of the effects of genotype (WT HF x KO HF) and exercise (sedentary x trained) by using the two-way ANOVA followed by Bonferroni post hoc test. For comparison between two variables, the Student T test was used. The analyses were performed using the software GraphPad Prism version 6.01 (La Jolla, CA). Results are presented as mean ± standard error of mean. The significance level adopted was $p < 0.05$.

RESULTS

Aerobic training avoids exacerbated weight gain in both genotypes and decreases calorie in KO animals

Despite the occurrence of body weight gains in the same proportion (with no statistically significant differences between the WT HF and KO HF) (Figure 1A), the KO HF mice showed a higher calorie intake in the sedentary groups

Table 1. Effect of PPARγ deletion and aerobic training on variables of body composition, lipid profile, and serum adiponectin of obese mice. All values are mean ± standard error of the mean (n = 8–10 / group) (two-way ANOVA followed by Bonferroni post-test $p < 0.05$).

	WT HF	KO HF	WT HFT	KO HFT
Body weight (g)	33.50 ± 4.94[a]	34.94 ± 3.04[b]	28.82 ± 2.30	31.18 ± 4.12
SAT (%)	1.97 ± 0.77	2.47 ± 1.34	1.89 ± 0.63	1.78 ± 1.04
RAT (%)	0.97 ± 0.42	1.10 ± 0.42[b]	0.78 ± 0.60	0.67 ± 0.44
EAT (%)	4.46 ± 1.575	5.39 ± 1.51[b]	3.52 ± 1.53	3.01 ± 1.75
Liver (%)	4.11 ± 0.19	4.20 ± 0.53	3.65 ± 1.01	4.19 ± 0.22
GAST (%)	0.76 ± 0.12[ac]	0.89 ± 0.11	1.01 ± 0.13	0.96 ± 0.077
TC (mg.dL^{-1})	171.01 ± 9.18	159.52 ± 16.64	138.69 ± 8.40	137.87 ± 5.99
TAG (mg.dL^{-1})	136.29 ± 11.88	127.77 ± 5.81	124.05 ± 7.02	113.40 ± 3.42
NEFA (mg.dL^{-1})	0.766 ± 0.04	0.654 ± 0.01	0.712 ± 0.03	0.676 ± 0.03
Adiponectin (ng.mL^{-1})	5.20 ± 2.64	6.26 ± 0.511[b]	5.72 ± 0.32	4.76 ± 0.37

g = grams; % = relative weight; SAT = Subcutaneous adipose tissue; RAT = Retroperitoneal adipose tissue; GAST = Gastrocnemious; EAT = Epididimal adipose tissue; TC = Total Cholesterol; TAG = triacylglycerol; NEFA = free fatty acids; a = different from WT HFT; b = different from KO HFT; c = different from KO HF.

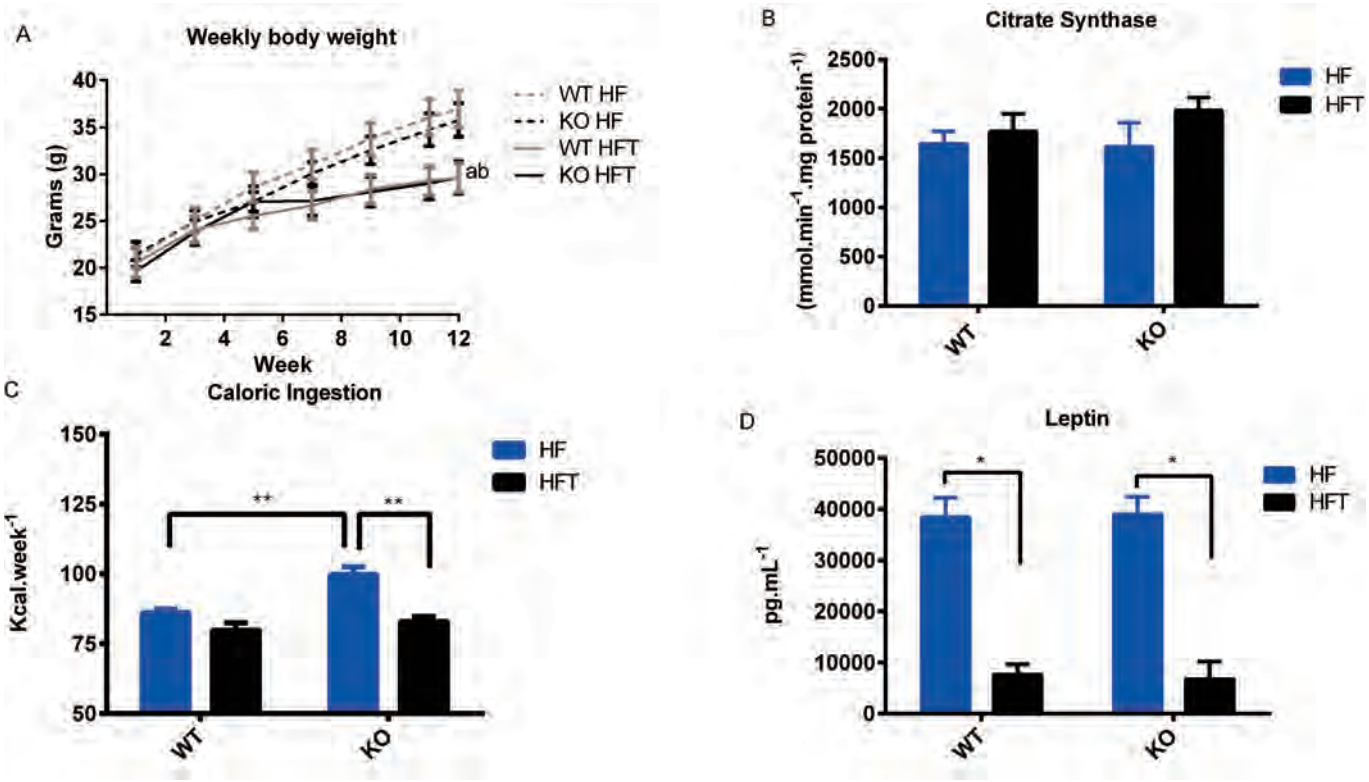

Figure 1. Effect of PPARγ deletion and aerobic training in obese mice. Weekly body weight (A), citrate synthase from the soleus muscle (B), caloric intake (C) and serum leptin concentration (D). All values are mean ± standard error of the mean (n = 8–10 per group). (Two-way ANOVA followed by Bonferroni post-test). a = different from WT HF; b = different from KO HF; * $p < 0.05$; ** $p < 0.01$.

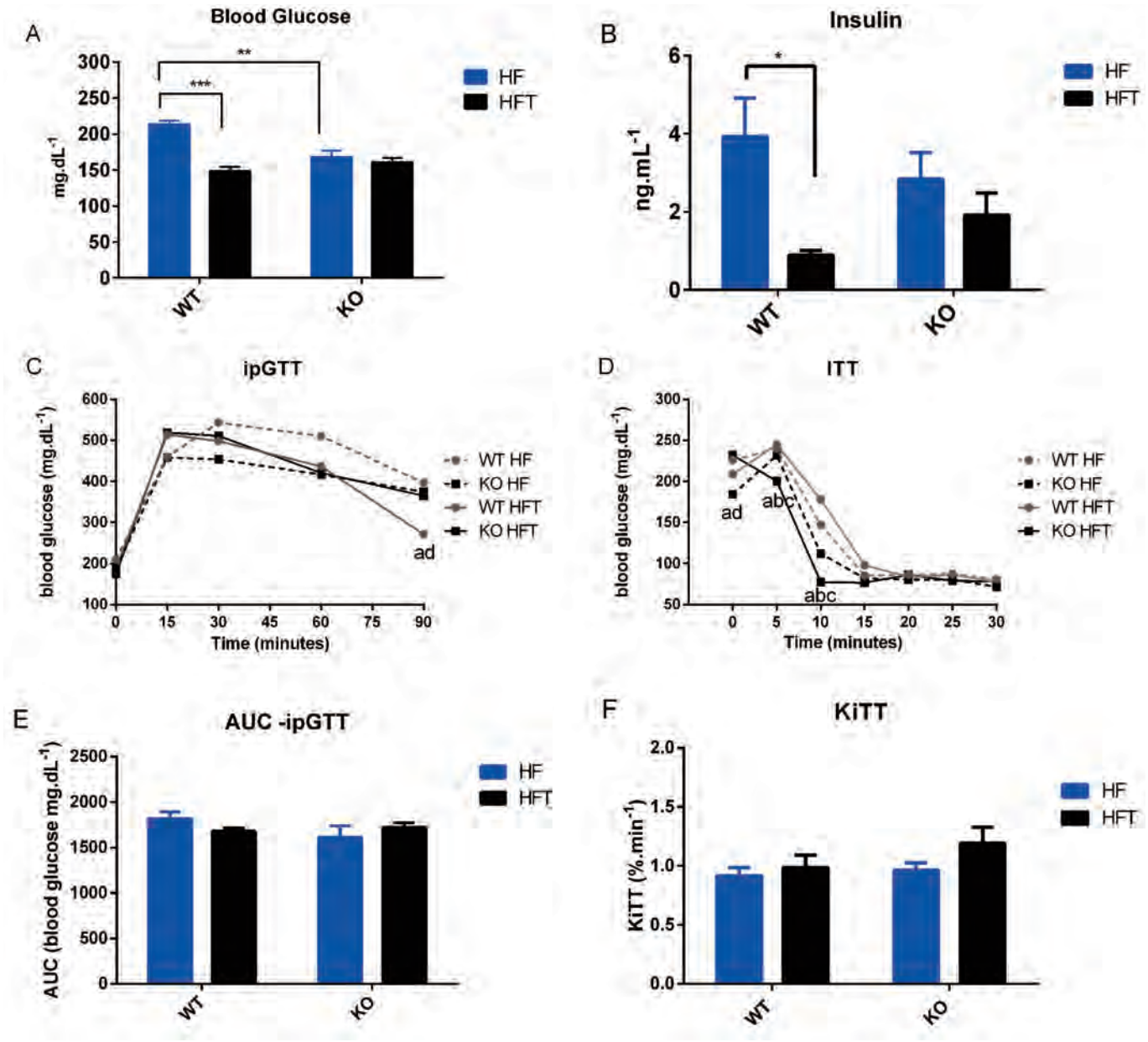

Figure 2. Glucose metabolism of WT and KO animals submitted to a high-fat diet and aerobic training. Glucose (A) and fasting insulin (B), intraperitoneal glucose tolerance test (ipGTT) (C), intraperitoneal insulin tolerance test (ipITT) (D), area under the AUC curve of the tolerance test (E) and glucose decay constant (KiTT) (F). All values are mean ± standard error of the mean (n = 5–6 per group). (ANOVA two-way followed by Bonferroni) a = different from WT HF. b = different from KO HF. c = different from WT HFT; d = different from KO HFT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

but aerobic training balanced the calorie intake in the genotypes (Figure 1C). In addition, leptin concentrations were equivalent between the genotypes with exercise-mediated decreases in both groups (Figure 1D).

Adipose tissues from the KO animals seems to be more susceptible to weight variances generated by exercise training, especially decreasing the ones located in the central region (retroperitoneal and epididymal) (Table 1). Neither the liver nor brown adipose tissue weights were affected by exercise *(data not shown)*. However, the gastrocnemius relative weight, which was naturally higher in the WT HF, was more enlarged in the training group (Table 1).

Aerobic training reduces fasting blood glucose and insulin in WT animals fed with a high-fat diet

Serum glucose and insulin concentrations in WT HFT animals were lower when compared to their sedentary pairs. In the KO HF, there was no significant effect by aerobic training (Figure 2A and B). With regards to the glucose tolerance and insulin resistance tests, exercise does not alter the area under the curve nor the glucose decay constant after insulin injection in any of the genotypes (Figure 2C and D).

Neither aerobic training nor the genotype affected the serum lipid concentrations (Table 1), however, adiponectin was lower in the KO HFT group when compared to their sedentary pairs (Table 1).

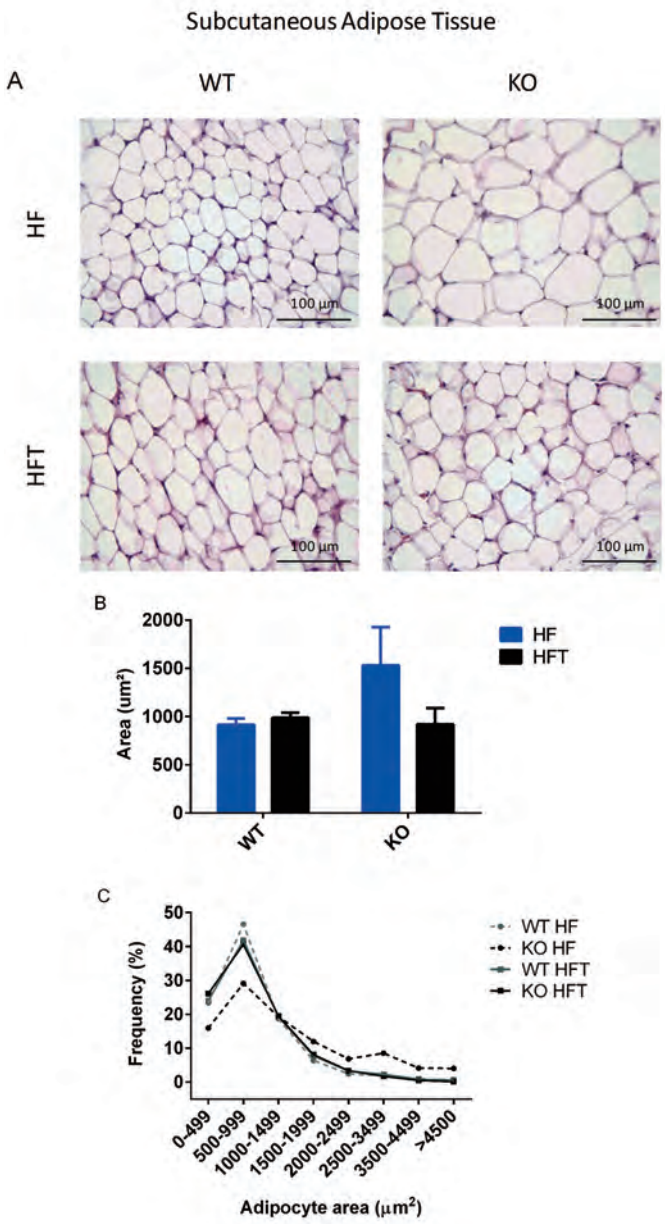

Figure 3. Morphology of adipose tissues of WT and KO animals fed with high-fat diet and submitted to aerobic training. Representative pictures of each group using 40 times amplification (A), mean area (B) and frequency of subcutaneous adipocyte distribution (C). The values were calculated from histological sections images using the ImagePro Plus Fiji version 6.0 program. All values are expressed as the mean ± standard error of the mean (n = 3 per group). (Two-way ANOVA followed by Bonferroni).

Aerobic training attenuates hypertrophy in subcutaneous adipose tissue of KO animals

Although not significant, the KO HF had a slightly higher adipocyte area in the subcutaneous adipose tissues but aerobic training did not prevent the expansion of this exacerbated area (Figure 3B and C).

Although the aerobic training did not contribute to the morphological alteration in the WT subcutaneous adipose tissues, the M1 marker CD11c was significantly reduced. However, even with normalization of subcutaneous adipose tissue area in the KO animals, macrophages maintained their M1/M2 markers balance (Figure 4B).

The IL-6 and adiponectin profiles were similar in both isolated adipocytes and total adipose tissue (adipocytes plus stromal portion) microenvironments. However, TNF-α seems to be essentially produced in the stromal portion of the tissue (Figure 5A and D). In addition, regardless of the non-reduction of M1 marker $CD11c^+$ as observed in the KO HFT (Figure 4B), exercise anti-inflammatory effects such as decreased TNF-α and IL-1β should be highlighted in this genotype (Figure 5D and H).

Aerobic training maintains anti-inflammatory effects in peritoneal macrophages despite of reduced M2 markers CD206 in the absence of PPARγ.

Aerobic training increased mainly M1 markers in both obese groups. In addition, M2 markers $CD206^+$ were lower in cells with PPARγ deletion when compared to WT (Figure 4A). The KO HFT showed a reduction of proinflammatory cytokines production such as TNF-α and IL-6 in 24h-cultured cells with LPS stimulation (Figure 6A and B). IL-10 production after LPS was lower in the KO macrophages of the trained animals compared to WT (Figure 6C). IL-1β showed higher values when LPS stimulated and increased due to the effect of aerobic training on WT macrophages (Figure 6F).

It was observed that LPS significantly increased the TLR-4 gene expression and that exercise prevents this phenomenon regardless of PPARγ (Figure 6H). The hypoxia inducible factor 1 alpha was higher in the KO HF when compared to the WT HF and KO HF LPS (Figure 6G).

DISCUSSION

In this study, different responses in macrophages subpopulation (peritoneal and adipose tissue-associated) were identified as a result of obesity and moderate aerobic training. Furthermore, PPARγ transcription factor has been shown to be necessary for modulating macrophages surface markers, though it plays a lesser degree of importance in the modulation of cytokines production. Since macrophages are plastic cells (39), it is reasonable that exercise can alter their function/secretion without undergoing significant phenotypic changes. This finding suggests the idea that when exercise is involved, not only macrophages phenotype but cell functionality (cytokine production) must be taken into account.

The KO HFT group showed a lower caloric intake despite the decrease in serum leptin concentrations which suggests that such animals may develop resistance to leptin when fed a high-fat diet, phenomenon that is also common in obese humans (40) and associated with hyperinsulinaemia and the risk of type 2 diabetes (41). Reduction in fasting insulin as well as improvement in glucose tolerance test in the WT HFT animals may be explained by an increase in glucose uptake in peripheral tissues such as skeletal muscle by insulin-independent pathways (42-43). In a study conducted simultaneously with the high-fat diet, exercise training improved insulin sensitive in the WT group (44) facilitated by the reduction in both fasting insulin and glucose.

In the knockout model for PPARγ in adipose tissue, high-fat diet did not induce a significant increase in body weight and insulin resistance (45). Compared to the data of the present study, which uses animals with the same gene deletion but

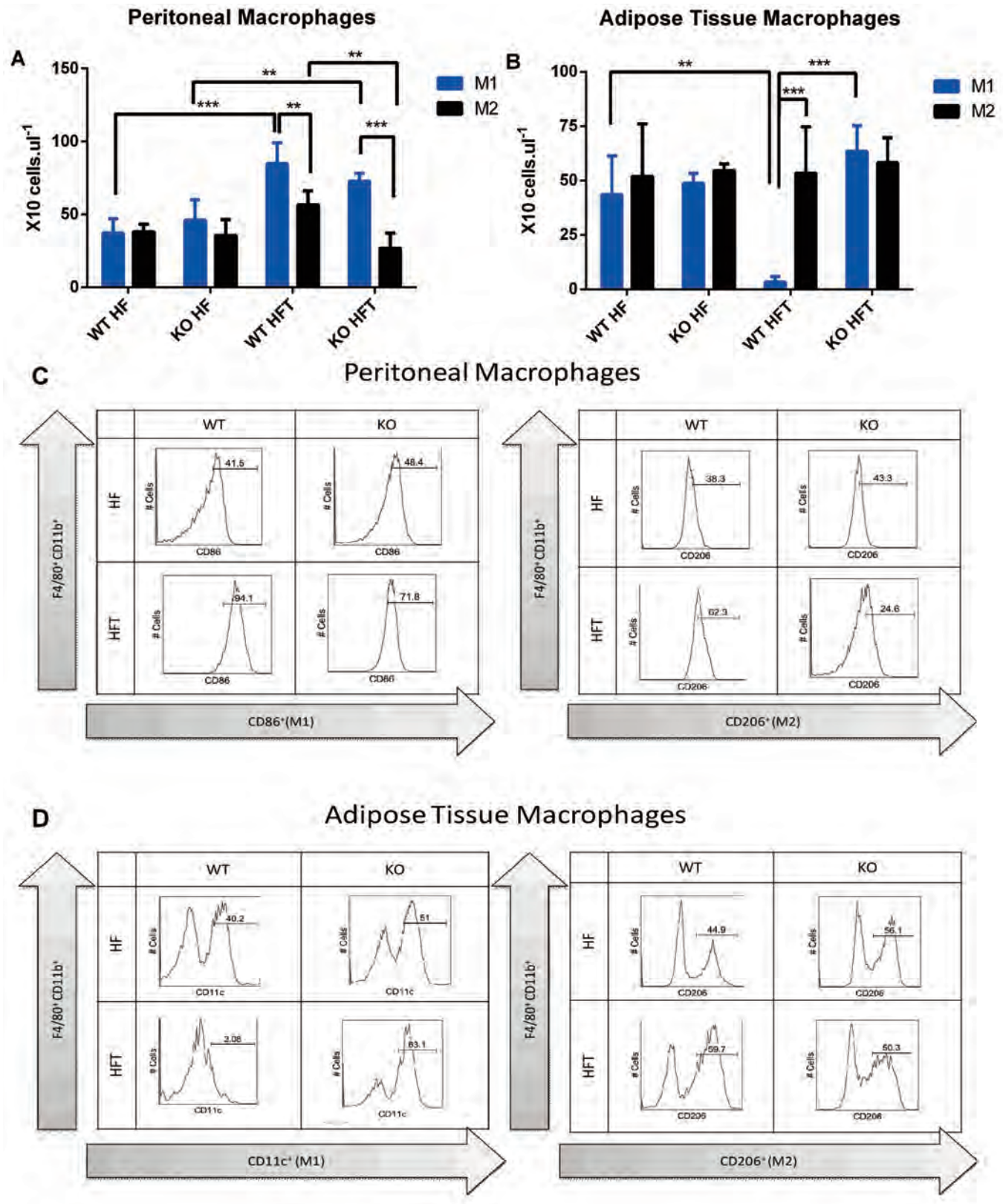

Figure 4. Phenotypic characterization of macrophages by flow cytometry. Effects of high fat-diet and aerobic training on peritoneal (A) and adipose tissue-associated (B) macrophages phenotypic markers in PPARγ conditional knockout (KO) and control (WT) mice. Quantification of markers (A and B) and histogram representation of the groups analysed (C and D). Leukocytes were identified by CD45. A sequential labeling strategy was used to identify populations expressing specific markers ($F4/80^+CD11b^+$), followed by identification of subpopulations with expression patterns: peritoneal macrophages M1 ($CD86^+$), M2 ($CD206^+$) and adipose tissue M1 ($CD11c^+$), M2 ($CD206^+$). (Two-way ANOVA followed by Bonferroni or t test for differences between M1 and M2) * $p < 0.05$; ** p, 0.01; *** $p < 0.001$

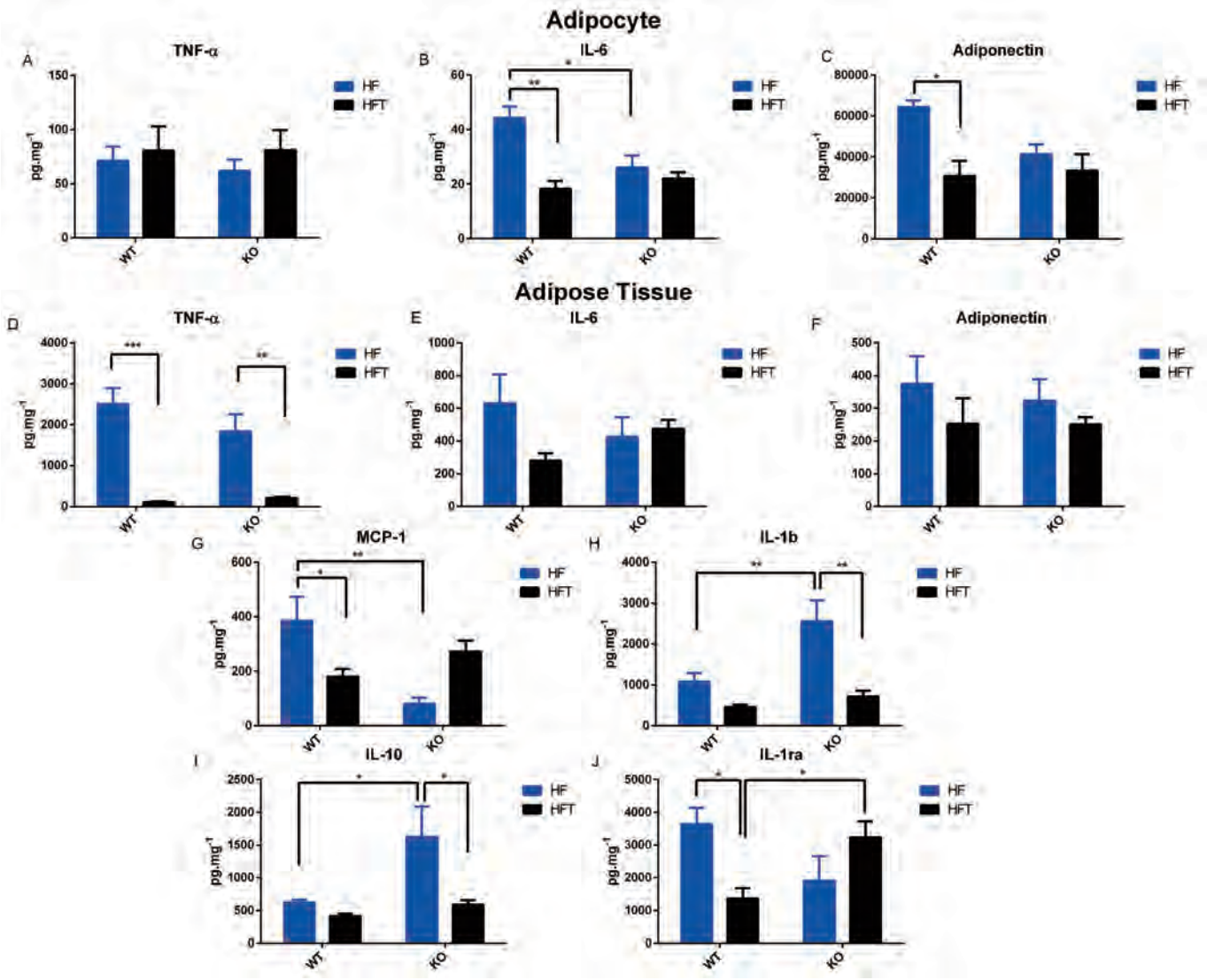

Figure 5. Isolated adipocytes and adipose tissue cytokines content of WT and KO mice fed with a high-fat diet and submitted to aerobic training. Adipocyte isolated from subcutaneous adipose tissue: tumor necrosis factor-α (TNF-α) (A), interleukin-6 (IL-6) (B) and adiponectin (C) and cytokine content in subcutaneous adipose tissue: TNF-α (D), IL-6 (E), adiponectin (F), monocyte chemotactic protein-1 (MCP-) (G), interleukin-1beta (IL-1β) (H), interleukin-10 (IL-10) (I) and interleukin-1 antagonist receptor (IL-1ra) (J). Cytokine concentrations were determined by ELISA and relativized by total proteins (n = 5 per group). Data are presented as mean ± standard error of the mean. (Two-way ANOVA followed by Bonferroni). * $p < 0.05$; ** p, 0.01; *** $p < 0.001$.

in myeloid cell lines, it was observed that the KO HFT animals had a more pronounced reduction in adipose tissue weight in the central regions (epididymal and retroperitoneal) compared to WT. Aerobic training is known to be a useful therapy for reducing visceral adiposity and one of the underlying mechanisms may be an increase in adipocyte triglyceride lipase expression (ATGL) related to an overexpression of PPARγ2 in adipose tissues (46). Other mechanisms that may explain an improvement in insulin sensitivity is the AMPK (Adenosine 5-Monophosphate-Activated Protein Kinase) activation, an energy sensor increases glucose uptake in an insulin-independent manner (47). AMPK activation in macrophages, either via exercise or pharmacological means (48), is also strongly related to an anti-inflammatory phenotype (49).

In relation to the serum variables, adiponectin was lower in KO HFT. A systematic review focused on the effects of exercise on serum adiponectin concentrations concluded that in humans adiponectin was acutely or chronically increased as an effect of aerobic training (50). Although adiponectin is produced in adipose tissues, PPARγ deletion in myeloid cells may have affected its serum concentration. In isolated adipocytes from WT HFT, adiponectin was significantly reduced compared to WT sedentary. This fact suggests that the reduction in WT adipocytes may be due to a decrease in adipose tissue mass, though the lack of PPARγ may also be involved. PPARγ is a potent regulator of adipogenesis and the incorporation of fatty acids by adipose tissues (51). In obesity, there is also an increase in the expression of adipose tissue–associated macrophages in animal (52) and human models (53). Thus, the results of cytokine content (total tissue and isolated adipocytes) as well as the immunophenotying, indicates that these cells may be a relevant way to examine PPARγ interferences.

The exact mechanism by which this inflammatory stimulus initiates insulin resistance and/or metabolic dysfunctions has not been fully elucidated, however, some studies have investigated the alleged low-grade chronic inflammation caused by

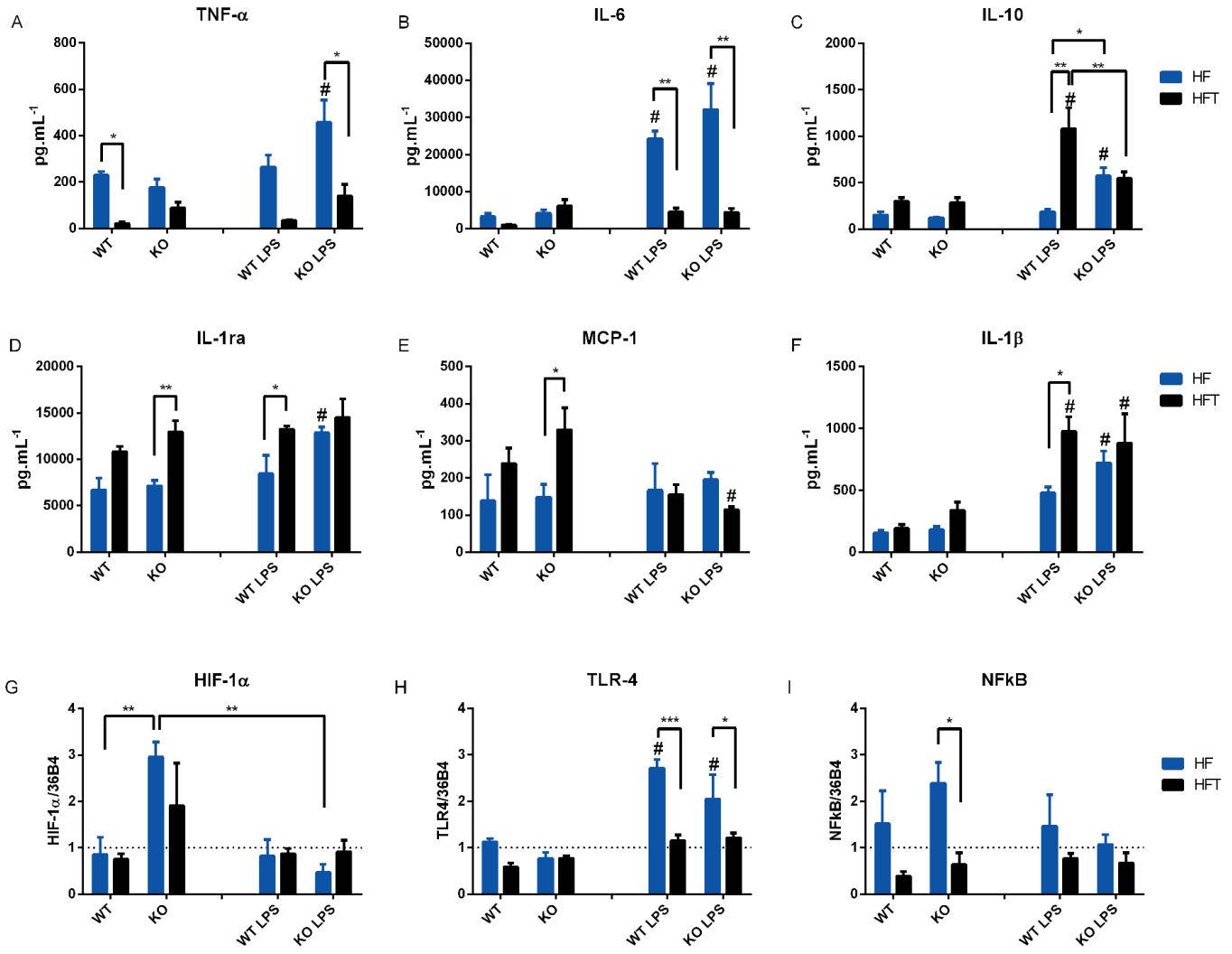

Figure 6. Peritoneal macrophages cytokines production and gene expression from WT and KO obese mice submitted to aerobic training stimulated or not by LPS (2.5 μg.mL^{-1}). Cytokines production: Tumor necrosis factor-α (TNF-α) (A), interleukin-6 (IL-6) (B), interleukin-10 (IL-10) (C), interleukin- -1ra (IL-1ra) (D), monocyte chemoattractant protein-1 (MCP-1) (E) and interleukin-1β (IL-1β) (F) were determined by ELISA in culture medium (n = 8–10 per group) and gene expression: hypoxia inducible factor 1-α (HIF-1α) (G), toll- like receptor 4 (TLR4) (H) and nuclear factor kappa B (NF-κB) (I) were obtained by polymerase chain reaction (n = 5 per group). Data are presented as mean ± standard error of the mean. (ANOVA Two-way followed by Bonferroni). * $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; # different from the same non-stimulated group.

obesity. Previous research has shown that adipose tissue macrophages from non-obese subjects present an M2 profile (54). In our model, the aerobic training reduced M1 marker CD11c^{+} only in WT HFT, in agreement with Kawanishi et al.'s findings which showed that the increase of CD11c^{+} by a high-fat diet was attenuated by chronic exercise due to the inhibition in M1 surface markers mRNA in adipose tissues of obese mice and TLR4 downregulation (55). In the present study, TLR4 gene expression in adipose tissues was not measured, though TLR4 downregulation was present in LPS stimulated peritoneal macrophages from the trained mice, indicating that the expression of the receptor is a strong regulator of exercise-induced immune response over obesity. One mechanism recently related to this theory is the irisin production, an adipomyokine induced by muscle contraction, and relevant for adipose tissue browning that may be associated with downregulation of downstream pathways of TLR4/MyD88 (56).

An important role has been suggested for proinflammatory cytokines derived from adipose tissue-associated macrophages, especially TNF-α and IL-1β (54). Reflecting what has been previously suggested our data showed that in the KO HF animals there was an increase of IL-1β in comparison with WT HF, which was reverted by the aerobic training. IL-1β plays a very important role in adipose tissues as it is associated not only with insulin resistance but with the secretion of proinflammatory cytokines (57). Secretion of IL-1β is mediated by the NLRP3 inflammatory pathway and the adipose tissue is believed to be a major contributor to serum IL-1β concentration (58). The KO HFT exhibited a lower IL-1β content in the adipose tissues, agreeing with a recent study that observed reduced NLRP3 and IL-1β gene expression via resistive and aerobic training, respectively (59) (Figure 7).

Aerobic training was also effective in lowering inflammatory cytokines such as TNF-α, MCP-1, and a tendency to reduce IL-6 in WT HFT. Surprisingly, there was no excess of MCP-1 in KO HF. However, studies have shown that increased MCP-1 in adipose tissues may be associated with insulin resistance. Since the KO HF animals exhibited a

milder insulin resistance, this may be one of the reasons why MCP-1 was unchanged (60).

Similar to adipose tissue-associated macrophages immunophenotyping, peritoneal macrophages markers were also not unbalanced by the absence of PPARγ or by the high-fat diet, but exercise was a main point to disturb the M1/M2 equilibrium. One assumption may be the constitutive secretion of proinflammatory cytokines previously observed in obese Zucker rats in response to LPS (61) that was possibly increased due to the HF diet (62). In addition, peritoneal macrophages isolated from the trained mice showed higher numbers of the M1 marker CD86 in cells from both genotypes. However, the M2 marker CD206 was lower in the KO HFT compared to WT HFT.

A previous study showed that proinflammatory cytokines such as TNF-α and IL-6 even under LPS stimulation had similar secretion patterns independently of PPARγ macrophages deletion (63), though the LPS dose (0.1ug.mL−1) was lower compared to the present study (2.5ug .mL−1). Chawala et al. have suggested that macrophages PPARγ is involved in inflammation due to the effect of its ligands on lipid metabolism, such as regulation of its target gene, CD36. However, in regards to cytokine production and the inflammatory response, the effects may be independent of PPARγ activation capacity (63).

The LPS-stimulated WT HFT group showed an anti-inflammatory response to aerobic training by reducing TNF-α and IL-6, while increasing IL-10 and IL-1ra. The possibility of a suppression of inflammatory responses may be due to a functional anti-inflammatory phenotype via the activation of AMPK, the negative regulation of IκB degradation and the positive regulation of Akt (49). In this context, a study evaluating the surface markers of monocytes after aerobic training reported that the profile of polarization markers was representative of a M2 subtype of macrophages, the "M2b," characterized by the production of M1-associated cytokines as well as high concentrations of IL-10 (27, 64). Although the same reduction in TNF-α and IL-6 have been observed in HFT KO, no improvement in M2 related cytokines such as IL-10 and IL-1ra was noted in the present study. In humans, studies have shown that low-intensity aerobic training may induce monocyte differentiation into M2 macrophages via PPARγ/PGC-1α/β (65), confirming the harmful effect of the anti-inflammatory action of exercise on the absence of PPARγ.

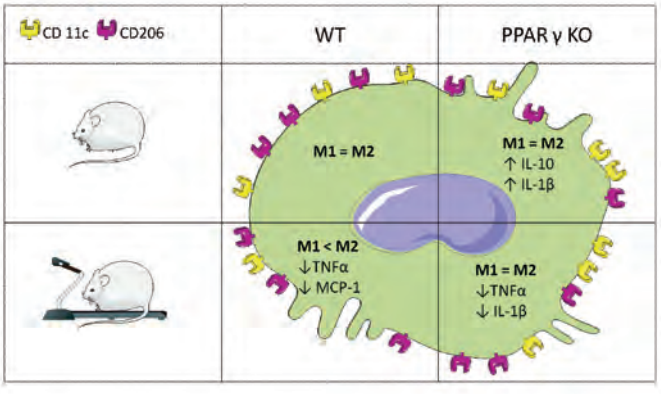

Figure 7. Exercise-mediated effects on subcutaneous adipose tissue of WT and PPARγ KO mice. Adipose tissue macrophages from WT exercised mice showed lower M1 marker (CD11c) compared to KO although the absence of PPARγ did not inhibit proinflammatory cytokines (TNF-α and IL-1β) content reduction. WT = wild type; PPARγ = peroxisome proliferator-activated receptor-gamma; KO = knockout; CD = cluster of differentiation; TNF-α = tumoral necrosis factor alpha; IL-1β =interleukin 1 beta; IL-10 = interleukin 10; MCP-1 = monocyte chemo attractant protein -1.

In endothelial cells, the role of PPARγ in the inhibition of MCP-1 and IFN-γ has been suggested by Murao, et al. (66), but little is known about its effects on macrophages. In the present study, there was a tendency for MCP-1 to decrease in the HFT KO group after stimulation of LPS in culture. One possible reason for this may be that the exercise induced LPS overflow into the circulation, resulting in autoimmunization or LPS tolerance (67). This is because TNF-α and IL- 6 did not increase in the presence of LPS in animals of both genotypes.

Activation of the TLR4/NF-κB pathway results in an increased expression of inflammatory cytokines (68).This increase can be explained by HF diet causing local inflammation, via TLR4/NF-κB, in the gut (69). However, this has not been previously explored in peritoneal macrophages in the context of exercise. Unchanged gene expression of inflammation-related factors such as HIF1α and TLR4 in non-LPS-stimulated peritoneal macrophages may be suggested as a possible explanation for the non-increase in proinflammatory cytokines in the KO HF compared to WT HF. However, the decrease in TLR4 gene expression in LPS-stimulated cells from trained mice, independently of the genotype, suggests that exercise was primordial in TLR4 mRNA downregulation. This is one of the reasons why training may have reduced inflammatory cytokines.

HIF-1α remained unchanged in WT animals and elevated in KO (without LPS), demonstrating that this factor does not appear to have an effect on the secretion of proinflammatory cytokines in peritoneal macrophages. Yet, this increase in HIF-1α expression may be signalling a change in cellular metabolism via a Krebs cycle accumulation of succinate, caused by the decline in the activity of the succinate dehydrogenase enzyme, and generating a subsequent stabilization in the transcription factor HIF-1α (70). Despite being well-known as a key regulator of glycolysis, HIF-1α seems not to be essential for the proinflammatory activation of adipose tissue-associated macrophages (71).

Each phenotype, M1 or M2, has a different metabolic signature. In other words, there is a different activation and metabolic pathways for each profile. While M2 macrophages exhibit high rates of oxidative phosphorylation and fatty acid oxidation, cells with M1 characteristics have predominant glycolytic metabolism (70). It is extremely important that studies and therapies involving macrophages focus on their metabolic reprogramming and the attendant effects on their profile. In fact, similarities among PPARs function may be an experimental dilemma when an isotype knockout is used due to its possible compensation/overexpression by others. In a study conducted by our group, a PPARγ-positive regulation was observed when palmitoleic acid supplementation was used in the culture medium of PPARα knockout peritoneal macrophages (72). Indicating that this regulatory effect could be one of the hypotheses for the anti-inflammatory effect of exercise in the absence of PPARγ macrophages.

Overall, moderate aerobic training protected both genotypes from weight gain, as well as reducing caloric intake

from KO animals. There were different responses in the surface markers of macrophages, depending on the local environment (peritoneum or adipose tissue). Aerobic training was able to support an anti-inflammatory profile in PPARγ knockout peritoneal macrophages with impaired M2 markers, possibly via a reduction in the expression of the TLR-4 gene.

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Key viral immune genes and pathways identify elite athletes with URS

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ABSTRACT

Purpose: *Habitual intense exercise may increase the incidence of upper respiratory symptoms (URS) in elite athletes. This study investigated whether immune gene expression could identify gene markers that discriminate athletes with a higher prevalence of URS.*
Methods: *This cross-sectional analysis of elite Australian athletes from various sports investigated whether athletes retrospectively reporting URS for two days or more in a month (n=38), had an altered immune gene expression profile compared with asymptomatic athletes (n=33). Peripheral blood samples were collected during Olympic selection events with corresponding URS data collected for the one-month period before sampling. Digital immune gene expression analysis was undertaken using the NanoString PanCancer Immune Profiling panel.*
Results: *Fifty immune genes were differentially expressed between the groups ($p<0.05$) and approximately 78% of these genes were more highly expressed in athletes reporting URS. Many of these genes were interferon-stimulated genes or genes involved in the Jak/Stat signalling pathway. Only interferon alpha inducible protein 27 (IFI27), an interferon stimulated gene involved in viral response, remained significantly higher in athletes reporting URS (log^2 fold-difference=2.49, odds ratio 1.02 per unit increase; $p<0.01$) post-adjustment and discriminated athletes reporting URS from asymptomatic athletes with 78% accuracy.*
Conclusion: *Expression of IFI27 could differentiate athletes reporting URS from asymptomatic athletes, a gene that is upregulated in the immune response to viral infection. Upregulation of viral signalling pathways provides novel information on the potential aetiology of URS in elite Olympic athletes.*

Key words: NanoString, elite athletes, digital immune gene expression, respiratory illness

INTRODUCTION

The chronic effects of habitual exercise on the immune system in elite athletes are well documented (43). Habitual, intense exercise can modulate various aspects of immune function associated with an increase in upper respiratory symptoms (URS), which can have a negative impact on sporting performance in elite athletes (14, 15, 32, 34). Preventing URS is a high priority for athletes and coaches underpinning extensive investigation for predictive biomarkers that indicate an increased risk of URS (7, 15, 17).

An increasingly common approach to enhance the understanding of immune regulation in disease is through multiparametric immune profiling, such as immune gene expression analysis (24, 36). Studying immune gene expression provides additional insight beyond simple phenotypic measurement of individual immune parameters as indicators of immune status. This approach accounts for the complex interaction between a diffuse biological network comprising a multitude of cells and molecules (23). Gene expression analysis has been utilised in exercise immunology to provide a deeper understanding of the immune-regulatory networks

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activated by acute exercise (29). Preliminary studies indicate changes in immune gene expression that might increase the risk of URS. In a study of marathon runners, increased expression of genes relating to a T-helper 2 (Th2) anti-inflammatory immune status was reported one week post-race (45). In endurance runners, a post-event reduction in toll-like receptor (TLR) transcripts from dendritic cell fractions isolated from whole blood has been reported (27). While these studies focus on the effects of acute exercise they provide evidence for exercise-induced immune regulation in support of the open-window theory that proposes a bout of intense exercise induces a transient period of immunosuppression lasting between three and 72 h (28). With regards to habitual exercise training, regular bouts of intense exercise likely act in a cumulative manner to induce a chronic anti-inflammatory immune phenotype (37, 42) that could compromise the ability of the immune system to prevent infection and viral re-activation (42).

URS are the most common illness in elite athletes and are the most common presentation to medical professionals during elite sporting events, such as the Olympic Games(11). While reports are mixed, illness during competition negatively impacts competitive performance (13, 33). Investigations of the etiology of URS in elite athletes during competition report a mix of viral, bacterial, allergic and airway irritation as key factors underpinning illness symptoms (15). Clearer identification of the basis of URS in athletes will inform strategies to reduce the risk of illness. To date, no study has examined the immune gene expression profiles of elite athletes reporting URS with reference to asymptomatic athletes. Characterising immune status in association with self-reported URS may provide key information on the inflammatory basis of disease and inform athlete management and treatment strategies to reduce illness negatively impacting athletic performance. A recognised limitation in elite athlete research is cohort size. We were able to compare URS data with immune gene expression in 71 athletes selected for a summer Olympic Games. The aim of this study was to determine whether immune gene expression profile of peripheral blood could differentiate athletes reporting URS for two days or more during the month prior to Olympic selection, from asymptomatic athletes.

METHODS

Design and participants

A cross-sectional design was employed to examine the prevalence of URS in Australian Olympic athletes and determine whether URS is associated with patterns of immune gene expression. Questionnaire and sample collection were undertaken during Olympic selection events that occurred over a three-month period, approximately six months (March – April 2016; Southern Hemisphere autumn) before the 2016 Rio Olympic Games. A total of 71 athletes provided a blood sample 24-48 hours following completion of their selection eventsand responded to the questionnaires, with enough detail specific to URS, for inclusion in this study. Some athletes had trained prior to sample collection. To account for the effects of training, three classifications were used as determined by the sports scientists on the research team: trained for fewer than 3 hours at moderate intensity pre-sampling; trained for fewer than 3 hours at light intensity pre-sampling; and had not trained in the 12 hours prior to sampling. Athletes were also asked to report whether they had used probiotic supplementation during the previous month and these responses were recorded simply as 'yes' or 'no' (Supplement 1). Athletes also provided self-reported training hours per week and described the number of hours spent undertaking endurance, strength and team-based training from which group mean values were calculated. Ethical approval for the study was granted by the Australian Institute of Sport Ethics Committee (Approval number 20160407) and the Griffith University Human Ethics Committee (HREC 2016/213). All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants provided written informed consent prior to data and sample collection as part of the Stay Healthy project and as described previously (11).

To determine the prevalence of URS, a self-reported, retrospective illness symptoms log was completed via an electronic athlete management system used regularly by the athletes, as previously described (44). The illness symptoms log gathered information regarding the number of days athletes experienced URS, such as a blocked or runny nose, sore throat or sneezing as well as symptoms of chest illness including coughing, sputum, chest congestion, wheezing or high temperature over the previous month (Supplement 1). Based on their responses, athletes were allocated to one of two groups; the URS group (n=38) and the asymptomatic group (n=33). Where athletes had reported URS for two or more days during the previous month, they were classified in the URS group. Athletes were classified as asymptomatic if they did not report URS in the previous month. Athletes who were experiencing symptoms at the time of sampling or who reported URS for a single day only in the previous month were not included in analyses.

Blood sample collection

A peripheral blood sample (2.5mL) was collected from an antecubital vein by a qualified phlebotomist, on a single occasion for the evaluation of messenger ribonucleic acid (mRNA). Samples were collected into an RNA PAXgene® tube (Pre-AnalytiX, Feldbachstrasse, Switzerland), kept at room temperature for two hours and stored at -80°C until analysis. Due to the limitations of samples being collected during an Olympic selection event the time of sampling and whether athletes were fasted could not be controlled.

RNA extraction and mRNA isolation

Total RNA was extracted using the commercially available Maxwell® RSC miRNA tissue kit (Promega, Wisconsin, USA) as per the manufacturer's protocol using the Maxwell® RSC Instrument (Promega, Wisconsin, USA). Extracted RNA was stored at -80°C until required for mRNA and micro RNA separation (miRNA). To separate mRNA and miRNA from total RNA the commercially available RNA Clean and Concentrator™ kit (Zymo Research, California, USA) was used as per manufacturer's instructions. Isolated mRNA was stored at -80°C. The LabChip GX Touch 24 (PerkinElmer®, Massachusetts, USA) was used to assess the concentration and quality of mRNA samples.

Gene expression analysis

Isolated mRNA was analysed for digital gene expression analysis of 730 immune genes and key inflammatory pathways [11, 12], using the nCounter® PanCancer Immune Profiling panel (NanoString Technologies, WA, USA) on the nCounter® Gene Expression Assay (NanoString Technologies, WA, USA) as per the manufacturer's instructions. The nCounter® PanCancer Immune Profiling panel (NanoString Technologies, WA, USA) includes 38 housekeeping genes, with full information available at nanostring.com. In brief, 100 ng of mRNA was hybridised with sequence-specific barcoded probes at 65 °C for 24 h before being placed into the NanoString Prep Station where the target-probe complex was immobilised on to the analysis cartridge. Cartridges were read by the nCounter Digital Analyser for digital counting of molecular barcodes corresponding to each target. Validation using polymerase chain reaction or other methods was not pursued due to the literature demonstrating the reproducibility and validity of digital gene expression analysis (1, 2, 10, 22).

Statistical analysis

Differences in group characteristics were evaluated in SPSS version 25 (IBM Computing, New York, USA). All data were evaluated for skewness and kurtosis (within the range of 0 ± 3) to confirm normal distribution. Continuous variables were assessed using a t-test and are presented as mean and standard variation (SD). Categorical variables are presented as count (n) and percent (%) and assessed using the Chi-square test for independence. The Yates' Continuity Correction was applied to compensate for two-by-two comparisons and Cramer's V was reported to show effect size (small 0.1; medium 0.3; large 0.5). The Phi correlation coefficient was used to indicate the degree of association between two variables. Adjustments for multiple comparisons were made using the Benjamini-Hochberg procedure and presented as a *q*-value. Statistical significance was indicated at the level of $p<0.05$ or $q<0.05$.

Immune gene expression data was evaluated using the Advanced Analysis Module in nSolver™ Analysis Software 4.0 (NanoString Technologies, WA, USA). The package includes modules enabling automated and optimised normalization using geNorm, differential gene expression analysis and Pathview plots analysis (5). Raw data was normalised using negative controls to account for systemic background noise and platform-associated sources of variation were normalised using positive controls. An optimized set of reference genes were used to adjust transcript counts relative to housekeeping genes included in the panel. Assessment of immune gene expression data was undertaken and confounding variables, sport and probiotic use, were adjusted for. Unsupervised hierarchical clustering was used to generate a heat map to visualize normalized data and view relationships between and within clusters of genes. Differential immune gene expression was determined between athletes reporting URS and asymptomatic athletes using multiple linear regression, and adjustment for multiple comparisons was performed using the Benjamini-Yekutieli false discovery rate (FDR). The Pathview Plots Module was used to map differential gene expression to known protein-based KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways.

RESULTS

Prevalence of URS in Olympic athletes

Responses to the URS log indicated that 53% of athletes reported URS and/or chest symptoms for two or more days in the month prior to sampling. Sixty-three percent of athletes reported URS for 2-5 days, 19% of athletes experienced URS for 6-9 days and 18% for more than 10 days, respectively. Thirty-three out of 71 athletes reported no URS and/or chest symptoms during the previous month. These results determined the two groups within this study for subsequent gene expression analysis: the URS group (n=38) and the asymptomatic group (n=33).

Group characteristics

The group characteristics are described in Supplement 2; groups did not differ significantly in age, gender distribution or self-reported training load. The URS and asymptomatic groups were significantly different with regards to the distribution of sports (p=0.04, Cramer's V = 0.01, small effect size). Benjamini-Hochberg adjusted post-hoc analysis revealed that, between the groups, the significantly different sport was water polo (p=0.02) with only one water polo player categorized in the asymptomatic group compared to 11 within the URS group. The URS group reported significantly higher probiotic use (p=0.007, Cramer's V = 0.03, small effect size) from which 53% of athletes indicated probiotic use in the previous month compared to 21% of athletes from the asymptomatic group.

Differential gene expression

A total of 305 immune genes within the Pan Cancer Immune Profiling Panel were expressed above background and unsupervised hierarchic clustering of these genes could not separate the groups (Supplement 3). Prior to adjustment, 50 immune genes were differentially expressed ($p<0.05$) between athletes reporting URS and asymptomatic athletes. Of these 50 genes, 36 were upregulated (72%) and 14 were downregulated (28%) in athletes reporting URS in comparison with athletes reporting no URS. Following Benjamini-Yekutieli adjustment only one gene, *IFI27*, remained significant ($p=6.7*10^{-04}$; 2.49 Log^2 fold-difference, confidence interval (CI) 1.67-3.31), see Supplement 4 for the full results table. The top 40 most significant genes can be viewed at Figure 1.

Results from the differential expression analysis were overlaid on various KEGG pathways to identify perturbed pathways and returned a plot for the JAK-STAT pathway (Figure 2). Multiple genes and gene families were over-expressed in the JAK-STAT pathway in the athletes reporting URS compared to asymptomatic athletes prior to Benjamini-Yekutieli adjustment. The Pathview module also returned plots for immune processes known to be involved in the response to influenza A (Supplement 5) and the Herpes Simplex Virus (Supplement 6). The genes common to all three Pathview plots are central to the JAK-STAT pathway and have primary roles in immune regulation.

Gene expression and self-reporting URS

Logistic regression was performed to assess the effects of probiotic use, sport of the athlete and *IFI27* expression on the

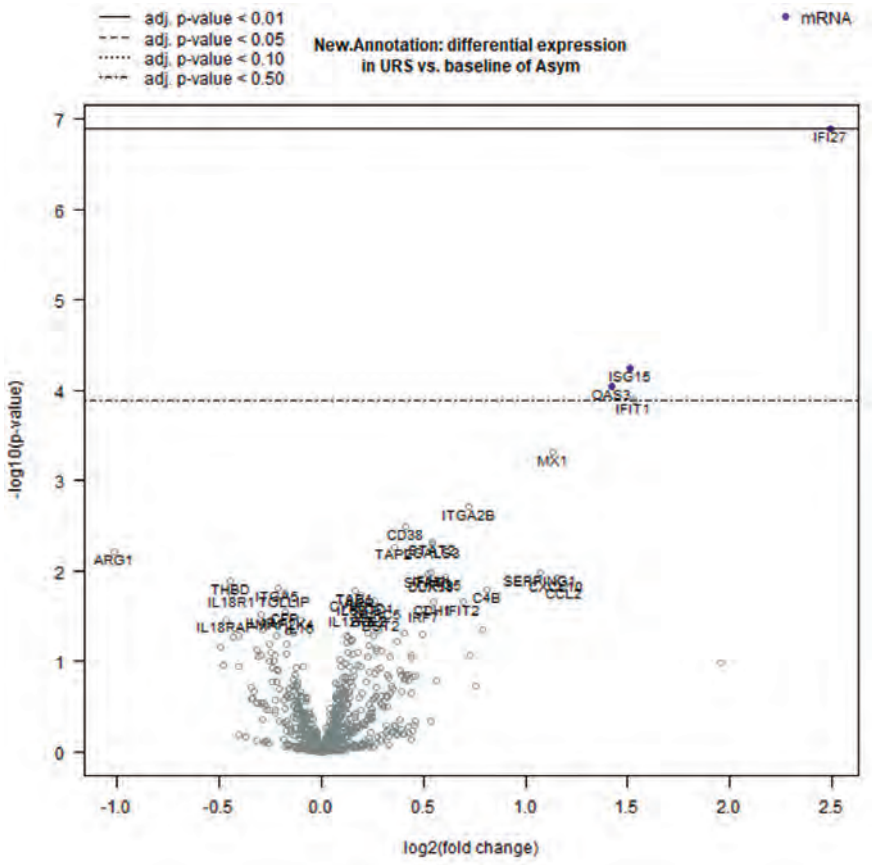

Figure 1: Volcano plot displaying the top 40 differentially expressed genes between athletes reporting URS compared to asymptomatic athletes. Highly differentially expressed genes, by (log^2) fold-difference fall to either side of the plot and highly significantly different genes fall at the top of the plot. *IFI27*, an interferon signalling gene, is shown in the top right corner with a log^2 fold-difference of 2.49, p=6.7*10^-4.

likelihood that athletes would report URS (Table 1). The logistic regression model was statistically significant $\chi^2(df=6, n=71) = 31.59$, p=<0.001 indicating that the model could distinguish between athletes reporting URS and asymptomatic athletes. The model explained between 35% (Cox and Snell R square) and 47% (Nagelkerke R square) of the variance in URS and correctly classified 78% of cases. The only variable within the model that achieved statistical significance was *IFI27* (Odds ratio (OR)=1.02, p=0.01, 95% CI, 1.004 to 1.03). For every unit increase in *IFI27* mRNA, athletes were 1.02 times more likely to report URS in the previous month.

DISCUSSION

Highly multi-parametric immune gene expression profiling in elite athletes during Olympic selection resulted in two novel discoveries. For the first time we have identified that athletes reporting URS could be differentiated from asymptomatic athletes with 78% accuracy by the expression of one gene, *IFI27.* The expression of this gene, an interferon-stimulated gene, is increased during the viral innate immune response via interferon signalling and increased mitochondrial activity (6). Secondly, in light of recent findings from Tang *et al.*, that *IFI27* is able to differentiate viral URS from other URS aetiologies (41) our results provide unique insight into the aetiology of URS in an elite athlete cohort in the lead up to an Olympic selection event. Furthermore, our observation of an increased expression of the immune anti-viral genes *MX1, OAS3, IFIT2* and I*SG15,* in the current study (prior to Benjamini-Yekutieli adjustment) were also reported as being related to viral infection by Tang et al. Our findings provide preliminary evidence for a viral basis of URS in highly trained athletes and indicates that inflammatory processes may still have been resolving.

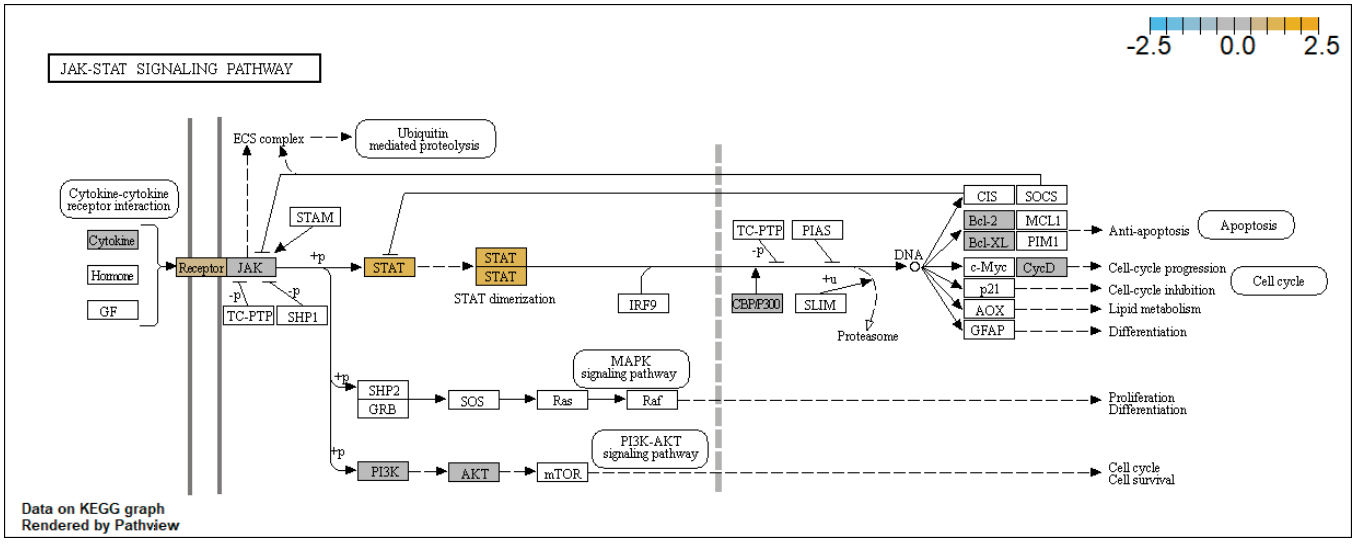

Figure 2: Genes from the STAT family and SOCS family are over expressed within the JAK-STAT pathway in athletes reporting URS compared to asymptomatic athletes. A KEGG diagram (Kyoto encyclopedia of genes and genomes) is a computerised representation of a biological pathway and its components. Genes and genes families within the plot and are represented within the Pan Cancer Immune Profiling Panel shown in colour or in grey. Genes and gene families known to be involved in the pathway but are not represented within the panel are shown in white. Genes and gene families that are over-expressed in the KEGG pathway are shown in shades of orange (log2 fold-difference 0 to 2.5).

Table 1 Logistic regression of probiotic supplementation, sport of the athlete and *IFI27* gene expression on the likelihood that athletes report URS (*indicates p<0.05)

							95% CI	
Variables	**B**	**SE**	**Wald**	**df**	***p*-value**	**Odds ratio**	**Lower**	**Upper**
***IFI27* gene expression**	0.18	0.007	6.27	1	0.01*	1.02	1.004	1.03
Probiotic supplementation	1.1	0.85	1.65	1	0.20	3.0	0.56	15.94
Sport *(Rowing as reference)*			2.73	4	0.60			
Rugby 7's	-0.06	1.09	0.003	1	0.96	0.94	0.11	8.01
Soccer	0.03	1.18	0.001	1	0.98	1.03	0.1	10.39
Triathlon	-20.12	2.84x10^4	5.0x10^-7	1	0.99	1.83x10^-9	0.0	0.0
Water polo	1.95	1.32	2.17	1	0.14	7.0	0.53	93.12
Constant	-1.56	1.25	1.57	1	0.21	0.21		

Other investigators have also pointed to a link between *IFI27* expression and infection with respiratory syncytial virus (12) and a number of other viruses (48), (31), (6). These associations indicate that *IFI27* expression is stimulated by a range of viruses and stressors and could explain, in part, why some of the athletes self-reported URS. To date, assessment of URS aetiology in athletes typically identify infective sources in approximately thirty percent of cases, which has prevented definitive recommendations for preventive interventions to reduce the risk of URS in athletic cohorts (38). Current paradigms in exercise immunology indicate that respiratory symptoms, particularly in athletes prone to recurrent illness, may be related to viral reactivation, although the role of viral reactivation in respiratory illness in athletes is conflicting (16, 18, 20). Our findings contribute to the evidence for a viral aetiology for URS in highly trained athletes. Future research could incorporate viral molecular profiling in conjunction with clinical investigation in longitudinal studies to inform diagnostic, treatment and prevention programs for URS in elite athlete cohorts.

The higher expression of immune genes in the JAK-STAT pathway and interferon-stimulated genes (ISG's) are also associated with viral infection (41, 47). The JAK-STAT pathway is central to multiple immune processes via cellular membrane signalling that leads to the induction of immune gene expression (36). Interferons, cytokines, interleukins and hormones are all able to activate the JAK-STAT pathway with widespread downstream effects (30). A key mechanism of JAK-STAT activation is via interferon signalling which leads to the expression of ISG's (21). JAK-STAT upregulation was observed in the URS group in addition to two other KEGG pathways outlining genes known to be involved in the immune response to influenza A and herpes simplex infection. Identification of upregulated gene expression across these two viral response pathways is important as both viruses have been identified as sources of URS in athletes either through primary, unresolved or reactivated infection (8, 20, 35, 39). Several other studies involving non-athletes have identified a strikingly similar set of core genes that are common to the immune gene expression response elicited following viral inoculation (3, 9, 19, 26, 40, 46, 47).

Evaluating other respiratory pathologies also provides insights into immune-regulatory mechanisms that may be relevant in the context of URS in athletes. In patients with asthma, peripheral blood immune gene expression was measured from 166 blood samples taken following asthma exacerbations and compared to 1149 samples representing quiescent asthma in the same patients. This study reported upregulation of the JAK-STAT pathway and for ISG's. Upregulated genes included *STAT1, STAT2, IFI27, JAK1, JAK2, IRF7, MX1, IFI35, ISG15, OAS2, IFIH1,* and *OASL* (4). Many of the same genes in this study were significantly upregulated in athletes reporting URS in our study, including *IFI27*. Although patients experiencing asthma exacerbations were excluded from the study if diagnosed by a physician with an active illness, an accurate URS diagnosis is difficult (39). In individuals with allergic inflammation, a recent study compared the gene expression of PBMCs with or without antigen challenge from four subjects with diagnosed allergic inflammation compared to the cells from four healthy controls (25). Although the study involved a very small cohort, gene activation of the JAK-STAT pathway within PBMC's was observed in patients with allergic inflammation (25). The studies described above had recruited patients with diagnosed URS caused by either asthma, allergic inflammation or infection and all reported involved upregulation of the JAK-STAT pathway and ISG immune expression. Our results indicating up-regulation of *IFI27* was reported in patients following asthma exacerbation but not following allergic inflammation. The current results provide cohort-specific evidence for *IFI27* as a potential viral biomarker in highly trained athletes although the involvement of *IFI27* in other inflammatory pathways should be examined further.

Limitations

The main limitation of this study was that sampling conditions were not standardized. The variables that were not controlled included sex, diet, sport, the time of day samples were collected, whether the athletes had trained or were rested, whether athletes were fasted/non-fasted, the location athletes were sampled and by whom they were sampled. Many of these factors can alter the circulation / mobilisation of immune cells and, where possible, the statistical approach adjusted for those variables that were statistically significant between the groups. The

current findings should be confirmed using a more structured study design, ideally within a prospective-longitudinal study using athletes from multiple centers, to determine whether *IFI27* is related to URS. An additional limitation of this study was that the number of days between sample collection and when URS were experienced was not recorded. A record of when URS was last experienced could be used to inform statistical evaluation and further interpretation of the results.

Conclusion

Athletes reporting URS were able to be differentiated from asymptomatic athletes for the first time by a higher expression of the *IFI27* immune gene involved in the response to viral infection and multiple genes within the JAK-STAT pathway. The results give important insight into the aetiology of URS in athletes. The results present an opportunity for further research aiming to refine diagnostic, treatment and URS prevention programs in elite athletes.

Contribution: The data presented were collected as part of the "Stay Healthy" project which was led by MKD in collaboration with AJC, DBP, NV, DH, GW, RA, LMB, BL, MT, DW, GL, SP, SH, MW, AWC and NPW. The study was undertaken by CC as part of her PhD research and she played a central role in all stages of the research and manuscript writing. AWC, NPW and AJC were her supervisors, they conceived the study design and guided all stages of the work. JV and AJC guided laboratory analyses. All authors contributed with the logistics of "Stay Healthy" project as well as the interpretation of the study results, the editorial process and the revision of the manuscript.

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Conflicts of Interest: Nil

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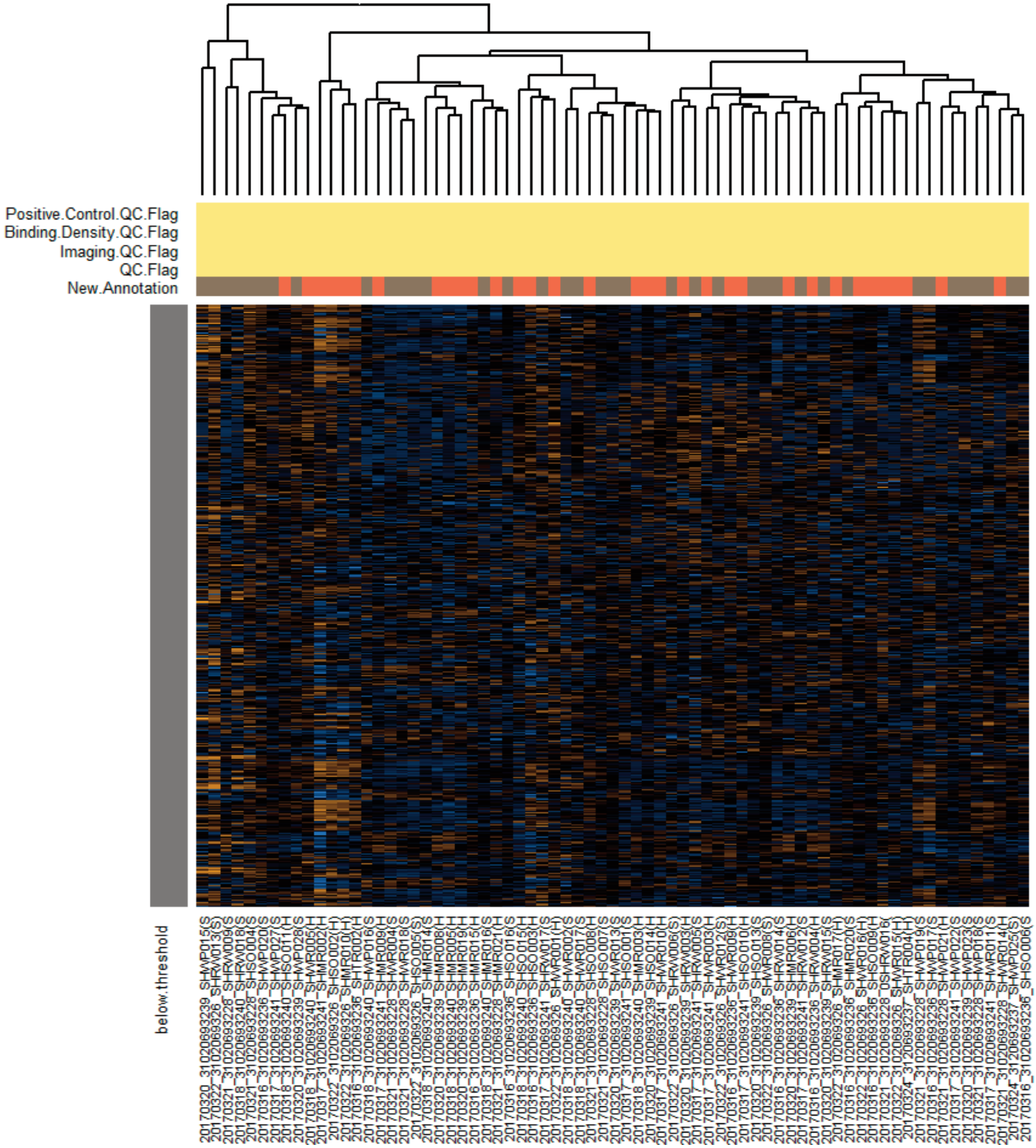
Supplement 1: Illness symptoms log and group characteristics questionnaire	**Answer**
Q1. In the last month, have you had upper respiratory symptoms such as blocked or runny nose, sore throat or sneezing?	Yes/No
Q1-1. How many days of FULL TRAINING with upper respiratory symptoms?	Number of days
Q1-2. How many days of MODIFIED TRAINING with upper respiratory symptoms?	Number of days
Q1-3. How many days were there where you COULD NOT TRAIN due to upper respiratory symptoms?	Number of days
Q2. In the last month, have you had chest infection symptoms such as coughing, sputum, chest congestion, wheezing or high temperature?	Yes/No
Q2-1. How many days of FULL TRAINING with chest infection symptoms?	Number of days
Q2-2. How many days of MODIFIED TRAINING with chest infection symptoms?	Number of days
Q2-3. How many days were there where you COULD NOT TRAIN due to chest infection symptoms?	Number of days
Q4. In the last month, have you taken probiotic supplements?	Yes/No
Q5. When was the last time you trained and at what intensity did you train?	Open question
Q6. Describe your normal amount of training in a week?	Open question

Supplement 2: Group characteristics; sex, the main sport of the athletes, the time and intensity of the training session immediately prior to sampling, age and self-reported training load (* indicates $p<0.05$)

	Asym (n=33) n (%)	**URS (n=38) n (%)**	**X^2**	**Adj. p-value**	**Yates' continuity correction**	**Phi coefficient**	**q-value**
Sex							
Male	13 (39.4)	8 (21.1)	0.09		0.15	0.20	-
Female	20 (60.6)	30 (78.9)					
Sport							
Triathlon	2 (6.1)	0 (0)		0.17			
Rugby 7's	19 (57.6)	11 (28.9)		0.05			
Water polo	1 (3.0)	11 (28.9)	0.04*	0.02*	-	0.46	-
Rowing	3 (9.1)	9 (23.7)		0.17			
Soccer	8 (24.2)	7 (18.4)		0.55			
Time and intensity of the training session immediately prior to sampling							
>12 hrs; overnight rest	21 (64)	18 (47)					
<3 hrs; light session	8 (24)	7 (18)	0.09		-	0.26	-
<3 hrs; typical session	4 (12)	13 (34)					
Probiotic use							
Yes	7(21.2)	21(55.3)	0.003		0.007*	0.35	-
No	26(78.8)	17(44.7)					
	Asym mean(SD)	**URS mean(SD)**	**Mean difference**		**SE of difference**	**p-value**	**q-value**
Age	25.1 (4.4)	23.8 (3.5)	1.33		0.94	0.16	0.32
Self-reported training hrs/week							
Resistance	4.2 (1.3)	4.2(1.5)	0.1		0.48	0.90	0.9
Endurance	14.9 (10.8)	10.1 (9.6)	4.8		5.0	0.35	0.47
Team	8.4 (2.5)	9.6 (1.9)	1.2		0.74	0.11	0.32

Supplement 3: Unsupervised hierarchic clustering of 305 immune genes that were above background and differentiated URS (n=38, grey) and asymptomatic athletes (n=33, coral), within the horizontal row above the heat map. Each column within the heat map represents a sample and each row represents a gene. The horizontal yellow bar at the top of the map represents the quality control (QC) standards (positive control, binding density and imaging) the yellow colour indicates that all sample passed QC. The grey column to the left of the map represents the gene count threshold and the grey colour indicates that all genes met the minimum expression of 50 transcripts. Within the heat map, the data is plotted as a z-score - orange is indicative of high gene expression and blue is indicative of low gene expression. Despite some minor clustering of URS subjects to the right of the map no substantial differences in overall immune gene expression could separate the groups.



Supplement 4: Adjusted differential gene expression between the URS and Asym group. Most genes reaching $p<0.05$ were expressed at a higher frequency in the URS group. Only *IFI27* remained significant following adjustment for multiple comparisons BY.$p<0.05$.

Gene	Log^2 FC	Linear Fold-difference	Confident limit Log^2		P-value	BY.*p*.value
			Lower	Upper		
IFI27	2.49	5.62	1.67	3.31	$1.28*10^{-07}$	$6.7*10^{-04}$
ISG15	1.51	2.85	0.83	2.20	$5.71*10^{-05}$	0.15
OAS3	1.42	2.68	0.76	2.09	$9.03*10^{-05}$	0.16
IFIT1	1.53	2.89	0.80	2.27	$1.30*10^{-04}$	0.17
MX1	1.13	2.19	0.53	1.73	$4.90*10^{-04}$	0.51
ITGA2B	0.72	1.65	0.28	1.16	$1.93*10^{-03}$	1
CD38	0.41	1.33	0.15	0.68	$3.18*10^{-03}$	1
STAT2	0.54	1.46	0.18	0.91	$4.80*10^{-03}$	1
LGALS3	0.54	1.46	0.18	0.91	$5.12*10^{-03}$	1
TAP2	0.36	1.28	0.12	0.61	$5.41*10^{-03}$	1
ARG1	-1.02	0.49	-1.73	-0.32	$6.13*10^{-03}$	1
SERPING1	1.07	2.10	0.28	1.87	0.010	1
IFIH1	0.53	1.45	0.14	0.93	0.011	1
STAT1	0.52	1.43	0.13	0.91	0.011	1
IFI35	0.60	1.52	0.15	1.06	0.012	1
DDX58	0.54	1.45	0.13	0.95	0.012	1
CXCL10	1.15	2.22	0.28	2.03	0.012	1
THBD	-0.45	0.73	-0.79	-0.10	0.013	1
CCL2	1.19	2.28	0.26	2.12	0.014	1
ITGA5	-0.21	0.86	-0.38	-0.05	0.015	1
C4B	0.81	1.75	0.17	1.45	0.016	1
TAB1	0.16	1.12	0.03	0.29	0.017	1
TOLLIP	-0.18	0.88	-0.33	-0.03	0.018	1
IL18R1	-0.44	0.74	-0.80	-0.08	0.018	1
APP	0.19	1.14	0.04	0.35	0.019	1
CYLD	0.14	1.10	0.03	0.25	0.020	1
NOD1	0.26	1.20	0.04	0.48	0.022	1
IFIT2	0.69	1.62	0.11	1.27	0.022	1
CDH1	0.55	1.46	0.09	1.01	0.022	1
IL6ST	0.18	1.13	0.03	0.32	0.023	1
NLRC5	0.28	1.22	0.04	0.52	0.025	1
IRF7	0.50	1.42	0.07	0.93	0.026	1
CFP	-0.18	0.88	-0.34	-0.02	0.028	1
BCL2	0.23	1.18	0.03	0.44	0.030	1
IL12RB1	0.18	1.13	0.02	0.34	0.030	1
IL4R	-0.30	0.81	-0.56	-0.03	0.031	1
MAP2K4	-0.18	0.89	-0.33	-0.02	0.032	1
BST2	0.30	1.23	0.03	0.57	0.034	1
IL18RAP	-0.47	0.72	-0.90	-0.04	0.035	1
IL16	-0.10	0.93	-0.20	-0.01	0.035	1
FLT3LG	0.16	1.12	0.01	0.31	0.037	1
CD83	0.24	1.18	0.02	0.46	0.038	1
CD97	-0.21	0.87	-0.40	-0.01	0.040	1
BID	-0.29	0.82	-0.56	-0.01	0.045	1
ISG20	0.28	1.21	0.01	0.55	0.046	1
SIGLEC1	0.79	1.73	0.03	1.55	0.046	1
SELPLG	-0.17	0.89	-0.33	-0.01	0.047	1
UBC	-0.14	0.91	-0.27	0.00	0.049	1
THBS1	0.40	1.32	0.01	0.79	0.049	1
IRF4	0.23	1.17	0.01	0.45	0.049	1
PBK	0.49	1.41	0.01	0.97	0.05	1
TNFSF14	-0.22	0.86	-0.44	0.00	0.05	1

HLADOB	0.25	1.19	0.00	0.50	0.05	1
IL1R2	-0.41	0.76	-0.81	0.00	0.05	1
TRAF3	0.12	1.09	0.00	0.24	0.05	1
ITGA4	0.13	1.09	0.00	0.26	0.05	1
S100A12	-0.44	0.74	-0.87	0.00	0.05	1
POU2F2	0.15	1.11	0.00	0.30	0.06	1
HLADMA	0.15	1.11	0.00	0.30	0.06	1
BCL2L1	0.37	1.29	-0.01	0.75	0.06	1
IL2RG	0.12	1.09	0.00	0.25	0.06	1
STAT5B	-0.17	0.89	-0.35	0.01	0.06	1
TNFRSF10C	-0.25	0.84	-0.52	0.01	0.07	1
LRP1	0.24	1.18	-0.01	0.48	0.07	1
IL17A	-0.50	0.71	-1.02	0.03	0.07	1
TAP1	0.27	1.20	-0.02	0.56	0.07	1
CXCR6	-0.32	0.80	-0.66	0.03	0.08	1
F13A1	0.27	1.20	-0.02	0.55	0.08	1
LCN2	0.29	1.23	-0.03	0.62	0.08	1
FOS	-0.25	0.84	-0.53	0.03	0.08	1
CD36	0.18	1.13	-0.02	0.38	0.08	1
SMAD3	0.13	1.09	-0.01	0.27	0.08	1
ICAM3	-0.17	0.89	-0.36	0.02	0.08	1
CD74	0.15	1.11	-0.02	0.32	0.08	1
SLC11A1	-0.30	0.82	-0.63	0.04	0.09	1
IDO1	0.73	1.66	-0.09	1.55	0.09	1
CEBPB	-0.23	0.86	-0.48	0.03	0.09	1
ROPN1	0.44	1.35	-0.06	0.93	0.09	1
MFGE8	0.22	1.17	-0.03	0.48	0.09	1
PRG2	0.34	1.26	-0.05	0.72	0.09	1
BCL6	-0.31	0.81	-0.67	0.04	0.09	1
ITGB3	0.44	1.36	-0.07	0.94	0.09	1
MAP4K2	0.09	1.06	-0.01	0.19	0.10	1
FUT7	-0.26	0.84	-0.55	0.04	0.10	1
CXCR1	-0.23	0.85	-0.51	0.04	0.10	1
HLADRB4	1.96	3.89	-0.37	4.28	0.10	1
IKBKE	0.11	1.08	-0.02	0.25	0.11	1
HAMP	-0.48	0.72	-1.06	0.10	0.11	1
CD40	0.18	1.13	-0.04	0.40	0.11	1
CD1E	-0.41	0.75	-0.90	0.09	0.11	1
PNMA1	0.14	1.10	-0.03	0.30	0.11	1
CYBB	0.19	1.14	-0.04	0.43	0.12	1
IKBKB	0.10	1.07	-0.02	0.22	0.12	1
TRAF6	-0.09	0.94	-0.20	0.02	0.12	1
TREM1	-0.24	0.85	-0.54	0.06	0.12	1
TFRC	0.29	1.22	-0.07	0.64	0.12	1
RELA	-0.13	0.92	-0.28	0.03	0.12	1
TNFSF10	0.28	1.21	-0.07	0.62	0.12	1
TLR4	-0.22	0.86	-0.50	0.06	0.12	1
LTA	0.16	1.11	-0.04	0.36	0.13	1
IL6	0.39	1.31	-0.11	0.88	0.13	1
CXCR2	-0.21	0.86	-0.48	0.06	0.13	1
IL15	0.24	1.18	-0.07	0.54	0.13	1
CCRL2	0.26	1.20	-0.08	0.60	0.14	1
IL13	0.41	1.33	-0.13	0.95	0.14	1
IL15RA	0.32	1.25	-0.10	0.74	0.14	1
MST1R	0.39	1.31	-0.12	0.91	0.14	1
IL2	0.42	1.34	-0.13	0.98	0.14	1
MAPK3	-0.13	0.91	-0.31	0.04	0.14	1
ICOS	0.16	1.11	-0.05	0.36	0.15	1
CCL14	0.45	1.36	-0.15	1.04	0.15	1
SELE	0.46	1.38	-0.16	1.08	0.15	1
ANP32B	0.12	1.08	-0.04	0.27	0.15	1

IL11RA	0.14	1.10	-0.05	0.34	0.15	1
CD209	0.33	1.26	-0.12	0.77	0.15	1
HLADMB	0.12	1.08	-0.04	0.28	0.15	1
CD99	0.10	1.07	-0.04	0.24	0.15	1
IFNGR1	-0.14	0.91	-0.33	0.05	0.16	1
SOCS1	0.41	1.33	-0.16	0.98	0.16	1
SAA1	0.41	1.32	-0.15	0.96	0.16	1
LAMP3	0.56	1.47	-0.22	1.34	0.16	1
IL17B	0.43	1.35	-0.17	1.03	0.17	1
FPR2	-0.22	0.86	-0.53	0.09	0.17	1
GNLY	-0.24	0.85	-0.57	0.10	0.17	1
CDKN1A	0.22	1.16	-0.09	0.52	0.17	1
CD244	-0.12	0.92	-0.28	0.05	0.17	1
CTLA4	0.15	1.11	-0.06	0.35	0.17	1
ILF3	0.07	1.05	-0.03	0.16	0.18	1
TAPBP	0.12	1.09	-0.05	0.29	0.18	1
TNFSF8	0.09	1.06	-0.04	0.21	0.18	1
TNFSF12	-0.11	0.92	-0.28	0.05	0.18	1
CD3G	0.12	1.09	-0.06	0.30	0.19	1
CD58	-0.13	0.92	-0.31	0.06	0.19	1
PSMB9	0.15	1.11	-0.07	0.37	0.19	1
TFEB	-0.12	0.92	-0.30	0.06	0.19	1
XCR1	0.35	1.27	-0.16	0.85	0.19	1
C2	0.76	1.69	-0.36	1.87	0.19	1
IFNAR1	-0.14	0.91	-0.34	0.07	0.19	1
PSMB10	0.12	1.09	-0.06	0.30	0.19	1
CD63	-0.12	0.92	-0.30	0.06	0.19	1
CRP	-0.35	0.79	-0.86	0.17	0.19	1
MASP1	-0.34	0.79	-0.86	0.17	0.19	1
TNFSF13B	0.27	1.20	-0.14	0.67	0.20	1
ICAM2	0.09	1.06	-0.04	0.22	0.20	1
C3AR1	-0.21	0.87	-0.52	0.11	0.20	1
IFITM2	-0.21	0.87	-0.52	0.11	0.20	1
CD40LG	0.10	1.07	-0.05	0.26	0.20	1
TLR1	-0.16	0.90	-0.39	0.08	0.21	1
CCL15	0.35	1.27	-0.19	0.88	0.21	1
PTGS2	-0.14	0.91	-0.34	0.07	0.21	1
PSEN1	-0.10	0.93	-0.26	0.06	0.22	1
CD180	0.14	1.10	-0.08	0.36	0.22	1
CTCFL	0.39	1.31	-0.23	1.00	0.22	1
HLADRB3	0.12	1.09	-0.07	0.31	0.22	1
THY1	-0.33	0.79	-0.86	0.20	0.22	1
CD274	0.44	1.35	-0.26	1.13	0.22	1
CFB	0.34	1.26	-0.20	0.88	0.22	1
ITK	0.10	1.07	-0.06	0.27	0.23	1
LILRB1	0.13	1.10	-0.08	0.34	0.23	1
TAL1	0.20	1.15	-0.12	0.51	0.23	1
TBK1	0.10	1.07	-0.06	0.26	0.23	1
TFE3	-0.17	0.89	-0.43	0.10	0.23	1
TRAF2	0.08	1.06	-0.05	0.21	0.23	1
CD33	-0.11	0.92	-0.30	0.07	0.24	1
TCF7	0.10	1.07	-0.06	0.26	0.24	1
NFKBIA	-0.10	0.93	-0.26	0.06	0.24	1
MAPK11	0.27	1.20	-0.17	0.70	0.24	1
MAPK14	-0.15	0.90	-0.39	0.09	0.24	1
LTF	0.30	1.23	-0.19	0.79	0.24	1
MAPKAPK2	-0.10	0.93	-0.28	0.07	0.24	1
CD276	0.31	1.24	-0.21	0.84	0.24	1
FCER1A	-0.17	0.89	-0.47	0.12	0.24	1
ADORA2A	0.09	1.06	-0.06	0.23	0.24	1
BTK	0.10	1.07	-0.06	0.26	0.25	1

CCL19	0.32	1.25	-0.22	0.86	0.25	1
ATG16L1	0.07	1.05	-0.05	0.18	0.25	1
CD28	0.12	1.09	-0.08	0.32	0.25	1
IFI16	0.19	1.14	-0.13	0.50	0.25	1
CLEC7A	0.14	1.10	-0.10	0.37	0.25	1
GTF3C1	0.10	1.07	-0.07	0.27	0.25	1
SPO11	0.33	1.25	-0.23	0.88	0.25	1
TNFRSF17	0.26	1.20	-0.18	0.71	0.25	1
CTSW	-0.16	0.90	-0.42	0.11	0.25	1
CASP8	-0.08	0.95	-0.21	0.06	0.25	1
IL18	-0.17	0.89	-0.45	0.12	0.26	1
PIN1	0.08	1.05	-0.05	0.21	0.26	1
IFNA8	-0.34	0.79	-0.91	0.24	0.26	1
CD96	0.09	1.06	-0.06	0.24	0.26	1
BCL10	-0.08	0.95	-0.21	0.06	0.26	1
CCL21	-0.34	0.79	-0.93	0.25	0.26	1
ELANE	0.25	1.19	-0.19	0.69	0.26	1
RUNX1	0.08	1.06	-0.06	0.22	0.26	1
TNFRSF11A	-0.23	0.86	-0.62	0.17	0.27	1
HLADPA1	0.11	1.08	-0.08	0.31	0.27	1
LILRA5	-0.19	0.88	-0.52	0.14	0.27	1
LILRB2	-0.12	0.92	-0.32	0.09	0.27	1
NFATC1	0.08	1.06	-0.07	0.23	0.27	1
TNFRSF8	-0.12	0.92	-0.32	0.09	0.28	1
IFNAR2	-0.08	0.95	-0.21	0.06	0.28	1
CD6	0.09	1.06	-0.07	0.25	0.28	1
IRF8	0.09	1.06	-0.07	0.25	0.28	1
IRAK2	-0.11	0.92	-0.32	0.09	0.29	1
CD34	0.29	1.22	-0.24	0.82	0.29	1
VCAM1	-0.32	0.80	-0.90	0.27	0.29	1
EPCAM	-0.29	0.82	-0.84	0.25	0.29	1
C9	-0.28	0.82	-0.80	0.24	0.29	1
CASP1	0.13	1.09	-0.11	0.36	0.30	1
IL2RA	0.13	1.10	-0.11	0.38	0.30	1
MAP2K2	0.07	1.05	-0.06	0.20	0.30	1
CSF3R	-0.13	0.91	-0.37	0.11	0.30	1
TNFRSF1A	-0.11	0.93	-0.31	0.10	0.30	1
C6	0.29	1.22	-0.26	0.84	0.30	1
STAT4	0.09	1.06	-0.08	0.25	0.30	1
CEACAM1	0.24	1.18	-0.21	0.68	0.30	1
HLADRA	0.09	1.06	-0.08	0.26	0.31	1
TLR8	-0.14	0.91	-0.41	0.13	0.31	1
DOCK9	0.10	1.07	-0.09	0.29	0.31	1
CD4	0.10	1.07	-0.09	0.28	0.31	1
LTBR	-0.11	0.93	-0.33	0.10	0.31	1
LY86	0.09	1.06	-0.08	0.26	0.32	1
NFATC4	-0.28	0.83	-0.81	0.26	0.32	1
MAGEC1	0.32	1.25	-0.31	0.96	0.32	1
NLRP3	-0.12	0.92	-0.35	0.11	0.32	1
NFKB2	0.08	1.05	-0.07	0.23	0.33	1
TNFAIP3	0.08	1.06	-0.08	0.24	0.33	1
PLA2G1B	0.31	1.24	-0.30	0.91	0.33	1
MR1	0.11	1.08	-0.11	0.33	0.33	1
PSMB8	0.10	1.07	-0.10	0.31	0.33	1
ZAP70	0.08	1.06	-0.08	0.23	0.33	1
CLEC4C	0.25	1.19	-0.25	0.74	0.33	1
TLR2	-0.13	0.92	-0.38	0.13	0.33	1
CD47	0.04	1.03	-0.04	0.12	0.34	1
S100A8	-0.18	0.88	-0.54	0.19	0.34	1
RELB	0.12	1.08	-0.12	0.35	0.34	1
MNX1	-0.26	0.84	-0.79	0.27	0.34	1

NRP1	0.19	1.14	-0.20	0.58	0.34	1
CD14	-0.12	0.92	-0.36	0.12	0.34	1
NUP107	0.07	1.05	-0.08	0.22	0.34	1
MPPED1	-0.23	0.85	-0.70	0.24	0.35	1
BATF	-0.10	0.93	-0.32	0.11	0.35	1
CD3E	0.07	1.05	-0.08	0.22	0.35	1
CCL3L1	-0.27	0.83	-0.82	0.29	0.35	1
CCL5	0.10	1.07	-0.11	0.32	0.35	1
C1QBP	0.06	1.05	-0.07	0.20	0.36	1
CCND3	-0.06	0.96	-0.20	0.07	0.36	1
IL6R	-0.09	0.94	-0.30	0.11	0.36	1
MEF2C	0.10	1.07	-0.12	0.31	0.37	1
CCL3	-0.23	0.86	-0.72	0.27	0.37	1
LAMP2	-0.09	0.94	-0.27	0.10	0.37	1
PYCARD	-0.09	0.94	-0.29	0.11	0.37	1
CX3CL1	0.25	1.19	-0.29	0.79	0.37	1
FYN	0.06	1.04	-0.07	0.18	0.37	1
ATG10	-0.08	0.94	-0.27	0.10	0.38	1
TLR6	-0.11	0.93	-0.36	0.14	0.38	1
ENTPD1	-0.13	0.92	-0.40	0.15	0.38	1
CD3D	0.08	1.05	-0.09	0.24	0.38	1
PECAM1	-0.08	0.95	-0.26	0.10	0.38	1
NOD2	0.13	1.09	-0.16	0.42	0.38	1
EWSR1	0.04	1.03	-0.05	0.12	0.38	1
IFITM1	0.16	1.12	-0.20	0.52	0.38	1
CARD9	0.09	1.06	-0.11	0.29	0.39	1
IL1RAP	-0.12	0.92	-0.40	0.15	0.39	1
PPARG	0.23	1.18	-0.29	0.76	0.39	1
PIK3CD	-0.06	0.96	-0.21	0.08	0.39	1
ANXA1	-0.06	0.96	-0.19	0.07	0.39	1
REL	0.06	1.04	-0.08	0.19	0.40	1
TLR10	-0.08	0.95	-0.27	0.11	0.40	1
TANK	0.09	1.06	-0.12	0.30	0.41	1
IFNL2	0.24	1.18	-0.32	0.79	0.41	1
NCF4	-0.11	0.93	-0.35	0.14	0.41	1
CD8B	-0.11	0.93	-0.36	0.14	0.41	1
IL17F	0.24	1.18	-0.32	0.80	0.41	1
IL8	0.16	1.12	-0.23	0.55	0.42	1
FCER1G	-0.14	0.91	-0.47	0.19	0.42	1
HCK	-0.09	0.94	-0.31	0.13	0.42	1
IRF5	0.09	1.06	-0.12	0.30	0.42	1
LIF	0.25	1.19	-0.35	0.84	0.42	1
JAK2	0.10	1.07	-0.14	0.33	0.42	1
CR1	-0.12	0.92	-0.43	0.18	0.42	1
IFNB1	0.22	1.17	-0.32	0.77	0.43	1
TICAM1	-0.07	0.95	-0.25	0.10	0.43	1
SPANXB1	0.25	1.19	-0.36	0.85	0.43	1
NOTCH1	-0.09	0.94	-0.30	0.13	0.43	1
BLK	0.14	1.10	-0.21	0.49	0.43	1
IRAK4	-0.05	0.96	-0.19	0.08	0.43	1
PTPRC	-0.11	0.93	-0.37	0.16	0.44	1
PDCD1LG2	0.39	1.31	-0.59	1.36	0.44	1
ALCAM	0.08	1.05	-0.12	0.27	0.44	1
CD27	0.07	1.05	-0.10	0.24	0.44	1
IRAK1	-0.05	0.97	-0.16	0.07	0.44	1
CXCL13	0.23	1.17	-0.36	0.81	0.45	1
LILRA1	-0.07	0.95	-0.27	0.12	0.45	1
KLRK1	-0.08	0.94	-0.30	0.13	0.45	1
CXCR4	-0.06	0.96	-0.20	0.09	0.45	1
TXNIP	-0.05	0.96	-0.20	0.09	0.45	1
CD5	0.07	1.05	-0.11	0.24	0.45	1

USP9Y	-0.29	0.82	-1.03	0.45	0.45	1
SH2B2	-0.09	0.94	-0.33	0.15	0.45	1
ETS1	0.06	1.04	-0.10	0.23	0.45	1
C7	0.24	1.18	-0.38	0.85	0.45	1
CCL11	-0.21	0.87	-0.75	0.33	0.45	1
ITGB1	0.06	1.04	-0.10	0.23	0.46	1
MSR1	0.46	1.38	-0.76	1.68	0.46	1
AICDA	-0.17	0.89	-0.62	0.28	0.46	1
EP300	0.04	1.03	-0.07	0.15	0.46	1
CXCL11	0.54	1.45	-0.89	1.96	0.47	1
LTB	0.05	1.04	-0.09	0.19	0.47	1
CXCL16	-0.09	0.94	-0.33	0.15	0.47	1
ECSIT	0.08	1.06	-0.14	0.29	0.47	1
IGF2R	-0.09	0.94	-0.33	0.16	0.47	1
TNFSF11	-0.19	0.88	-0.69	0.32	0.48	1
MAP3K5	0.06	1.04	-0.11	0.24	0.48	1
PASD1	0.21	1.15	-0.36	0.77	0.48	1
FUT5	0.21	1.15	-0.36	0.78	0.48	1
ULBP2	0.17	1.13	-0.30	0.64	0.48	1
LRRN3	0.13	1.09	-0.23	0.49	0.48	1
TIGIT	0.10	1.07	-0.17	0.36	0.48	1
CCR1	0.13	1.10	-0.24	0.50	0.48	1
HSD11B1	-0.17	0.89	-0.65	0.31	0.49	1
SYT17	-0.17	0.89	-0.65	0.31	0.49	1
SBNO2	-0.09	0.94	-0.36	0.17	0.49	1
IL26	-0.16	0.90	-0.61	0.29	0.49	1
CD22	0.08	1.06	-0.16	0.32	0.50	1
TLR5	-0.16	0.90	-0.61	0.29	0.50	1
CCR7	0.08	1.05	-0.14	0.29	0.50	1
CCL22	0.21	1.16	-0.40	0.82	0.50	1
MAF	0.06	1.04	-0.12	0.24	0.51	1
KIR3DL1	0.35	1.28	-0.69	1.40	0.51	1
IL13RA1	-0.08	0.95	-0.32	0.16	0.51	1
TNFRSF11B	0.21	1.16	-0.42	0.85	0.51	1
KLRB1	-0.10	0.94	-0.38	0.19	0.51	1
CD24	0.07	1.05	-0.15	0.30	0.51	1
CCR6	0.08	1.06	-0.17	0.34	0.51	1
KIR Activating Subgroup 2	0.31	1.24	-0.62	1.24	0.51	1
C8G	0.14	1.10	-0.27	0.54	0.51	1
IL22	0.20	1.15	-0.40	0.80	0.52	1
IL1RN	0.12	1.09	-0.25	0.49	0.52	1
TNFSF4	0.09	1.06	-0.18	0.36	0.52	1
KLRC1	0.12	1.09	-0.25	0.49	0.52	1
KIR Inhibiting Subgroup 1	0.45	1.37	-0.92	1.82	0.52	1
BTLA	0.06	1.04	-0.12	0.24	0.52	1
MAGEA12	0.18	1.13	-0.37	0.73	0.52	1
FCGR2B	0.10	1.07	-0.21	0.41	0.52	1
C1QB	0.36	1.29	-0.76	1.48	0.53	1
PRM1	0.18	1.13	-0.37	0.73	0.53	1
KIR_ Inhibiting Subgroup 2	0.43	1.34	-0.91	1.76	0.53	1
CD207	0.17	1.13	-0.37	0.72	0.54	1
CCR2	0.06	1.04	-0.13	0.25	0.54	1
ITGAL	0.03	1.02	-0.07	0.14	0.54	1
FCGR2A	-0.09	0.94	-0.37	0.19	0.54	1
CXCR5	0.08	1.06	-0.17	0.33	0.54	1
RORA	0.06	1.04	-0.12	0.24	0.54	1
MRC1	-0.12	0.92	-0.52	0.27	0.54	1
CD84	0.05	1.04	-0.12	0.23	0.54	1

HMGB1	0.02	1.02	-0.05	0.09	0.54	1
ICAM1	-0.08	0.95	-0.34	0.18	0.54	1
TBX21	-0.08	0.95	-0.33	0.17	0.55	1
YTHDF2	0.03	1.02	-0.07	0.14	0.55	1
VEGFA	-0.08	0.95	-0.34	0.18	0.55	1
IL3	0.19	1.14	-0.42	0.80	0.55	1
LCP1	-0.06	0.96	-0.24	0.13	0.55	1
PIK3CG	-0.04	0.97	-0.16	0.09	0.55	1
CD86	0.06	1.04	-0.14	0.26	0.56	1
IL24	-0.13	0.91	-0.58	0.31	0.56	1
IGLL1	0.17	1.12	-0.40	0.73	0.57	1
MEFV	-0.07	0.95	-0.31	0.17	0.57	1
SMPD3	0.32	1.25	-0.78	1.43	0.57	1
MME	-0.10	0.93	-0.44	0.24	0.57	1
CCR5	-0.07	0.95	-0.32	0.18	0.57	1
CFI	0.17	1.12	-0.40	0.73	0.57	1
IFNA7	0.14	1.10	-0.33	0.60	0.57	1
MARCO	0.39	1.31	-0.96	1.74	0.57	1
IL7R	0.05	1.04	-0.12	0.22	0.57	1
NFKB1	-0.05	0.97	-0.21	0.12	0.58	1
CSF2RB	-0.07	0.96	-0.30	0.17	0.58	1
CFD	0.15	1.11	-0.39	0.70	0.58	1
CTSL	0.44	1.36	-1.15	2.04	0.59	1
CD19	0.07	1.05	-0.19	0.34	0.59	1
SLAMF7	0.08	1.05	-0.20	0.35	0.59	1
APOE	0.15	1.11	-0.39	0.69	0.59	1
MAGEA1	-0.17	0.89	-0.79	0.45	0.59	1
LAMP1	-0.03	0.98	-0.15	0.09	0.59	1
IL25	-0.15	0.90	-0.70	0.40	0.59	1
CD53	-0.04	0.97	-0.21	0.12	0.59	1
TREM2	0.14	1.10	-0.37	0.65	0.59	1
IFNA17	0.14	1.10	-0.36	0.63	0.60	1
MAPK8	-0.03	0.98	-0.16	0.09	0.60	1
ATF1	-0.03	0.98	-0.12	0.07	0.60	1
IL1RL2	0.14	1.10	-0.39	0.68	0.60	1
MAVS	-0.03	0.98	-0.15	0.09	0.61	1
IL23A	0.07	1.05	-0.19	0.33	0.61	1
ITGA1	0.43	1.35	-1.20	2.06	0.61	1
CYFIP2	-0.02	0.99	-0.11	0.06	0.61	1
LYN	-0.06	0.96	-0.28	0.17	0.61	1
CMA1	0.14	1.10	-0.40	0.67	0.61	1
SPP1	0.24	1.18	-0.69	1.18	0.61	1
TYK2	-0.03	0.98	-0.17	0.10	0.62	1
IL22RA1	0.16	1.11	-0.45	0.76	0.62	1
C8B	0.14	1.10	-0.40	0.68	0.62	1
CCR3	-0.09	0.94	-0.46	0.27	0.62	1
CD1C	-0.06	0.96	-0.30	0.18	0.62	1
VEGFC	0.32	1.25	-0.93	1.56	0.62	1
CDH5	0.12	1.09	-0.36	0.61	0.62	1
CCL8	0.35	1.27	-1.04	1.74	0.62	1
KLRD1	-0.07	0.96	-0.33	0.20	0.63	1
CKLF	-0.07	0.96	-0.33	0.19	0.63	1
NFATC2	0.04	1.03	-0.13	0.21	0.63	1
LILRB3	-0.06	0.96	-0.31	0.19	0.63	1
PRAME	0.14	1.11	-0.44	0.73	0.63	1
SYCP1	-0.15	0.90	-0.78	0.47	0.63	1
RIPK2	0.05	1.04	-0.16	0.26	0.63	1
PDGFRB	0.41	1.32	-1.25	2.06	0.63	1
TARP	-0.08	0.95	-0.39	0.24	0.64	1
ITGAE	0.04	1.03	-0.13	0.21	0.64	1
SIGIRR	0.04	1.02	-0.11	0.18	0.64	1

CX3CR1	0.06	1.04	-0.19	0.31	0.64	1
BST1	-0.06	0.96	-0.32	0.19	0.64	1
PDCD1	0.36	1.29	-1.15	1.88	0.64	1
C1S	0.10	1.08	-0.33	0.54	0.64	1
IL17RB	-0.11	0.93	-0.55	0.34	0.64	1
MICA	0.07	1.05	-0.22	0.36	0.64	1
ARG2	0.40	1.32	-1.29	2.09	0.65	1
IL34	-0.10	0.93	-0.52	0.32	0.65	1
CARD11	0.04	1.03	-0.15	0.23	0.65	1
CLEC6A	0.37	1.29	-1.22	1.96	0.65	1
CCL28	-0.14	0.91	-0.73	0.46	0.65	1
FLT3	-0.41	0.76	-2.18	1.37	0.66	1
ABCB1	-0.05	0.97	-0.26	0.16	0.66	1
SELL	0.06	1.04	-0.21	0.33	0.66	1
MYD88	-0.04	0.97	-0.24	0.15	0.66	1
ITGA6	0.04	1.03	-0.14	0.23	0.66	1
CTSG	0.30	1.23	-1.02	1.62	0.66	1
TNFRSF13C	0.06	1.04	-0.21	0.33	0.66	1
SMAD2	-0.02	0.99	-0.12	0.08	0.66	1
GZMM	0.05	1.03	-0.17	0.26	0.66	1
CAMP	-0.08	0.95	-0.45	0.29	0.67	1
CD48	0.04	1.03	-0.13	0.21	0.67	1
IRF1	0.05	1.04	-0.19	0.30	0.67	1
EBI3	-0.11	0.93	-0.63	0.40	0.67	1
MERTK	-0.37	0.77	-2.08	1.34	0.67	1
MAP2K1	-0.03	0.98	-0.14	0.09	0.67	1
CDK1	0.33	1.26	-1.22	1.88	0.68	1
CD160	-0.08	0.95	-0.45	0.29	0.68	1
CD2	0.03	1.02	-0.13	0.20	0.68	1
SH2D1B	-0.06	0.96	-0.34	0.22	0.68	1
CD1B	0.12	1.08	-0.44	0.67	0.69	1
CEACAM8	0.27	1.21	-1.04	1.58	0.69	1
EGR1	0.10	1.07	-0.37	0.56	0.69	1
CXCL1	-0.06	0.96	-0.34	0.22	0.69	1
JAM3	0.09	1.06	-0.34	0.52	0.69	1
IFNA2	0.12	1.09	-0.48	0.72	0.69	1
PSMB7	0.02	1.01	-0.08	0.13	0.70	1
GZMK	0.05	1.03	-0.20	0.30	0.70	1
CD46	0.03	1.02	-0.13	0.19	0.70	1
MASP2	0.12	1.09	-0.49	0.73	0.70	1
NEFL	0.24	1.18	-0.97	1.44	0.70	1
MCAM	0.10	1.07	-0.42	0.62	0.70	1
HLADPB1	0.03	1.02	-0.14	0.20	0.71	1
IL3RA	0.08	1.06	-0.34	0.50	0.71	1
CHUK	-0.03	0.98	-0.17	0.12	0.71	1
MBL2	-0.11	0.93	-0.70	0.47	0.71	1
LCK	0.03	1.02	-0.12	0.18	0.71	1
CXCL14	-0.11	0.93	-0.69	0.47	0.71	1
GPI	-0.03	0.98	-0.16	0.11	0.71	1
IL1RAPL2	0.08	1.05	-0.33	0.48	0.71	1
ITGAM	-0.04	0.97	-0.25	0.18	0.72	1
ATF2	0.02	1.01	-0.08	0.11	0.72	1
TPSAB1	-0.19	0.88	-1.25	0.88	0.73	1
BMI1	0.03	1.02	-0.12	0.17	0.73	1
CCL26	0.11	1.08	-0.49	0.70	0.73	1
SLAMF1	0.04	1.03	-0.18	0.25	0.74	1
MAP3K1	-0.02	0.98	-0.16	0.11	0.74	1
CD247	0.03	1.02	-0.13	0.19	0.74	1
LBP	0.10	1.07	-0.50	0.71	0.74	1
KIR3DL2	0.44	1.36	-2.16	3.04	0.74	1
JAK3	0.04	1.02	-0.18	0.25	0.74	1

DUSP4	0.08	1.06	-0.41	0.58	0.74	1
A2M	0.09	1.06	-0.45	0.62	0.74	1
CD37	-0.02	0.98	-0.17	0.12	0.75	1
HLAC	-0.06	0.96	-0.41	0.30	0.75	1
BIRC5	0.22	1.16	-1.12	1.55	0.75	1
IL1R1	-0.32	0.80	-2.26	1.63	0.75	1
IL17RA	-0.04	0.98	-0.25	0.18	0.75	1
CXCL6	-0.27	0.83	-1.96	1.42	0.75	1
PPBP	0.06	1.05	-0.35	0.47	0.76	1
STAT6	-0.02	0.98	-0.17	0.12	0.76	1
HLAG	-0.06	0.96	-0.46	0.33	0.76	1
CD9	0.04	1.03	-0.23	0.31	0.76	1
PVR	-0.04	0.97	-0.29	0.22	0.76	1
AKT3	-0.02	0.99	-0.17	0.12	0.77	1
C5	0.29	1.22	-1.62	2.19	0.77	1
TLR7	0.30	1.23	-1.70	2.30	0.77	1
CXCR3	0.04	1.03	-0.21	0.28	0.77	1
IL12B	0.08	1.06	-0.46	0.63	0.77	1
CD79B	-0.03	0.98	-0.25	0.19	0.78	1
PRF1	-0.03	0.98	-0.28	0.21	0.79	1
KIR3DL3	-0.09	0.94	-0.74	0.56	0.79	1
ATG5	0.02	1.01	-0.11	0.14	0.79	1
MS4A1	0.04	1.03	-0.23	0.30	0.79	1
IFNA1	-0.08	0.94	-0.69	0.52	0.79	1
MAGEC2	0.08	1.06	-0.50	0.66	0.79	1
CD1D	-0.02	0.98	-0.19	0.14	0.79	1
TNFRSF9	-0.28	0.82	-2.36	1.79	0.79	1
MUC1	0.23	1.17	-1.45	1.91	0.79	1
IL13RA2	0.09	1.06	-0.56	0.74	0.79	1
FOXJ1	-0.28	0.83	-2.31	1.76	0.79	1
CCL18	0.07	1.05	-0.42	0.55	0.79	1
PMCH	-0.08	0.95	-0.64	0.49	0.79	1
CTSS	0.02	1.02	-0.16	0.21	0.79	1
C1QA	0.14	1.10	-0.91	1.19	0.80	1
KLRC2	-0.05	0.97	-0.40	0.30	0.80	1
CCL25	-0.06	0.96	-0.54	0.42	0.80	1
TGFB1	-0.02	0.99	-0.13	0.10	0.80	1
KLRF1	-0.04	0.97	-0.33	0.25	0.80	1
CCL27	-0.06	0.96	-0.53	0.41	0.80	1
ZNF205	0.07	1.05	-0.48	0.62	0.80	1
LAG3	0.04	1.03	-0.28	0.36	0.81	1
RRAD	0.07	1.05	-0.47	0.60	0.81	1
IL5	0.08	1.06	-0.55	0.71	0.81	1
STAT3	-0.02	0.98	-0.22	0.17	0.81	1
IL2RB	-0.03	0.98	-0.26	0.20	0.81	1
S100B	0.08	1.06	-0.56	0.72	0.81	1
CD80	0.20	1.15	-1.42	1.82	0.81	1
IRF2	0.02	1.02	-0.17	0.22	0.81	1
TNFRSF1B	-0.02	0.99	-0.18	0.14	0.81	1
ADA	0.02	1.01	-0.15	0.19	0.81	1
DUSP6	-0.02	0.98	-0.23	0.18	0.82	1
TNFSF13	-0.02	0.98	-0.22	0.17	0.82	1
ATG7	0.02	1.01	-0.15	0.18	0.82	1
TNFRSF4	0.27	1.20	-2.04	2.57	0.82	1
HLADQA1	-0.26	0.83	-2.51	1.99	0.82	1
SLAMF6	-0.02	0.99	-0.22	0.17	0.82	1
CXCL5	0.06	1.04	-0.47	0.59	0.82	1
RUNX3	-0.02	0.99	-0.18	0.14	0.83	1
MS4A2	-0.17	0.89	-1.63	1.30	0.83	1
CCL7	-0.07	0.95	-0.67	0.53	0.83	1
LY9	0.02	1.01	-0.14	0.18	0.83	1

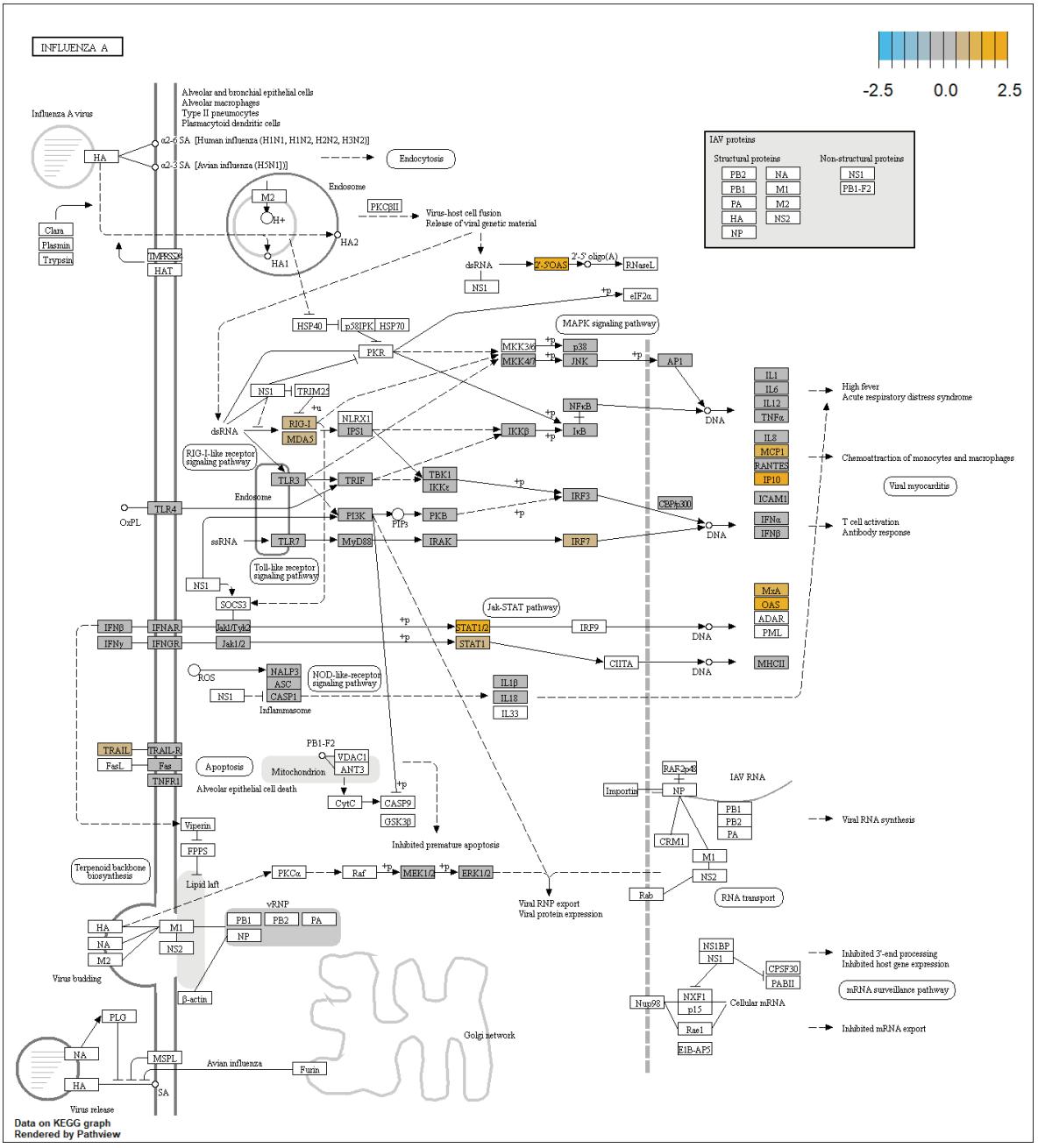
CCL4	0.03	1.02	-0.22	0.28	0.83	1
SYK	-0.02	0.99	-0.19	0.15	0.83	1
CCL20	0.33	1.25	-2.76	3.42	0.84	1
NOS2A	0.06	1.05	-0.55	0.68	0.84	1
TICAM2	-0.03	0.98	-0.27	0.22	0.84	1
ITGAX	-0.02	0.98	-0.25	0.20	0.84	1
IL27	0.17	1.12	-1.42	1.75	0.84	1
GZMA	-0.02	0.98	-0.26	0.21	0.84	1
CXCL9	0.20	1.15	-1.72	2.12	0.84	1
LILRA4	0.16	1.12	-1.37	1.69	0.84	1
TNFSF18	0.06	1.04	-0.51	0.62	0.84	1
CLEC4A	0.02	1.02	-0.20	0.24	0.84	1
IL7	0.22	1.16	-1.90	2.33	0.84	1
ITGB2	0.02	1.01	-0.14	0.17	0.84	1
CEACAM6	0.12	1.09	-1.08	1.33	0.84	1
CHIT1	-0.17	0.89	-1.84	1.51	0.84	1
IL32	-0.02	0.99	-0.23	0.19	0.85	1
PTGDR2	-0.04	0.97	-0.47	0.38	0.85	1
PLA2G6	0.01	1.01	-0.12	0.15	0.85	1
MAGEA4	0.05	1.04	-0.50	0.61	0.85	1
IGF1R	-0.02	0.99	-0.21	0.18	0.86	1
FN1	0.05	1.04	-0.54	0.65	0.86	1
IL19	-0.05	0.97	-0.61	0.51	0.86	1
CSF3	0.04	1.03	-0.44	0.53	0.86	1
SPINK5	-0.05	0.96	-0.64	0.53	0.86	1
CMKLR1	-0.03	0.98	-0.36	0.30	0.86	1
HAVCR2	0.02	1.01	-0.17	0.21	0.86	1
CASP10	0.20	1.15	-1.98	2.38	0.86	1
GATA3	0.18	1.14	-1.87	2.24	0.86	1
IL9	0.05	1.04	-0.55	0.66	0.86	1
CXCL3	-0.18	0.88	-2.19	1.84	0.86	1
TP53	-0.01	0.99	-0.14	0.12	0.86	1
C3	0.25	1.19	-2.63	3.13	0.87	1
CD200	0.03	1.02	-0.28	0.33	0.87	1
DDX43	-0.12	0.92	-1.47	1.23	0.87	1
MAPK1	0.02	1.01	-0.19	0.23	0.87	1
TTK	0.13	1.09	-1.38	1.63	0.87	1
REPS1	0.02	1.01	-0.19	0.23	0.87	1
IL1RL1	-0.09	0.94	-1.24	1.05	0.87	1
FEZ1	-0.17	0.89	-2.18	1.85	0.87	1
GZMB	-0.02	0.98	-0.33	0.28	0.87	1
MAP3K7	0.01	1.00	-0.08	0.10	0.88	1
CREB5	-0.02	0.99	-0.29	0.25	0.88	1
TIRAP	-0.02	0.99	-0.21	0.18	0.88	1
EOMES	-0.14	0.91	-1.84	1.57	0.88	1
F2RL1	-0.03	0.98	-0.37	0.32	0.88	1
SPACA3	-0.04	0.97	-0.62	0.53	0.88	1
CD163	0.02	1.02	-0.29	0.34	0.88	1
MAGEB2	0.04	1.03	-0.46	0.54	0.88	1
IL21R	0.02	1.01	-0.21	0.25	0.88	1
CSF2	-0.05	0.97	-0.64	0.55	0.88	1
TNFRSF18	0.16	1.12	-1.91	2.23	0.88	1
TNFRSF12A	0.20	1.15	-2.42	2.81	0.88	1
IL23R	0.10	1.07	-1.18	1.37	0.88	1
IRF3	-0.01	0.99	-0.20	0.17	0.88	1
ELK1	0.13	1.09	-1.55	1.80	0.88	1
BAGE	-0.04	0.98	-0.51	0.44	0.88	1
SPA17	0.20	1.15	-2.43	2.83	0.88	1
SPN	0.01	1.01	-0.16	0.18	0.89	1
TLR3	-0.11	0.92	-1.67	1.44	0.89	1
AMICA1	-0.01	0.99	-0.20	0.18	0.89	1

TNFRSF13B	-0.13	0.92	-1.91	1.65	0.89	1
C8A	0.04	1.03	-0.54	0.62	0.89	1
PSEN2	0.23	1.17	-2.98	3.44	0.89	1
PRKCE	0.17	1.13	-2.23	2.58	0.89	1
GAGE1	0.19	1.14	-2.44	2.82	0.89	1
S100A7	0.04	1.03	-0.52	0.60	0.89	1
LTK	-0.11	0.93	-1.68	1.46	0.89	1
IKBKG	-0.01	0.99	-0.16	0.14	0.89	1
DMBT1	0.17	1.13	-2.36	2.70	0.90	1
PRKCD	-0.01	0.99	-0.20	0.18	0.90	1
BAX	-0.01	1.00	-0.10	0.09	0.90	1
CD79A	0.02	1.01	-0.24	0.27	0.90	1
CD59	-0.02	0.99	-0.30	0.27	0.90	1
RPS6	0.01	1.01	-0.19	0.21	0.90	1
XCL2	0.02	1.01	-0.31	0.35	0.91	1
IL5RA	-0.07	0.95	-1.33	1.18	0.91	1
OSM	0.08	1.06	-1.32	1.48	0.91	1
IFNG	0.09	1.06	-1.43	1.60	0.91	1
MIF	0.01	1.01	-0.14	0.16	0.91	1
CR2	0.16	1.12	-2.69	3.02	0.91	1
ITGB4	-0.10	0.93	-1.89	1.69	0.91	1
CD1A	0.12	1.08	-1.92	2.16	0.91	1
CLEC5A	0.09	1.06	-1.48	1.66	0.91	1
HLAA	-0.01	0.99	-0.19	0.17	0.91	1
CCL13	0.16	1.12	-2.65	2.96	0.91	1
SH2D1A	-0.01	0.99	-0.21	0.19	0.91	1
IL11	-0.03	0.98	-0.64	0.58	0.92	1
RAG1	0.03	1.02	-0.44	0.49	0.92	1
ITCH	-0.01	1.00	-0.10	0.09	0.92	1
FADD	-0.13	0.91	-2.56	2.30	0.92	1
TPTE	-0.02	0.99	-0.43	0.39	0.92	1
CD44	-0.01	1.00	-0.11	0.10	0.92	1
CTAG1B	-0.10	0.93	-1.93	1.74	0.92	1
IL22RA2	0.03	1.02	-0.51	0.57	0.92	1
CD70	0.09	1.06	-1.61	1.79	0.92	1
RORC	0.02	1.01	-0.32	0.35	0.92	1
KIT	-0.11	0.93	-2.30	2.08	0.92	1
TNFRSF10B	-0.01	0.99	-0.20	0.18	0.92	1
JAK1	0.00	1.00	-0.10	0.09	0.92	1
CD164	-0.01	1.00	-0.16	0.15	0.93	1
CCR4	0.12	1.09	-2.44	2.68	0.93	1
PLAUR	-0.01	0.99	-0.27	0.25	0.93	1
MAGEA3	-0.03	0.98	-0.61	0.55	0.93	1
SEMG1	0.11	1.08	-2.26	2.48	0.93	1
TNF	0.01	1.01	-0.16	0.17	0.93	1
COLEC12	0.03	1.02	-0.52	0.57	0.93	1
IL12RB2	-0.09	0.94	-2.08	1.90	0.93	1
KLRG1	-0.02	0.99	-0.36	0.33	0.93	1
CREB1	0.01	1.01	-0.16	0.18	0.93	1
COL3A1	-0.13	0.92	-2.98	2.73	0.93	1
CD68	-0.01	0.99	-0.21	0.19	0.93	1
HLAB	-0.01	1.00	-0.18	0.16	0.93	1
CD81	-0.01	1.00	-0.18	0.16	0.94	1
IL4	0.05	1.04	-1.32	1.43	0.94	1
CCR9	0.06	1.04	-1.37	1.48	0.94	1
TMEFF2	-0.02	0.99	-0.56	0.52	0.94	1
IRGM	-0.06	0.96	-1.82	1.69	0.94	1
CREBBP	-0.10	0.93	-2.93	2.73	0.95	1
CTSH	-0.01	1.00	-0.17	0.16	0.95	1
EGR2	-0.09	0.94	-2.64	2.46	0.95	1
ATG12	0.10	1.07	-2.76	2.96	0.95	1

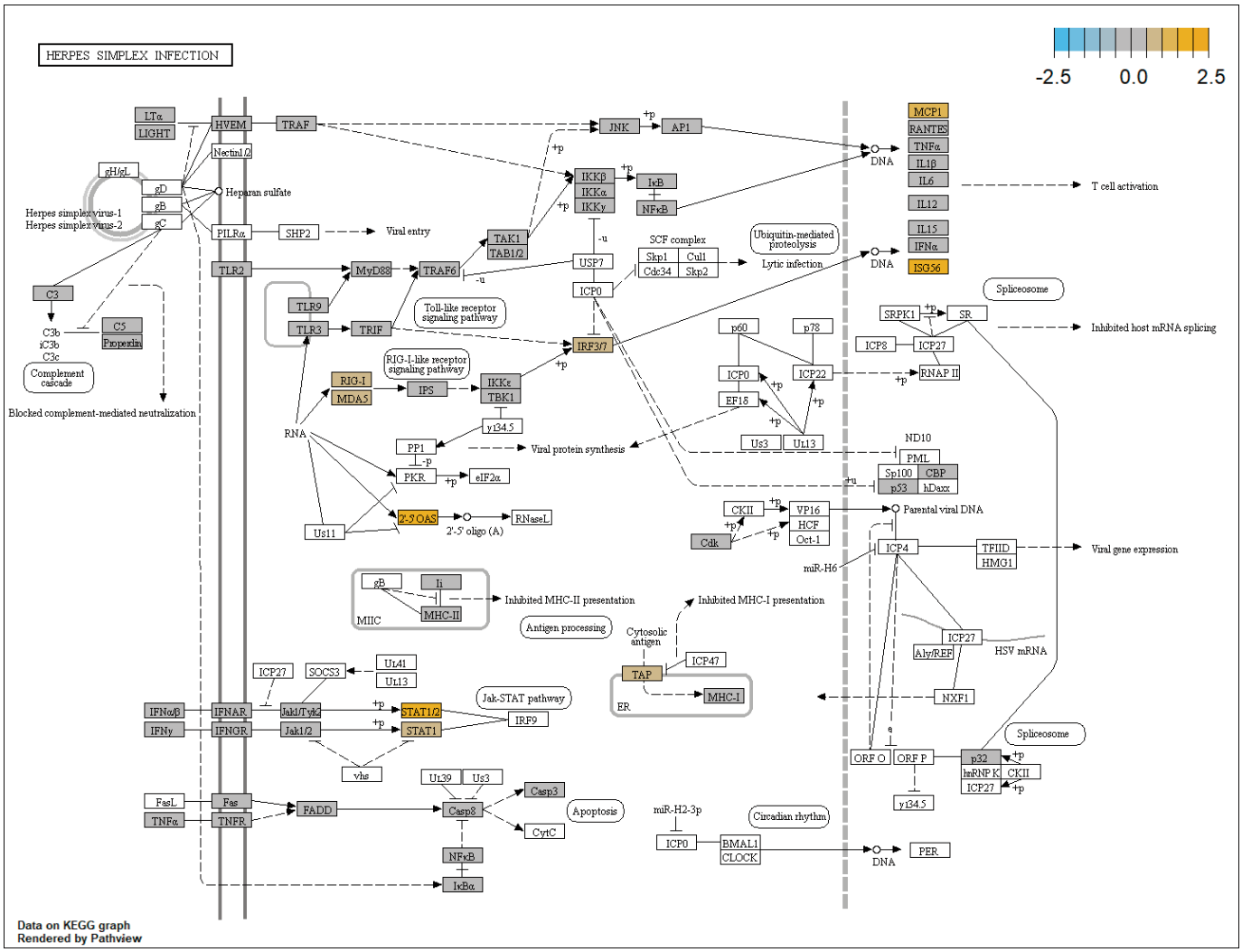
POU2AF1	0.07	1.05	-2.06	2.20	0.95	1
AXL	0.02	1.01	-0.44	0.47	0.95	1
GZMH	0.01	1.01	-0.39	0.42	0.95	1
FAS	0.01	1.01	-0.30	0.32	0.95	1
FCER2	0.01	1.01	-0.29	0.31	0.95	1
ENG	0.09	1.06	-2.61	2.79	0.95	1
PDGFC	0.09	1.06	-2.53	2.71	0.95	1
AMBP	0.02	1.01	-0.62	0.66	0.95	1
HLADQB1	-0.06	0.96	-1.82	1.71	0.95	1
CXCL12	0.02	1.01	-0.60	0.64	0.95	1
CD55	0.12	1.09	-3.65	3.90	0.95	1
DPP4	-0.11	0.93	-3.65	3.42	0.95	1
IL21	-0.02	0.99	-0.55	0.52	0.95	1
TNFRSF14	0.00	1.00	-0.15	0.16	0.95	1
IL1B	-0.01	0.99	-0.39	0.36	0.95	1
IL10RA	0.00	1.00	-0.11	0.11	0.95	1
PSMD7	0.00	1.00	-0.08	0.08	0.96	1
C1R	0.08	1.06	-2.99	3.15	0.96	1
TXK	0.00	1.00	-0.18	0.19	0.96	1
NFATC3	0.00	1.00	-0.10	0.09	0.96	1
F12	-0.04	0.97	-1.72	1.63	0.96	1
C4BPA	0.03	1.02	-1.05	1.10	0.96	1
DEFB1	0.01	1.01	-0.54	0.57	0.96	1
BLNK	0.06	1.04	-2.57	2.68	0.97	1
PLAU	-0.05	0.97	-2.17	2.08	0.97	1
IL12A	-0.04	0.97	-1.85	1.78	0.97	1
SERPINB2	0.04	1.03	-2.09	2.18	0.97	1
TGFB2	-0.01	1.00	-0.45	0.44	0.97	1
CSF1	0.04	1.02	-2.20	2.27	0.98	1
CXCL2	-0.03	0.98	-1.89	1.83	0.98	1
CCL1	0.04	1.03	-2.50	2.58	0.98	1
TNFSF15	0.10	1.07	-6.98	7.17	0.98	1
TLR9	-0.07	0.96	-5.19	5.06	0.98	1
CTAGE1	-0.01	0.99	-0.65	0.63	0.98	1
FCGR1A	0.01	1.01	-0.64	0.65	0.98	1
CD3EAP	0.04	1.03	-3.28	3.36	0.98	1
INPP5D	0.00	1.00	-0.12	0.13	0.98	1
CSF1R	-0.02	0.98	-2.30	2.25	0.98	1
SSX1	-0.01	1.00	-0.57	0.55	0.98	1
ICAM4	-0.02	0.98	-2.40	2.35	0.98	1
NCR1	0.00	1.00	-0.29	0.28	0.99	1
CCL24	-0.01	1.00	-0.70	0.68	0.99	1
ATM	0.03	1.02	-2.77	2.82	0.99	1
HRAS	0.03	1.02	-2.94	2.99	0.99	1
ST6GAL1	0.00	1.00	-0.15	0.15	0.99	1
LY96	0.00	1.00	-0.36	0.36	0.99	1
MICB	0.00	1.00	-0.21	0.21	0.99	1
HLAE	0.00	1.00	-0.15	0.15	0.99	1
CCL16	0.00	1.00	-0.60	0.59	0.99	1
IL1A	0.00	1.00	-0.56	0.57	0.99	1
CD8A	0.00	1.00	-0.28	0.27	0.99	1
CASP3	0.00	1.00	-0.10	0.10	0.99	1
AIRE	0.01	1.01	-2.02	2.05	0.99	1
KIR_Activating Subgroup_1	-0.01	1.00	-0.91	0.89	0.99	1
NT5E	-0.01	0.99	-2.00	1.98	0.99	1
FOXP3	0.01	1.01	-1.78	1.80	0.99	1
CLU	0.00	1.00	-0.57	0.56	0.99	1
FCGR3A	0.00	1.00	-0.28	0.28	0.99	1
ICOSLG	0.01	1.01	-2.38	2.40	0.99	1
NCAM1	-0.01	0.99	-2.10	2.08	0.99	1

ITGA2	-0.02	0.99	-3.84	3.81	0.99	1
LAIR2	0.00	1.00	-0.18	0.18	0.99	1
CCL17	0.00	1.00	-0.58	0.59	0.99	1
IL10	0.01	1.01	-2.99	3.01	1.00	1
CCL23	0.00	1.00	-1.36	1.37	1.00	1
CD7	0.00	1.00	-0.19	0.19	1.00	1
PAX5	0.00	1.00	-1.87	1.87	1.00	1
CT45A1	0.00	1.00	-1.74	1.75	1.00	1
ABL1	0.00	1.00	-5.41	5.40	1.00	1
SSX4	0.00	1.00	-5.40	5.40	1.00	1

Supplement 5: A KEGG pathway of the genes involved in the immune response to influenza A. Genes shown in white are known to be involved in the pathway however, but not represented within the Pan Cancer Immune Profiling Panel. Genes and gene families that are over-expressed in the KEGG pathway are shown in shades of orange (log2 fold-difference 0 to 2.5). The genes upregulated in athletes reported URS are as follow: *RIG1* (retinoic acid inducible gene 1) and *MDA5* (melanoma differentiation associated protein 5) translation yields proteins involved in the pattern recognition of viruses, the *OAS* genes (oligoadenylate synthase) leads to synthesis of protein capable of degrading viral RNA, *IRF3* (interferon regulatory factor 3) genes products stimulates transcription of interferon genes while the product of the *MCP1* (monocyte chemotactic protein 1) and *IP10* genes (interferon-inducible cytokine10) are involved in T cell activation and migration of monocytes. *TRAIL* (TNF-related apoptosis-inducing ligand), a gene whose protein is involved in initiation of apoptosis was also upregulated.



Supplement 6: A KEGG pathway of the genes involved in the immune response to herpes simplex virus. Genes shown in white are known to be involved in the pathway however, are not represented within the Pan Cancer Immune Profiling Panel. Genes and gene families that are over-expressed in the KEGG pathway are shown in shades of orange (log2 fold-difference 0 to 2.5). The genes upregulated in athletes who reported URS are as follow: *RIG1* (retinoic acid inducible gene 1) and *MDA5* (melanoma differentiation associated protein 5) translation yields proteins involved in the pattern recognition of viruses, the *2'-5'OAS* (oligoadenylate synthase 1) gene leads to synthesis of protein capable of degrading viral RNA, *IRF3* (interferon regulatory factor 3) gene products stimulates transcription of interferon genes while the product of the *MCP1* (monocyte chemotactic protein 1) and *ISG56* genes (interferon stimulated gene 56) are involved in T cell activation, monocytes activation and migration. *ISG56* is also involved in sensing single stranded (viral) RNA.



The mobilisation of early mature CD56^{dim}CD16^{bright} NK cells is blunted following a single bout of vigorous intensity exercise in Type 1 Diabetes

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ABSTRACT

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease that targets and destroys insulin-secreting pancreatic beta cells. Although T cell mediated, a number of other immune cells are also critically involved in co-ordinating the events leading to T1D. Specifically, innate subsets play an important role in the pathogenesis of T1D. NK cells are one of the first cell types to infiltrate the pancreas, causing damage and release of beta cell antigens. Previous work in our group has shown differential mobilisation of highly differentiated CD8$^+$ T cells during vigorous intensity exercise in T1D compared to a control cohort. Here, we aimed to explore exercise-induced mobilisation of other cell types involved in T1D pathogenesis. In this study, we investigated the effects of a single bout of vigorous (80% predicted VO_2 max) intensity exercise on innate cell mobilisation in T1D and control participants. T1D (N=12, mean age 33.2yrs, predicted VO_2 max 32.2 ml.kg.min^{-1}, BMI 25.3 kg.m^{-2}) and control (N=12, mean age 29.4yrs, predicted VO_2 max 38.5 ml.kg.min^{-1}, BMI 23.7 kg.m^{-2}) male participants completed a 30-minute bout of cycling at 80% predicted VO_2 max in a fasted state. Peripheral blood was collected at baseline, immediately post-exercise, and 1 hour post-exercise. NK cell subsets mobilised during vigorous intensity exercise in both control and T1D participants. However, mature NK cells, defined as the CD56^{dim}CD16^{bright} subset, displayed a lower percentage increase following vigorous intensity exercise in T1D participants (Control: 185.12%, T1D: 97.06%). This blunted mobilisation was specific to early mature NK cells (KIR$^+$) but not later differentiated NK cells (KIR$^+$CD57$^+$). Myeloid lineage subsets mobilised to a similar extent in both control and T1D participants. In conclusion, vigorous exercise mobilises innate immune cells in people with T1D albeit to a different extent to those without T1D. This mobilisation of innate immune cells provides a mechanistic argument to support exercise in people with T1D where it has the potential to improve surveillance for infection and to modulate the autoimmune response to the beta cell.

Key Words: Exercise, Physical activity, Type 1 Diabetes, Innate immunity, Natural Killer cells

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INTRODUCTION

Type 1 Diabetes (T1D) results from beta cell destruction caused by autoreactive T cells. Pancreatic lymphocyte infiltration results in a hallmark inflammation within the pancreas referred to as insulitis (57). In the initial stages of insulitis, a mixed leukocyte population including CD4$^+$ T cells, CD8$^+$ T cells, natural killer (NK) cells, Dendritic cells (DC), B cells, and macrophages infiltrate the pancreas (49, 67, 69, 81). Trafficking of CD45$^+$ cells from lymphoid organs to the pancreas, specifically to the region of insulitis, has been demonstrated by cell tracking in mice (49). CD4$^+$ T cell, CD8$^+$ T cell, and B cell populations are seen to migrate into the pancreas. NK cells and B cells make up much lower proportions of pancreatic lymphocyte populations (67). However, NK cells are one of the first cell types to infiltrate the pancreas, even before autoreactive T cell infiltration occurs (2, 8, 29, 64). The presence of NK cells results in damage and release of beta cell antigens. Myeloid lineage subsets then present beta cell antigens to autoreactive T cells and thus begins the autoreactive cascade. As demonstrated by these mixed immune populations infiltrating the pancreas in T1D, a range of immune cell subsets are involved in the pathogenesis of T1D.

T1D is an immune mediated disorder and the primary treatment is insulin replacement. However insulin replacement does not achieve the level of diabetes control required to protect patients from the complications of this condition (44). Whilst significant effort has been invested in trying to prevent and cure T1D, little progress has been made to date (75). Exercise has clear immunomodulatory effects which could unlock the potential for immunotherapy of T1D (43). However, to do so, we need to understand the effects of exercise on the immune system in T1D. Previous work in our group has shown differential mobilisation of highly differentiated CD8+ T cells during vigorous intensity exercise in T1D, a subset highly involved in the pathogenesis of T1D (23). This study however did not address the effect of exercise on innate immune cells. Because innate immune cells are some of the first cells to infiltrate the pancreatic islet in T1D, we also wanted to characterise the effect of exercise on innate immune cell mobilisation in people with T1D.

Innate cells in T1D

NK cells home to the diabetic pancreas before T cell infiltration or beta cell destruction occurs. NK cells have been

detected from day 1 in the pancreatic infiltrate of the non-obese diabetic (NOD) mouse, before inflammation becomes established (8, 64). A high percentage of NK cells are also detected at weeks 4-5 in the NOD pancreas, i.e. the prediabetic stage. Both NK cells and T cells were then detected in later weeks 9-10 (8). Furthermore, NK cells were present in the pancreas of NOD-Rag (no B or T cells) mice indicating NK cells infiltrate the pancreas independent of T cells and established inflammation, and is believed to be the first event in insulitis (8).

NK cell infiltration into the pancreas drives T1D onset. Increased intra-pancreatic NK cells accelerated the onset of diabetes in NOD mice and NK cell associated cytokines were hyper-expressed in the pancreas from mice with accelerated diabetes onset (2). In addition to this, intra-pancreatic NK cells influence diabetogenic T cell function. Activated NK cells within the pancreas of NOD mice produce large amounts of IFN-γ, thus promoting effector function of diabetogenic $CD4^+$ T cells (29). Furthermore, depletion of NK cells has prevented T1D in animal models, supporting their involvement in T1D pathogenesis (2, 30, 64).

NK cells within the islet have a distinct phenotype, differing to those found in the spleen and peripheral blood (74). Pancreatic NK cells are in an activated state indicted by increased expression of CD25, CD69, and PD-1, coupled with down-regulation of CD62 ligand (CD62L). Pancreatic NK cells also express higher killer cell lectin-like receptor subfamily G member 1 (KLRG1) than splenic NK cells, indicating their high proliferation state (74), and lower natural-killer group 2-member D (NKG2D) expression, a natural cytotoxicity receptor, was also reported (74).

Myeloid subsets, DC and macrophages, can also be detected within the pancreas during insulitis in diabetic donors (54, 69). DCs (defined as lineage negative, $HLA\text{-}DR^+$ (48)) are present in the islets of diabetic donors and have a clear role in the pathogenesis of T1D (69, 81). DC present beta cell antigens to autoreactive T cells, driving T cell trafficking to the pancreas (78). In NOD mice, DCs are efficient antigen presenting cells (APC). DCs effectively stimulated GAD, a beta cell autoantigen, reactive T cell proliferation in *in vitro* co-cultures (52). In another study, plasmacytoid DCs (pDC) were shown to present immune complexes to $CD4^+$ T cells more efficiently than conventional DCs (cDCs), suggesting a possible pathogenic role of pDCs in T1D onset (3).

Macrophages have also been implemented in the pathogenesis of T1D. Macrophages are recruited to the pancreas by $CD4^+$ T cells (13). One week after adoptive transfer of $CD4^+$ T cell into NOD.SCID (no B or T cells) mice, macrophages were detected in pancreatic infiltrates. Diabetogenic $CD4^+$ T cells recruit macrophages through C-C Motif Chemokine Ligand (CCL)1 secretion. This interacts with and recruits activated $CCR8^+$ macrophages (13). Macrophage infiltration is also mediated through CCL2 expression on beta cells. CCL2 promotes recruitment of macrophages from the bone marrow to the islets. Furthermore, CCL2 receptor inactivation prevents macrophage recruitment (54).

Acute exercise and innate cells

A significant amount of work on the effects of exercise on immunity has been undertaken in non-T1D cohorts. Acute exercise causes significant immune cell mobilisation, of which NK cells are the most exercise responsive lymphocyte subset (35, 56, 73). Fully differentiated $CD8^+$ T cells are the next most significantly mobilised cell subset (12, 79), with lower levels of mobilisation among $CD4^+$ T cells and B cells (12, 34, 72).

NK cells are phenotypically identified as $CD3^-CD56^+$ (21, 68). NK cells can be further divided based on their CD16 expression into functionally different subsets; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56d^{im}CD16^{dim}$ and $CD56^{bright}CD16^-$ (65). During maturation, NK cells become $CD56^{dim}$, and lose NKG2A expression (7). This is followed by increased killer immunoglobulin receptor (KIR) expression, with a gradual increase in CD57 (7). CD57 is a marker of highly mature, highly differentiated NK cells, and is expressed by highly cytotoxic $CD56^{dim}CD16^{bright}$ NK cells (47). Lack of Inhibitory C-type lectin receptor A (NKG2A) and expression of KIRs independently correlated with reduced proliferation, and co-expression of CD57 was associated with a completely abolished proliferative response to cytokines. This is evidenced by $KIR^+CD57^-NKG2A^-$ proliferate more than $KIR^+CD57^+NKG2A^-$.

NK cells are the most responsive lymphocyte subset to acute exercise due to their high beta-adrenergic receptor expression resulting in their preferential intensity-dependant mobilisation in response to adrenaline during acute exercise (5, 25, 50, 82). Of the NK cells mobilised, $CD56^{dim}CD16^{bright}$ NK cells and those with a highly differentiated phenotype ($CD57^+$ KIR^+ $NKG2A^{lo}$) are preferentially redeployed and demonstrate the largest increase post-exercise (6, 76). This is followed by a larger decrease below baseline during the recovery period. Following exercise, $CD56^{bright}$ subsets return to baseline levels, whereas $CD56^{dim}$ decrease below baseline levels (6, 76). There have also been reports of IL-2Rβ ($CD122^+$) and IL-2Rα ($CD25^+$) NK cells increasing following exercise. CD25 is expressed on $CD56^{bright}$ NK cells in comparison to CD122 which is constitutively expressed on all naïve NK cells (56, 73).

DCs also increase in the peripheral blood in response to physical stress (9, 24, 38, 60). In particular, there is an increase in DCs expressing adhesion molecules CCR5 and CD62L. Circulating DCs also display reduced toll-like receptor (TLR) responsiveness after acute exercise, as evidenced by a less pronounced upregulation of activation markers, HLA-DR and CD86. Therefore this indicates the mobilisation of DCs which may be less prone to drive inflammatory processes following exercise (24). In a recent study by Brownet al., (2018), 9 healthy males completed a 20-minute cycling bout at 80% VO_2 max. DCs were reported to increase by 150% following exercise. In this study, there was a preferential mobilisation of plasmacytoid DCs (pDC) ($CD303^+$) over than myeloid DCs (mDC) ($CD303^-$) during exercise. Within the mDC subsets, $CD1c^-CD141^-$ cells showed the largest exercise-induced mobilisation, with a stepwise pattern observed for

$CD1c^+CD141^-$, $CD1c^+CD141^+$, $CD1c^-CD141^+$ cells. It was also reported that $CD205^-$ mDC, DCs capable of recognising apoptotic and necrotic cells, were the most exercise responsive. All DC subsets returned to resting levels within 30 minutes following exercise cessation (9).

Other mononuclear cells also respond to exercise. Monocytes mobilise in an exercise intensity-dependent manner, with mature monocytes ($CD14^{low}$) increasing the most (33, 71, 80). Granulocytes also increase, with the majority of these being neutrophils. Neutrophils increase immediately post-exercise and fall below baseline during the 1 hour post-exercise recovery period, this is followed by an increase 2 hours post-exercise referred to as the "second wave" (10, 46, 66). Vigorous acute exercise also increases hematopoietic stem and progenitor cells (HSPC) post-exercise (4, 27).

Acute exercise and T1D

To date, there is a limited amount of research investigating the effects of exercise in people with T1D. This research is predominantly focused in two areas. First, exercise training in T1D has been shown to mediate improvements in beta cell function through increased insulin content and insulin secretion (43, 58, 59). We have previously hypothesised that an exercise training programme has the potential to modulate beta cell loss in people newly diagnosed with T1D (59). We have tested this hypothesis in a pilot randomised controlled trial (43, 58). This study showed that beta cell function, when corrected for the changes in insulin sensitivity that accompany physical exercise, appears to be preserved in people with T1D. Furthermore, exercise training in streptozotocin-induced T1D mice significantly increased insulin content and insulin secretion compared to sedentary mice (39).

Second, exercise modulates immunity in T1D (18, 23, 61). Two studies show that exercise training in NOD mice reduced immune cell infiltration into the pancreas and insulitis. These are the only two exercise studies in a model of T1D to demonstrate the modulatory effects of exercise on islet immunity (18, 61). Recently, our group has shown that acute vigorous intensity exercise causes intensity-dependant lymphocytosis in T1D. However, we observed an impaired mobilisation of highly differentiated $CD8^+$ EMRA T cells during vigorous intensity exercise in T1D (23). These are amongst the subsets which are directly involved in the pathogenesis of T1D. Furthermore, these subsets express high levels of the beta-2-adrenergic receptor and mobilise in response to adrenaline during acute exercise. This has led us to hypothesise that the adrenaline response during vigorous exercise may be impaired in T1D. Reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported previously, resulting in a dampened adrenaline response (31, 41, 77). During acute exercise, increased beta-adrenoceptor density and sensitivity of lymphocytes is noted in healthy participants. However, patients with congestive heart failure (CHF) who exhibited reduced beta-adrenoceptor density and sensitivity, displayed a blunted lymphocyte increase following acute exercise (51). Therefore, a similar effect may be seen in T1D and may impact exercise-induced lymphocytosis of exercise responsive subsets.

In this study, we describe the effects of vigorous intensity exercise on innate cell subsets in T1D, with a particular focus on the most exercise responsive subset, NK cells. We also investigated the adrenaline response during exercise in T1D to gain insight into the mechanisms of any differential lymphocyte mobilisation between T1D and controls.

METHODS

Participants

Ethical approval was granted by the Preston Research Ethics Committee (REC) for this study. Twelve controls and twelve T1D participants were recruited. All participants were male and between 16-65 years of age. Male only participants were chosen to minimise differences in immune cell phenotypic and functional capacity evident in females due to higher oestrogen levels (19, 36, 45). Participant baseline characteristics are reported in Table 1. T1D participants had a clinical diagnosis of T1D, were on basal bolus insulin regime or insulin pump therapy, competent in carbohydrate content estimation of meals, were willing to test glucose through capillary testing, and were able to recognise hypoglycaemic symptoms before blood glucose fell to 3.9mmol/L. Participants did not have a history of cardiac disease or other significant illness that would prevent attendance at the study site. All T1D participants did not have active proliferative diabetic retinopathy, autonomic neuropathy, or history of severe hypoglycaemia requiring third party assistance within the 3 months prior to the study. Discrepancies in participant numbers for the outcome measures presented in this study are due to low sample volume or missing reagents on the day of an individual's visit. Exact number of participants for each outcome measure can be found in Table and Figure legends.

Experimental design

Participants had one enrolment visit, where baseline demographics and anthropometric assessment were carried out (Table 1). These visits were undertaken in the NIHR/Wellcome Trust Clinical Research Facility at the University of Birmingham. Blood pressure and heart rate data were collected following 10min rest and using an Omron Professional Blood Pressure monitor. All equipment, including those for measuring height and weight are regularly calibrated for accuracy as per CRF protocol. During the enrolment visit, each participant completed a non-fasted incremental sub-maximal (85% HRmax) cycle ergometer test to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for the subsequent exercise visits to adjust for individual fitness (16). The enrolment visit and exercise visits were separated by a minimum of one week. Participants were asked to abstain from vigorous exercise 24 hours prior to each exercise visit. Participants were also required to record a food diary for the 24 hours prior to each exercise visit. Participants were advised to use these diaries to ensure that the same foods were consumed in the 24 hours prior to each exercise bout. The exercise visits started at 8.30am for all participants and consisted of a thirty-minute bout of cycling at 80% predicted VO_2 max. An initial fasting blood sample was taken for each participant once the cannula was inserted. The participant was then allowed to rest for a further 20 minutes before preparing

Table 1 Baseline Characteristics of T1D and control participants

	[1]Control	[2]T1D
	mean±SD	
Age (years)	28.8±4.6	33.2±9.7
Weight (Kg)	74.5±8.7	80.8±15.6
Height (cm)	155.6±54.8	177.63±7.3
BMI (kg/m2)	23.5±2	25.3±3.9
Waist circumference (cm)	86.3±6.5	90.3±12.3
Hip circumference (cm)	90.1±7	94.8±8.6
Chest Circumference (cm)	94.1±3.8	98.6±12.4
Waist-hip ratio	0.95±0.03	0.94±0.06
Body fat (%)	16.6±4.8	21.1±6.2
HbA1c*		65±12.4
Disease duration*		13.5±8.9
VO_2 Max	38.5±5.4	32.2±9.3
CMV index	0.42±0.47	0.51±0.5
Glucose (mmol)	5.24±0.48	8.91±3.15
Heart Rate (bpm)	68.1±8.12	74.4±14.37
Systolic BP (mmHg)	123.3±8.31	128.9±18.77
Dystolic BP (mmHg)	71.7±10.9	78.6±10.63
Number of cigarettes (per wk)#		
0	10	11
1-5	2	1
Alcohol intake (units per wk)#		
0	2	3
1-5	3	4
6-10	4	1
11-20	1	3
21-40	2	0
Job related PA (min/wk)	325±470	557±1221
Transportation PA (min/wk)	313±237	240±190
House Maintenance (min/wk)	129±122	135.45±129
Sport and Leisure PA (min/wk)	140±86	285.5±283
Light PA (min/wk)	42.5±45.2	81±91
Moderate PA (min/wk)	7.5±18.7	64.5±132
Vigorous PA (min/wk)	90±97	140±12
Time sitting (hrs/wk)	48±18	39.73±12
Stress score (1 year)	4.33±3.3	4.5±4.3
Stress score (1 month)	10.25±8.1	11.83±10.3
Stress score (visit 1)	7.36±9.86	5.67±9
Stress score (visit 2)	4.18±3	4±5

Mean and standard deviation values for baseline characteristics in control and T1D participants.

Note:
number of participants
[1]controls n=12
[2]T1D n=12

for the acute exercise bout. Fasting blood samples were collected intravenously at immediately pre-exercise, immediately post-exercise, and 1 hour post-exercise. Pre and post-exercise samples were taken whilst the participant was sitting on the cycle ergometer and sampling was strictly timed using a stopwatch. Timing for the immediately post-exercise sample was crucial because lymphocytes egress from peripheral blood within minutes of exercise cessation (70). At all 3 visits, all participants completed an international physical activity questionnaire (IPAQ) (37) and perceived stress questionnaires; the life scale events questionnaire (17), perceived stress scale (20), the undergraduate stress questionnaire (22), self-perceived health status (53), and the Pittsburgh sleep quality index (11).

Sample processing

All blood samples were processed under identical conditions using the same laboratory reagents and apparatus. Blood samples for immunophenotyping analysis were taken in lithium heparin vacuette tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany) and placed on a roller at room temperature to ensure constant mixing of the blood sample until processing. All sample processing was initiated within 2 hours of blood-draw. Haematological measures were conducted on 25µl of whole blood using an automated coulter counter (ABX Micros ES 60, HORIBA Medical). Relative cell number (cells/µl) of immune cell subsets was then calculated from this.

Whole blood staining

The whole blood staining protocol was optimised prior to the start of the study. The protocol was adapted from the Clinical Immunology Service, University of Birmingham. Red blood cells were lysed by preparing whole blood in 4ml aliquots and washed with 16ml Ammonium Chloride lysis buffer (16g Ammonium Chloride (326372, Sigma-Aldrich, Dorset, UK), 2g sodium hydrogen carbonate (S/4240/60, Fisher scientific Ltd, Loughborough, UK), 0.2g EDTA (E5134, Sigma-Aldrich, Dorset, UK), and 2L ddH2O). The sample was centrifuged at 1000g for 5 minutes. Pelleted cells were resuspended in 10mls RPMI-1640 (R0833, Sigma-Aldrich, Dorset, UK) (supplemented with 2% FBS) and centrifuged at 1000g for 5 minutes. Cells were then counted and resuspended to a concentration of $1x10^6$ cells/ml. Cells were stained with appropriate antibodies listed below and incubated in dark at 4°C for 20 minutes. Stained cells were fixed with 500µl 1X BD FACS lysing solution (containing 14% formaldehyde) (349202, BD Biosciences, Wokingham, UK) and incubated in dark at 4°C for a further 15 minutes. Fixed cells were washed (centrifuged at 1000g for 5 minutes) in 2ml phosphate-buffered saline (PBS). Pelleted cells were resuspended in 500µl PBS and stored at 4°C until flow cytometry analysis. The stability of fixed stains was assessed and confirmed that cells could be stored up to 24 hours at 4°C before flow cytometry analysis. All samples were analysed using BD LSR Fortessa X-20. Parent populations (i.e. lymphocytes) were selected based on their size on FSC/SSC dot plots. Doublets were omitted by selecting the linear population shown on FSC-A/FSC-H dot plots prior to recording. Events to record were set to 100,000 within the parent singlet population gate. Compensation was carried out monthly using compensation beads and single stained cells. A negative control (unstained whole blood) was run for each experiment.

Innate cell subset analysis

Two multicolour flow cytometry panels were designed to enable phenotypic analysis of leukocyte subsets using the following mAbs; **Panel 1 (NK cells):** anti-CD3 PE-Cy7 (UCHT1), anti-CD16 PE-CF594 (3G8), anti-CD18 (LFA-1) APC (6.7), anti-CD25 PE (M-A251) anti-CD56 BV510 (NCAM16.2), anti-CD57 BB515 (NK-1), anti-CD122 BV421 (131411), anti-CD158a (KIR) BV711 (HP-3E4), Live/Dead

fixable viability stain 780 APC-Cy7. **Panel 2 (Dendritic cells and monocytes):** anti-CD11c BV510 (B-ly6), anti-CD14 BV711 (MPhiP9), anti-CD16 PE-CF594 (3G8), CD123 BV421 (7G3), HLA-DR BV786 (G46-6), Lineage cocktail 2 (CD3 – SK7, CD14 – MoP9, CD19 – SJ25C1, CD20 – L27, CD56 – NCAM16.2) FITC, Live/Dead fixable viability stain 780 APC-Cy7.

Data analysis

FlowJo version 10 (FlowJo LLC, Oregon) was used to analyse flow cytometry data. Doublets were removed using FSC-A versus FSH-H. Dead cells positive for the viability stain were removed, and lymphocytes were gated based on size on SSC-A versus FSC-A dot plot. NK cells were selected as $CD3^-$ and further selected on $CD56^+/CD16^{+/-}$ expression as follows: $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD16^{dim}$, $CD56^{bright}CD16^-$, $CD56^{dim}CD16^{dim}$, and $CD56^-CD16^+$ (Figure 1). Cell surface expression of CD25, CD57, CD122, KIR, NKG2A, and LFA-1 was examined on the two most common NK cell subsets; $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$. Mature NK cells were $CD56^{dim}CD16^{bright}$ and defined as early mature (KIR^+) and highly-differentiated ($KIR^+CD57^+/NKG2A^{lo}$). t-SNE analysis was performed on concatenated samples from each time-point

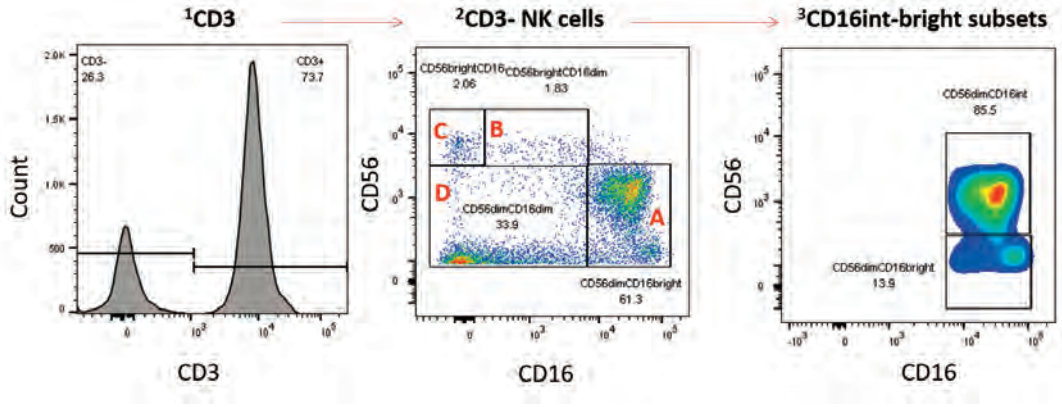

Figure 1. Representative flow cytometry gating strategy for NK cell subpopulations. CD3 negative lymphocytes -> CD56+ NK cells. NK cell subsets: A. CD56dim CD16bright, B. CD56bright CD16dim, C. CD56bright CD16-, D. CD56dim CD16dim. CD56dim CD16bright can be divided into CD16intermediate and CD16bright

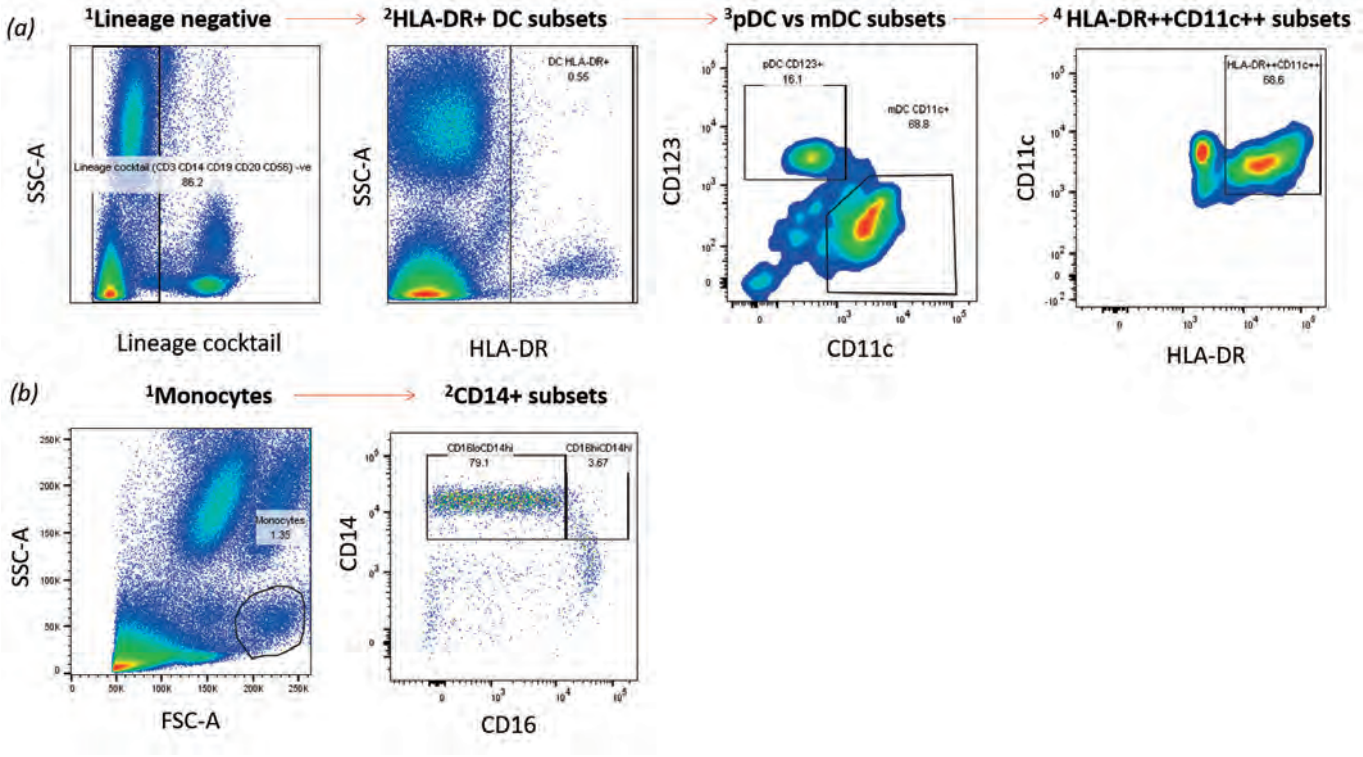

Figure 2. Representative flow cytometry gating strategy for DC and monocyte populations (a)Lineage negative -> HLA-DR+ DC subsets-> plasmacytoid vs myeloid DC subsets-> HLA-DR++CD11c++ DC subsets (b)Monocytes ->CD16loCD14hi and CD16hiCD14hi monocyte subsets.

during vigorous intensity exercise in control and T1D participants. A down sample population of $CD3^{-}CD56^{+}$ NK cells was selected up to 15,000 events. t-SNE analysis was run using phenotypic markers of NK cell subsets (CD56, CD16, CD57, KIR). Cell surface markers which are not core phenotypic markers and with variable expression level (CD25, CD122, LFA-1) were applied once the t-SNE plot was calculated.

Dendritic cells were selected as Lineage- 2 (CD3-, CD14-, CD19-, CD20-, CD56 -) and further selected on HLA-DR expression. Further subdivisions of dendritic cells were selected as $CD11c^{+}$ (myeloid) and $CD123^{+}$ (plasmacytoid) (Figure 2). DC subsets during vigorous exercise only is shown in T1D due to missing data. Monocytes were selected based on size on FSC-A/SSC-A and further selected on $CD14^{high}/CD16^{high/lo}$ expression (Figure 2).

Plasma isolation and adrenaline measurement

Blood was collected in EDTA vacuette tubes (454209, Greiner Bio-one GmbH, Frickenhausen, Germany) and immediately placed on ice. The blood was centrifuged at 1500RPM for 10 minutes at 4°C. The plasma (top layer) was removed using sterile pipette tips and placed in sterile Eppendorf tubes. Aliquots were made to avoid deterioration of the serum by freeze-thaw cycles. Plasma was stored at -80°C until use. Adrenaline (pg/mL) was measured at pre-exercise, post-exercise, and 1 hour post-exercise plasma samples using EPI (epinephrine/adrenaline) ELISA kit (E-EL-0045, Elabscience, Texas, USA).

Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM, Chicago) and GraphPad Prism version 7 (GraphPad Software, California). Firstly, normality tests were performed on all data using Q-Q plots in SPSS. Data which was not normally distributed was logged and normality tests were repeated, confirming all subsets to have normal distribution. Main effects of exercise are described as changes over time. Changes immediately post-exercise and 1 hour post-exercise are compared to baseline values and are reported in tables for each group under the heading "contrast". P values were reported as sphericity assumed however where Mauchly's test of sphericity was violated, i.e. $p \leq 0.05$, Greenhouse-Geisser corrected value was used. Student T-tests were performed on baseline characteristics. The p values, F values, and degrees of freedom (df) are reported in tables as [F = (df, df error) value, p-value]. Data are presented in tables as mean ± standard deviation (SD). P values ≤0.05 were considered significant. Significantly mobilised subsets in both control and T1D groups, that also demonstrated a blunted egress immediately post-exercise in T1D group, are highlighted in the results tables in bold.

RESULTS

Participants anthropometric and physiological characteristics are shown in Table 1. No statistically significant differences between groups were found for anthropometric and physiologic characteristics. The statistical analysis for innate cell subpopulations during vigorous intensity exercise are summarised in Tables 2-5.

Reduced NK cell mobilisation in T1D participants following vigorous intensity exercise compared to control participants

As previously described, NK cells are the most sensitive subset to mobilisation during acute exercise. In this study, NK cells significantly mobilised during vigorous intensity exercise in both the control (p=0.021) and T1D (p=0.005) group, significantly increasing immediately post-exercise in both groups independently (T1D: p=0.044, control: p=0.021). However, the magnitude of the response immediately post-exercise was higher in control participants (T1D: 100.49%, control: 174.13%) (Table 2).

NK cells, in particular mature CD56dimCD16bright subsets, display blunted mobilisation during vigorous intensity in T1D

NK cell subsets were defined using the classical cell surface markers CD56 and CD16 as shown in Figure 2; $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$, and $CD56^{bright}CD16^{-}$. The mean, standard deviation, and statistical analyses are displayed in Table 2.

Table 2. NK cell subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	[1]Controls T1	[1]Controls T2 mean±SD	[1]Controls T3	[b]Time	[a]Δ%	[2]T1D T1	[2]T1D T2 mean±SD	[2]T1D T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
Total NK cells (CD56+)	164.21±101.76	450.18±345.08	119.25±72.19	**$F_{(1.1, 7.1)}$= 8.584, p=0.021**	174.13	183.28±62.26	367.47±173	154.55±40.17	**$F_{(2, 8)}$= 11.212, p=0.005**	100.49	**$F_{(1, 11.4)}$= 14.696, p=0.002**	$F_{(1, 11.4)}$= 0.118, p=0.748
- $CD56^{dim}CD16^{bright}$	72.85±34.95	207.70±106.46	53.35±43.13	**$F_{(1.1, 7.5)}$= 27.545, p=0.001**	185.12	104.38±47.47	205.69±120.49	78.18±51.31	**$F_{(2, 12)}$= 3.929, p=0.049**	97.06	**F(1.4, 18.2)= 22.237, p<0.001**	$F_{(1.4, 18.2)}$= 2.113, p=0.159
- $CD56^{bright}CD16^{dim}$	3.63±3.14	5.62±4.52	3.28±2.55	**$F_{(2, 14)}$= 10.682, p=0.002**	54.79	3.35±1.32	5.31±2.83	2.45±0.82	$F_{(2, 12)}$= 3.340, p=0.070	58.34	**$F_{(1.4, 17.9)}$= 12.477, p=0.001**	$F_{(1.4, 17.9)}$= 0.662, p=0.473
- $CD56^{dim}CD16^{dim}$	14.19±12.58	30.29±26.43	11.93±9.884	**$F_{(1.2, 8.2)}$= 8.822, p=0.015**	113.78	14.81±6.47	20.51±12.72	20.60±22.54	$F_{(1.1, 8.3)}$= 0.385, p=0.689	38.54	**$F_{(2, 26)}$= 5.679, p=0.009**	$F_{(2, 26)}$= 2.657, p=0.089
- $CD56^{bright}CD16^{-}$	59.03±52.93	93.16±109.78	40.12±41.74	**$F_{(2, 14)}$= 13.011, p=0.001**	57.81	71.46±48.75	162.2±201.27	84.84±86.38	**$F_{(2, 12)}$= 6.493, p=0.012**	126.98	**F(2, 26)= 17.607, p<0.001**	$F_{(2, 26)}$= 0.471, p=0.630

Mean, standard deviation, and statistical analysis of NK cell sub populations for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant, and the percentage increase was considerably blunted in the T1D (<50 Δ% compared to control highlighted in red).

Note:
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Results were analysed using multiple regression analysis in control and T1D groups combined.
[1]Controls n=8, [2]T1D n=7

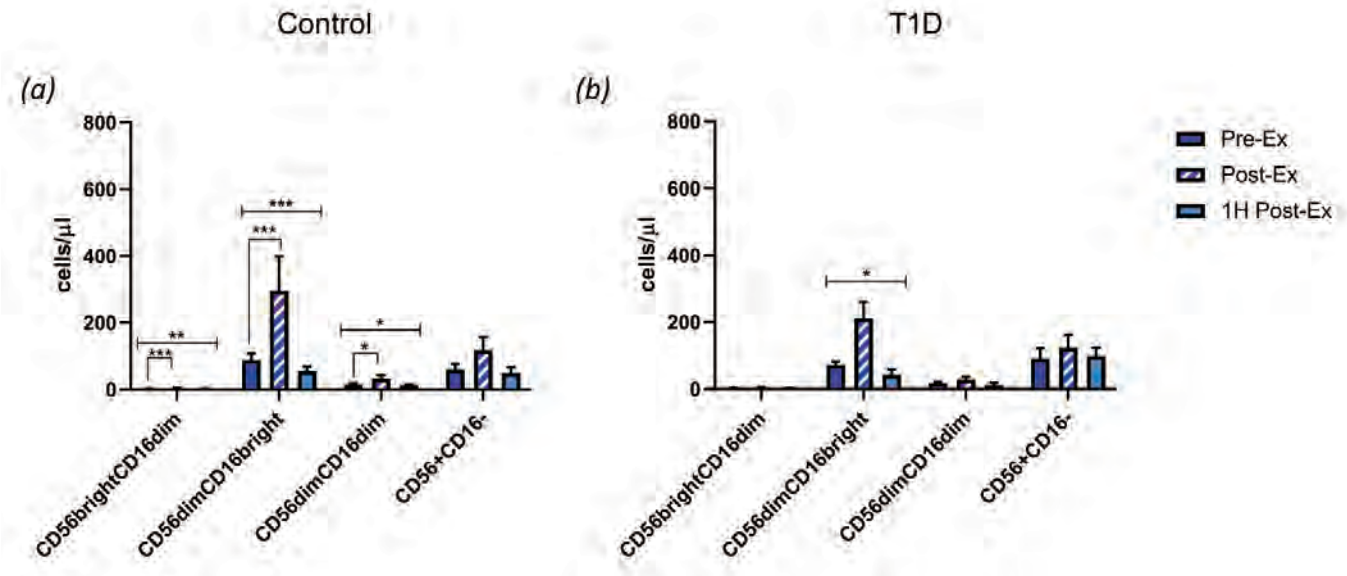

Figure 3. NK cell subsets during moderate and vigorous intensity exercise in control and T1D participants (a) NK cell subsets during vigorous exercise in control participants (b) NK cell subsets during vigorous exercise inT1D participants. Error bars represent SEM.
Note: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Typically, $CD56^{dim}CD16^{bright}$ NK cells demonstrate the largest response to acute exercise. This is further supported by the results of our study (Figure 3); the percentage increase of $CD56^{dim}CD16^{bright}$ NK cells is a minimum of 3-fold higher than that of the other subsets. $CD56^{dim}CD16^{bright}$ NK cells mobilised significantly during vigorous intensity exercise in both groups independently (T1D: p=0.049, controls: p=0.001) (Figure 3a and 3b). However, $CD56^{dim}CD16^{bright}$ NK cells significantly increased immediately post-exercise in the control group only (p=0.001). Furthermore, the magnitude of response post-exercise was much larger in control participants (T1D: 97.06%, control: 185.12%) (Table 2).

Interestingly, $CD56^{dim}CD16^{bright}$ NK cells were the only subset to significantly mobilise in the T1D group during vigorous intensity exercise, but to a lesser magnitude than the control group (Figure 3b). In the control group, $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$ all significantly mobilised during vigorous intensity exercise (Figure 3a).

Early mature $KIR^{+}CD56^{dim}CD16^{bright}$ NK cell subsets drive the blunted mobilisation during vigorous intensity exercise in T1D

$CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD56^{dim}$ NK cells are the most studied NK cell subpopulations. For this reason, we used a number of cell surface markers to define differentiation and migratory status of these NK cell subpopulations. CD57 and KIR can be used to define NK cells with a highly differentiated phenotype, with KIR being expressed first followed by co-expression with CD57 as NK cells reach maturation (6, 76). LFA-1 is an adhesion molecule involved in NK cell migration. Lastly, NK cells expressing both CD25 (IL-2Rα), also a marker of activation, and CD122 (IL-2Rβ/IL-15R), involved in NK cytokine signalling, have been shown to migrate during exercise previously (56, 73). The mean, standard deviation, and statistical analyses are displayed in Table 3.

Although a small subset, we measured all of the above surface markers on $CD56^{bright}CD16^{dim}$ cells. The results and statistical analysis are displayed in Table 3. $CD56^{bright}CD16^{dim}$ expressing KIR, albeit a small sub-group, are the only subset to mobilise in both groups during vigorous exercise (Control p=0.013, T1D p=0.045), with a similar percentage increase immediately following exercise in both groups. This subset may represent NK cells reaching early maturity as they begin to express KIR. However, we have focused mainly on the larger NK subpopulation $CD56^{dim}CD16^{bright}$ and our findings our described below.

All $CD56^{dim}CD16^{bright}$ NK cell subsets expressing the above surface markers, apart from LFA-1, significantly mobilised during vigorous intensity exercise in both groups. $LFA\text{-}1^{+}$ $CD56^{dim}CD16^{bright}$ NK cells did however significantly mobilise in the control group during vigorous intensity exercise (Table 3).

$CD56^{dim}CD16^{bright}$ NK cells make up the largest proportion of NK cells with a highly differentiated phenotype as defined by expression of KIR and CD57, whilst lacking NKG2A expression. As described previously, during NK cell maturation they lose NKG2A and gain KIR expression. This is followed by a gradual increase in CD57 expression as they become highly differentiated (7, 65). $KIR^{+}CD56^{dim}CD16^{bright}$ NK cells retain proliferative capacity, however $KIR^{+}CD57^{+}CD56^{dim}CD16^{bright}$ NK cells have a reduced proliferative capacity. It is well supported that mature, highly differentiated NK cells mobilise dramatically during acute exercise and we see this in both the control and T1D group in this study. Here we describe the differential mobilisation of early mature (KIR^{+}) and highly differentiated ($KIR^{+}CD57^{+}NKG2A^{lo}$) $CD56^{dim}CD16^{bright}$ NK cells in control and T1D participants.

$KIR^{+}CD56^{dim}CD16^{bright}$ NK cells significantly mobilised during vigorous intensity exercise in the control and T1D group

Table 3. CD56^{bright}CD16^{dim} and CD56^{bright}CD16^{dim} NK cell subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	[1]Controls T1	[1]Controls T2 mean±SD	[1]Controls T3	[b]Time	[a]Δ%	[2]T1D T1	[2]T1D T2 mean±SD	[2]T1D T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
CD56dimCD16bright												
LFA-1+	48.56±35.22	141.14±114.30	41.14±33.83	**$F_{(1,7.3)}$= 13.541, p=0.007**	190.63	70.92±49.46	118.81±73.34	56.46±52.87	$F_{(2,12)}$= 2.674, p=0.110	67.53	**$F_{(1.5,\ 20.1)}$= 14.133, p<0.001**	$F_{(1.5,\ 20.1)}$= 2.880, p=0.074
CD25+	21.97±30.85	84.88±141.73	8.87±9.28	**$F_{(1.1,\ 7.5)}$= 18.108, p=0.003**	286.20	38.03±28.55	140.89±137.34	15.90±19.26	**$F_{(2,\ 10)}$= 12.677, p=0.012**	270.47	**$F_{(1.2,\ 13.7)}$= 30.596, p<0.001**	$F_{(1.2,\ 13.7)}$= 0.483, p=0.623
CD122+	81.21±82.74	318.74±410.99	34.01±32.59	**$F_{(2,\ 14)}$= 7.416, p=0.006**	292.49	115.08±122.30	306.72±355.35	42.81±42.69	**$F_{(2,\ 10)}$= 12.227, p=0.002**	166.53	**$F_{(2,\ 24)}$= 17.600, p<0.001**	$F_{(2,\ 24)}$= 1.169, p=0.328
KIR+	56.60±31.70	165.51±100.50	24.35±13.53	**$F_{(2,\ 14)}$= 15.068, p<0.001**	192.40	62.55±87.75	108.98±123.43	14.14±14.16	**$F_{(2,\ 10)}$= 14.695, p=0.001**	74.24	**$F_{(2,\ 24)}$= 30.349, p<0.001**	$F_{(2,\ 24)}$= 0.529, p=0.596
KIR+CD57+	25.5±17.56	59.55±27.99	2.67±1.41	**$F_{(2,\ 4)}$= 70.295, p=0.001**	133.56	11.85±10.47	75.11±84.69	2.90±3.22	**$F_{(2,\ 8)}$= 10.081, p=0.012**	533.88	**$F_{(2,\ 10)}$= 29.023, p<0.001**	$F_{(2,\ 10)}$= 0.748, p=0.496
(KIR+CD57+)LFA1+	18.93±17.73	50.13±33.60	2.89±1.37	**$F_{(2,\ 4)}$= 83.110, p=0.001**	164.81	10.47±12.21	6.04±3.12	0.50±0.18	$F_{(2,\ 4)}$= 4.669, p=0.090	42.34	**$F_{(2,\ 8)}$= 27.895, p<0.001**	$F_{(2,\ 8)}$= 1.891, p=0.213
CD56brightCD16dim												
LFA-1+	1.88±2.83	2.88±3.92	1.30±1.49	$F_{(2,\ 12)}$= 2.383, p=0.134	52.99	1.73±1.88	1.97±2.74	1.23±1.16	$F_{(2,\ 10)}$= 0.616, p=0.559	13.80	$F_{(2,\ 22)}$= 1.110, p=0.347	$F_{(2,\ 22)}$= 1.850, p=0.181
CD25+	0.39±0.55	0.66±1.12	0.15±0.20	$F_{(1,\ 8.3)}$= 1.516, p=0.254	67.27	0.19±0.25	0.53±0.67	0.19±0.29	$F_{(1,\ 5.1)}$= 3.266, p=0.130	173.64	$F_{(1.1,\ 14.1)}$= 2.583, p=0.129	$F_{(1.1,\ 14.1)}$= 0.259, p=0.638
CD122+	2.74±2.12	4.58±2.96	2.14±1.69	**$F_{(2,\ 10)}$= 12.033, p=0.002**	66.92	3.27±2.01	6.93±5.30	2.77±2.19	$F_{(1.1,\ 8.8)}$= 3.546, p=0.069	112.19	**$F_{(1.2,\ 11.8)}$= 7.843, p=0.014**	$F_{(1.2,\ 11.8)}$= 0.704, p=0.439
KIR+	1.56±1.89	2.66±2.69	1.12±1.31	**$F_{(2,\ 12)}$= 8.546, p=0.005**	70.18	0.43±0.29	0.68±0.59	0.42±0.36	**$F_{(2,\ 10)}$= 4.312, p=0.045**	105.95	**$F_{(2,\ 22)}$= 10.687, p=0.001**	$F_{(2,\ 22)}$= 0.496, p=0.616
CD57+	0.087±0.05	0.15±0.15	0.06±0.04	$F_{(2,\ 6)}$= 1.148, p=0.378	66.92	0.03±0.05	0.15±0.06	0.07±0.08	$F_{(2,\ 8)}$= 3.893, p=0.082	424.26	$F_{(2,\ 12)}$= 2.684, p=0.109	**$F_{(2,\ 12)}$= 4.712, p=0.031**

Mean, standard deviation, and statistical analysis of CD56^{bright}CD16^{dim} and CD56^{bright}CD16^{dim} NK cell subset for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant, and the percentage increase was considerably blunted in the T1D (<50 Δ% compared to control highlighted in red).

Note:
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Results were analysed using multiple regression analysis in control and T1D groups combined.
Vigorous: [1]Controls n=8, [2]T1D n=6

(p<0.001, p=0.001 respectively). However, the percentage increase immediately following vigorous intensity exercise was blunted in the T1D group (Control 192.4%, T1D 74.24%).

The fully differentiated KIR^{+}CD57^{+} subset significantly mobilised during vigorous intensity exercise in the control and T1D group (p=0.001, p=0.012 respectively). Furthermore, highly differentiated NK cells (KIR^{+}CD57^{+}) with a migratory capacity (LFA-1^{+}) significantly mobilised during vigorous intensity exercise in the control group (p=0.001), but this was not significant in the T1D group.

In summary, highly differentiated, mature KIR^{+}CD57^{+} NK cells increase dramatically following vigorous intensity exercise in both the control and T1D groups. However, early mature KIR^{+} NK cells display a blunted increase following vigorous intensity exercise in the T1D group and this may drive the overall blunted mobilisation of CD56^{dim}CD16^{bright} NK cells observed.

Dimensionality reduction analysis of NK cell subsets supports observed blunted mobilisation of early mature KIR^{+}CD56^{dim}CD16^{bright} NK cell subsets in T1D

Dimensionality reduction using t-Distributed Stochastic Neighbour Embedding (t-SNE) algorithms is a powerful analysis technique that allows visualisation of multiple interactions on a 2D scale. t-SNE plots are formed using phenotypic markers to cluster similar populations. Markers which can change expression, for example adhesion molecules, can be assessed once the populations are defined. Here we have used t-SNE to observe changes within the CD56^{+} NK profile pre, immediately post, and 1 hour post vigorous intensity exercise (Figure 4). The NK cell subsets CD56^{dim}CD16^{bright} (blue), CD56^{bright}CD56^{dim} (green), CD56^{dim}CD16^{dim} (pink), and CD56^{bright}CD16^{-} (orange) are represented in t-SNE plots in Figure 4. The CD56^{dim}CD16^{bright} cluster is further divided into CD56^{dim}CD16$^{int\text{-}bright}$ (light blue) and CD56^{dim}CD16$^{bright+}$ (dark blue) to further delineate the

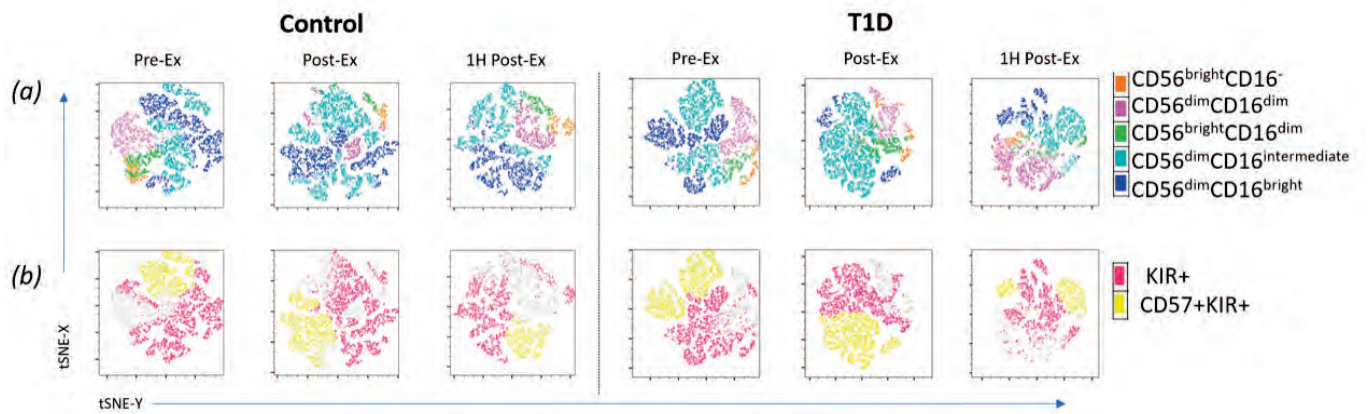

Figure 4. Representative concatenated t-SNE plots for NK cells during vigorous intensity exercise in control and T1D participants (a) NK cell populations CD56/CD16: CD56^{bright}CD56^{dim} (green), CD56^{dim}CD16^{dim} (pink), CD56^{bright}CD16^{-} (orange), CD56^{dim}CD16$^{int\text{-}bright}$ (light blue) and CD56^{dim}CD16$^{bright+}$ (dark blue) (b) maturity markers KIR/CD57: KIR^{+}NK cells (pink), CD57^{+}KIR^{+} NK cells (yellow)

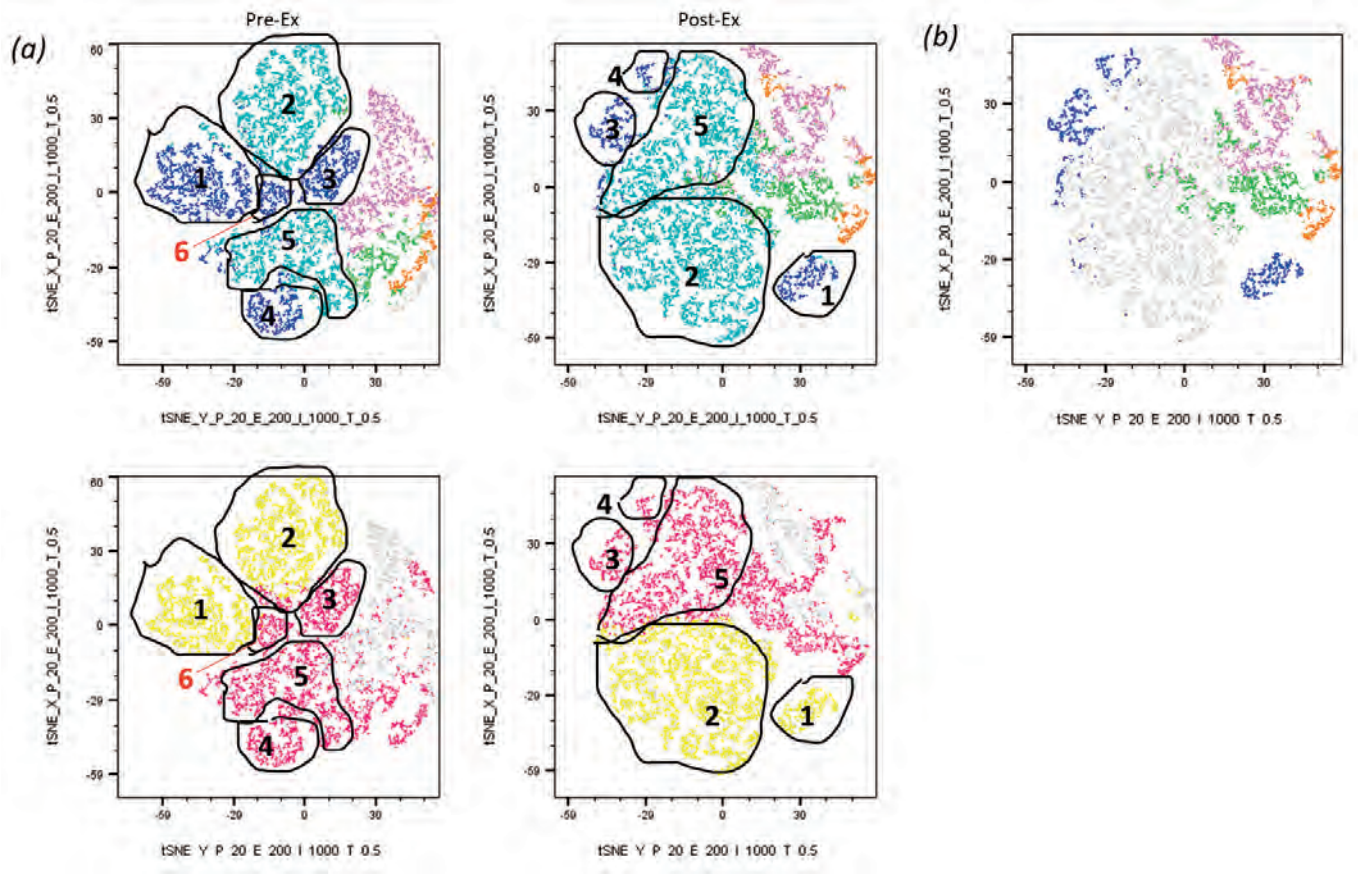

Figure 5. (a) Cluster definition (b) Confirms dark blue clusters not hidden behind light blue clusters.

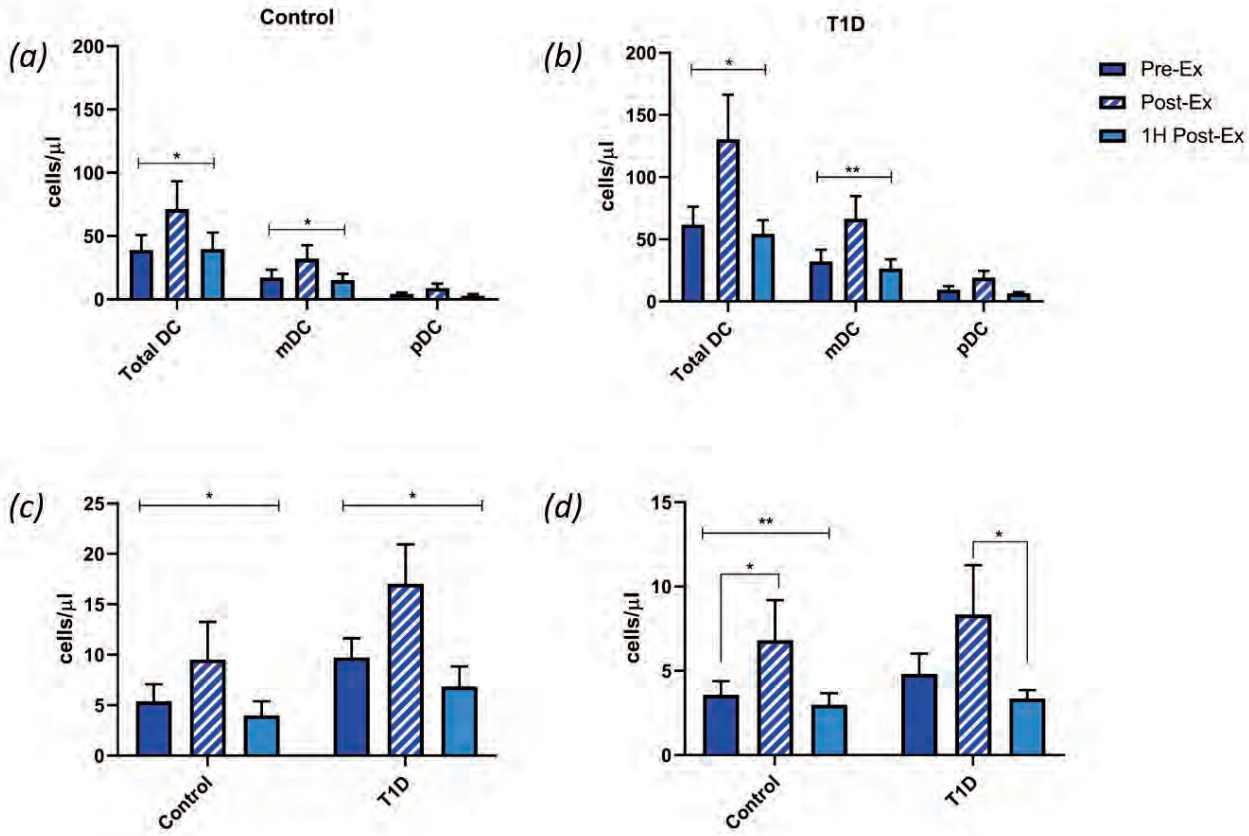

Figure 6. Dendritic cell subsets and monocytes during vigorous intensity exercise in control and T1D participants (a) DC subsets in control participants (b) DC subsets in T1D participants (c) HLA-DR^{hi} CD11c^{hi} mDC subsets during vigorous exercise in control and T1D participants (d) Total monocytes during vigorous exercise in control and T1D participants. Error bars represent SEM.
Note: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

changes observed within this subset during vigorous intensity exercise (Figure 4a).

At baseline, the combined CD16^{bright} subsets (blue) make up the bulk of the NK cell subsets in both groups (Figure 4a). Immediately post vigorous exercise, the CD56^{bright}CD16^{-} (orange), CD56^{bright}CD16^{dim} (green), and CD56^{dim}CD16^{dim} (pink) subsets are reduced, as the CD16^{bright} subsets (blue) have increased at this time point. Comparing the control group to the T1D group at this time point, it is obvious that the CD56^{dim}CD16$^{int\text{-}bright}$ (blue) subset is present post-exercise, but a proportion of the CD56^{dim}CD16^{bright} NK cells (blue) are absent, supporting the blunted effect reported above. Following recovery, 1 hour post-exercise, NK subsets return almost to normal in the control group, but the CD16^{bright} subset (blue) has only marginally returned to baseline levels in the T1D group. Here, the CD56^{dim}CD16^{dim} subsets (pink) represents are larger proportion of the NK cell subsets in the T1D group.

In Figure 4b, the pink clusters represent the early mature KIR^{+}NK cells and the yellow clusters represent the late mature CD57^{+}KIR^{+} NK cells. As expected, these populations reside mainly within the CD56^{dim}CD16^{bright} subsets (blue) shown in Figure 4a. Immediately post vigorous exercise, it is obvious that a proportion of KIR^{+}CD56^{dim}CD16^{bright} NK cluster is absent from the T1D group as shown by the appearance of an incomplete t-SNE plot. To further explain this, there are 6 clusters belonging to CD56^{dim}CD16^{bright} populations in the baseline sample (Figure 5). Four small clusters belong to the CD56^{dim}CD16^{bright} subset, one of which is CD57^{+}KIR^{+} (cluster 1 – yellow) and it is evident in the post-exercise t-SNE plot that there are only three clusters present, to which this missing cluster belongs to one of the three KIR^{+} populations identified at baseline (cluster 6). Both CD57^{+}KIR^{+} clusters (clusters 1 and 2) in yellow are present in the post-exercise plot, however cluster 1 which belongs to the CD56^{dim}CD16^{bright} subset, is dramatically reduced post-exercise in T1D (Figure 5a). At 1 hour post-exercise, KIR^{+} and CD57^{+}KIR^{+} NK cells return almost to normal in the control group, with a small decrease in CD57^{+}KIR^{+} NK cells as expected. This is also observed for the T1D group.

Using t-SNE algorithms to visualise changes in NK cell subsets during vigorous intensity exercise further highlights that the blunted increase of CD56^{dim}CD16^{bright} NK cells immediately post vigorous exercise is driven by a lack of early mature KIR^{+}CD56^{dim}CD16^{bright} NK cells. This dimensionality reduction analysis supports the findings using conventional flow cytometry gating analysis and enumeration of the populations during vigorous intensity exercise.

Myeloid lineage subsets mobilise similarly in control and T1D participants

The mean, standard deviation, and statistical analyses for dendritic cell (DC) and monocyte subsets are displayed in Table 4. Total DC mobilised during vigorous intensity exercise in both control (p=0.049) and T1D (p=0.027) groups, and to a similar magnitude in both groups (Figure 6a and 6b). Within the DC subsets, mDC preferentially mobilised during vigorous intensity exercise. mDC mobilised during vigorous intensity exercise in both control (p=0.045) and T1D (p=0.008) groups, and to a similar magnitude in both groups. This was driven by HLA-DR^{hi} CD11c^{hi} mDC, in both control and T1D groups (p=0.033, p=0.012 respectively). pDC did not significantly mobilise in either group. However, there was an evident percentage increase immediately following vigorous exercise in both groups.

Total monocytes significantly mobilised during vigorous intensity exercise in the control group (p=0.006). This was not found to be significant in the T1D group. However, there was an evident increase immediately post vigorous exercise.

Systemic adrenaline concentration during exercise is similar between groups

The adrenaline concentration during vigorous intensity exercise in control and T1D participants is shown in Figure 7.

Table 4. Myeloid subset mobilisation during vigorous intensity exercise in T1D and control participants

Subset	[1]Controls T1	[1]Controls T2 mean±SD	[1]Controls T3	[b]Time	[a]Δ%	[2]T1D T1	[2]T1D T2 mean±SD	[2]T1D T3	[b]Time	[a]Δ%	[c]Time (overall)	[c]Time*Group
Dendritic Cells (HLA-DR+)	38.84±37.82	71.26±65.78	39.76±38.96	**$F_{(2, 14)}$= 3.775, p=0.049**	83.5	58.81±36.54	122.29±90.70	52.1±25.40	**$F_{(2, 10)}$=5.315, p=0.027**	107.9	**$F_{(2, 24)}$= 9.978, p=0.001**	$F_{(2, 24)}$= 2.213, p=0.131
myeloid DC	17.29±19.6	32.02±32.39	15.30±14.46	**$F_{(2, 14)}$= 3.896, p=0.045**	85.2	30.48±21.38	61.75±42.62	24.83±17.20	**$F_{(2, 10)}$=8.105 p=0.008**	102.6	**$F_{(2, 24)}$= 13.293, p=0.000**	$F_{(2, 24)}$= 2.207, p=0.132
mDC(HLADR^{hi}CD11c^{hi})	5.39±5.06	9.52±11.2	3.98±4.20	**$F_{(2, 12)}$= 4.592, p=0.033**	76.8	9.04±4.66	16.22±8.99	6.49±4.51	**$F_{(2, 10)}$=7.213 p=0.012**	79.3	**$F_{(2, 22)}$= 11.899, p=0.000**	$F_{(2, 22)}$= 0.416, p=0.665
plasmacytoid DC(CD123+)	4.19±3.40	9.15±8.43	2.82±3.12	$F_{(1.5, 9.03)}$= 2.782, p=0.109	118.5	8.41±6.50	17.39±11.76	5.91±2.40	$F_{(1, 5)}$= 2.288, p=0.183	106.9	**$F_{(2, 16)}$= 5.342, p=0.017**	$F_{(2, 16)}$= 1.095, p=0.358
Monocytes	3.57±2.57	6.81±7.15	2.98±2.05	**$F_{(2, 14)}$= 7.436, p=0.006**	90.8	4.52±3.07	8.19±7.18	3.17±1.21	$F_{(2, 10)}$=2.212, p=0.160	81.1	**$F_{(2, 24)}$= 5.058, p=0.015**	$F_{(2, 24)}$= 1.305, p=0.290
CD16^{hi}CD14^{hi} monocytes	0.15±0.15	0.18±0.18	0.09±0.12	$F_{(2, 14)}$= 2.064, p=0.164	18.7	0.25±0.18	0.36±0.30	0.20±0.19	$F_{(2, 10)}$=1.165, p=0.351	40.5	$F_{(2, 24)}$= 2.642, p=0.092	$F_{(2, 24)}$= 0.525, p=0.598
CD16^{hi}CD14^{lo} monocytes	1.33±1.17	2.16±1.67	1.29±1.23	$F_{(2, 14)}$= 2.769, p=0.097	62.6	1.78±1.31	3.16±3.25	1.46±0.76	$F_{(2, 10)}$=1.435, p=0.283	77.7	$F_{(2, 24)}$= 2.976, p=0.070	$F_{(2, 24)}$= 0.968, p=0.394

Mean, standard deviation, and statistical analysis of myeloid subset for control and T1D participants during vigorous intensity exercise. Within subject's effect shown displayed under "time" and between subject's effects over time displayed under "time*group". Significant results highlighted in bold changes over time were statistically significant for both groups independently i.e. p values <0.05 were considered significant.

Note:
[a] Δ% Percentage change from baseline (T1) to immediately post exercise (T2) or 1 hour post exercise (T3).
[b] Results were analysed using multiple regression analysis in control and T1D groups independently.
[c] Results were analysed using multiple regression analysis in control and T1D groups combined.
Vigorous: [1]Controls n=8, [2]T1D n=6

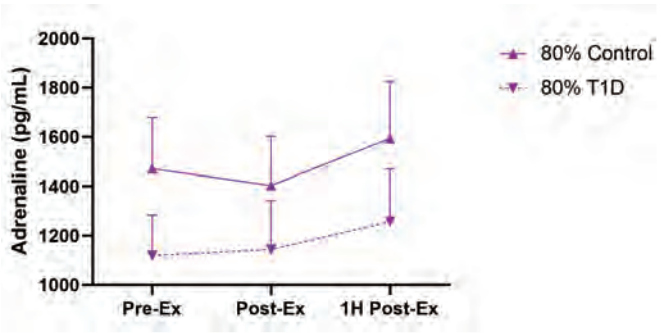

Figure 7. Adrenaline response during vigorous intensity exercise in control and T1D participants Total adrenaline concentration (pg/mL) during vigorous exercise in control and T1D participants. Error bars represent SEM. Control = 12, T1D = 12

Whilst there is a trend for lower adrenaline levels in T1D, this is not statistically significant. Nonetheless, these experiments need to be repeated with larger patient numbers, but we cannot currently exclude the possibility that lower basal adrenaline release in T1D participants may have an impact on the blunted NK cell mobilisation observed. It is notable that the baseline adrenaline concentration is abnormally elevated and therefore may mask the expected increase post-exercise in both groups (26, 28, 42).

DISCUSSION

This study has, for the first time, characterised the effects of acute exercise on the mobilisation of innate cell subsets in people with T1D. First, we aimed to investigate the effects of vigorous intensity exercise on innate cell subsets in T1D, with a particular focus on the most exercise responsive subset, NK cells. Second, we aimed to investigate the adrenaline response during exercise to gain insight into the mechanisms of differential lymphocyte mobilisation in T1D.

In this study we have demonstrated mobilisation of NK cell subsets in both control and T1D participants. Our findings are supported by previous studies also showing a preferential mobilisation of NK cells during acute exercise (5, 25, 50, 82). However, we show for the first time that this effect was blunted in the T1D participants. A strength to our study is that we have carried out a detailed characterisation of NK cell subsets during exercise including $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$, and $CD56^{bright}CD16^{-}$. We found that all NK subpopulations $CD56^{dim}CD16^{bright}$, $CD56^{bright}CD56^{dim}$, $CD56^{dim}CD16^{dim}$ and $CD56^{bright}CD16^{-}$ significantly mobilised during vigorous intensity exercise in controls. In T1D, only $CD56^{dim}CD16^{bright}$ NK cells significantly mobilised during vigorous intensity exercise. As demonstrated in previous studies, $CD56^{dim}CD16^{bright}$ NK cells demonstrate the largest response to acute exercise. The percentage increase of $CD56^{dim}CD16^{bright}$ NK cells is a minimum of 3-fold higher than that of the other subsets in our study. As seen with total NK cells, the magnitude of the response immediately following vigorous exercise was blunted in T1D participants.

Both $CD122^{+}$ (IL-2Rβ/IL-15R) and $CD25^{+}$ (IL-2Rα) $CD56^{bright}CD16^{dim}$ NK cells significantly mobilised during vigorous intensity exercise in control participants. However, only $CD122^{+}$ $CD56^{bright}CD16^{dim}$ NK cells subsets significantly mobilised during vigorous intensity exercise in T1D participants. Previous reports have shown mobilisation of $CD122^{+}$ and $CD25^{+}$ NK cells following exercise in healthy cohorts (56, 73). We have for the first time demonstrated this in T1D during vigorous intensity exercise. We have also demonstrated in our study that $CD56^{bright}CD16^{dim}$ NK cells with a migratory capacity ($LFA\text{-}1^{+}$) significantly mobilise following vigorous intensity exercise in the control group.

It has been well established that mature $CD56^{dim}CD16^{bright}$ NK cells with a highly differentiated phenotype mobilise dramatically during acute exercise (6, 76). In this study, we have examined the mobilisation of both early mature (KIR+) and highly differentiated ($KIR^{+}CD57^{+}$) NK cells during acute exercise. As expected, highly differentiated NK cells significantly mobilised during vigorous intensity exercise in the control and T1D group. Early mature NK cells (KIR^{+}) exhibited a similar effect. However, the percentage increase immediately following vigorous intensity exercise was blunted in the T1D group. In summary, highly differentiated, mature $KIR^{+}CD57^{+}$ NK cells increase dramatically following vigorous intensity exercise in both the control and T1D groups. However, the observed blunted increase of $CD56^{dim}CD16^{bright}$ NK cells is driven by early mature KIR^{+} subset. In this study we have used t-SNE visualisation to assess changes in NK cell subsets during vigorous intensity exercise to further delineate the evident blunting following vigorous exercise in T1D. Most of the data in the field of exercise immunology is presented in absolute numbers because changes in the proportions of populations are difficult to detect. The t-SNE plots allow us to identify and easily visualise subtle changes in subgroups that may not be obvious by directly looking at the proportion. Here we have further divided the $CD56^{dim}CD16^{bright}$ NK subsets into $CD56^{dim}CD16^{int\text{-}bright}$ and $CD56^{dim}CD16^{bright}$. Thus, further highlighting that the blunted increase in $CD56^{dim}CD16^{bright}$ NK cells is driven by the early mature KIR^{+} subgroup, specifically those within the $CD56^{dim}CD16^{bright}$ clusters. To further increase the strength of this powerful tool, more parameters need to be included in the phenotyping panels. Inclusion of more phenotypic markers such as other NK inhibitory and activation receptors may identify more subpopulations. In our study, multiple clusters reside within the mature NK cell populations suggesting some phenotypic differences within this subgroup. This could identify new exercise sensitive NK cell populations in future investigations on the effects of exercise on immune parameters in healthy and disease states. Although much work has been done to identify populations mobilised during exercise, deeper immunophenotyping analysis is necessary to broaden the scope of analysis and in particular, the investigations of exercise in disease states which have a skewed immune phenotype. Within the myeloid subsets, total DC mobilised during vigorous intensity exercise in both groups, and to a similar magnitude. Of the DC subsets, activated $HLA\text{-}DR^{hi}CD11c^{hi}$ mDC, preferentially mobilised during vigorous intensity exercise in both groups. Total monocytes significantly mobilised during vigorous intensity exercise in the control group. However, an increase in total monocytes immediately following exercise was evident in the T1D group. Previous studies have

also shown DC and monocyte mobilisation during acute exercise in healthy cohorts (9, 24, 33, 38, 60, 71, 80). In particular, a recent study demonstrated preferential mobilisation of pDCs during exercise in a healthy cohort (9). However, in our study we see a preferential mobilisation of mDC. This could possibly be due to differences in the phenotypic surface markers used; in our study we used CD11c to identify mDC and CD123 to identify pDC. In the aforementioned study, CD303- was used to identify mDC and $CD303^+$ to identify pDC. Additionally, the exercise bout lasted 20 minutes in the aforementioned study, whereas the acute exercise bout last 30 minutes in our study. This highlights how small differences in study design can impact on the variation of findings between research groups. The mechanisms through which differential mobilisation of lymphocyte subsets during exercise in T1D need to be defined to further understand the implications of acute exercise in T1D. NK cells are the most responsive lymphocyte subset to acute exercise due to their high beta-adrenergic receptor expression resulting in their preferential intensity-dependant mobilisation in response to adrenaline during acute exercise (5, 25, 50, 82). As seen in our previous work, exercise sensitive subsets including highly differentiated $CD8^+$ T cells which respond to adrenaline are also blunted. This leads us to the premise that the blunted effect is due to an impaired adrenaline response during vigorous intensity exercise in T1D (23). We measured systemic adrenaline during vigorous intensity exercise in both control and T1D participants. In this study, we found no statistical significant differences in systemic adrenaline between groups during exercise.

As a result, there are a number of points to consider surrounding the adrenaline response in our cohorts. The pre-exercise adrenaline concentration is abnormally elevated in both groups. Resting baseline adrenaline would be expected to be approximately 50-200pg/ml (26, 28, 42). However, in our study we found baseline adrenaline concentrations above 1000pg/ml. This is comparable to expected post-exercise adrenaline concentrations (42). Psychological stress is an important factor to consider when measuring adrenaline as it can result in dramatic increases in systemic adrenaline (26). Although we aimed to reduce psychological stress by taking an initial resting baseline sample 30 minutes prior to the pre-exercise sample, the stress of being in laboratory conditions may have caused an increase in adrenaline at this time point. In future studies, it may be better to increase the resting time or to have the participant sitting on the bike for a short period of time before taking the pre-exercise blood sample. Beginning with an elevated adrenaline level may have disguised the expected increase post-exercise in both groups and therefore makes it difficult to delineate differences in adrenaline release between control and T1D groups. Furthermore, although we aimed to recruit participants with similar activity levels, it is worth considering that the adrenaline response is different between trained and untrained individuals (40). Nonetheless, the lower basal adrenaline release in the T1D cohort may influence the blunted NK cell mobilisation observed in our study. Therefore, these experiments need to be repeated with larger patient numbers to fully elucidate the mechanism of differential mobilisation of certain lymphocyte subsets following vigorous intensity exercise in T1D. On the other hand, reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported previously, resulting in reduced adrenaline responses (31, 41, 77). Reduced beta-adrenoceptor density and sensitivity, causes a blunted lymphocyte increase during acute exercise (51). Therefore, exercise-induced mobilisation of exercise responsive subsets including highly differentiated $CD8^+$ T cells and early mature NK cells may be blunted due to a reduced lymphocyte beta-adrenoceptor density and sensitivity in T1D.

Early mature KIR^+ and highly differentiated mature KIR^+CD57^+ NK cells are important cytotoxic populations involved in immune surveillance. Both subsets are highly cytotoxic, however early mature $KIR^+CD56^{dim}CD16^{bright}$ are still proliferative compared to KIR^+CD57^+ NK cells. The blunted increase in early mature KIR^+ NK cells may be indicative of an increased risk of cancer associated with T1D (15, 62). Although a blunted increase is evident, there is still mobilisation of a proportion of early mature KIR^+ and complete mobilisation of highly mature KIR^+CD57^+ NK cells. Therefore, repeated bouts of acute vigorous intensity exercise may have positive implications in reducing the risk of cancer in T1D, particularly those with long-standing T1D. This is supported by previous studies investigating exercise training in cancer models in which immune surveillance was increased and tumour growth was reduced (63). NK cell infiltration into tumours was increased in trained mice. In this study, it was reported that adrenaline and IL-6 were imperative for NK cell mobilisation, redistribution, and activation to control tumour growth (63).

Preliminary data presented here provides evidence to investigate exercise training in T1D. Exercise-induced improvements in T1D following exercise training have been shown in NOD mice (61). NOD mice that were trained for 20 weeks demonstrated reduced immune cell infiltration into the pancreas and therefore a reduction in insulitis (61). NK cells are one of the first immune subsets to infiltrate the pancreas before diabetes onset, therefore reduced insulitis may be in part through a reduction in NK cell infiltration. Exercise training also acts as a preventative measure for atherosclerosis in people at risk such as people with T1D (1, 55). Together, this provides a strong basis to investigate exercise training in T1D to reduce pancreatic NK cell infiltration, reduce atherosclerosis risk, and increase immune surveillance. Respectively, these benefits may translate into a number of clinically relevant outcomes. Firstly, exercise may modulate the natural history of autoimmune T1D. We have recently published the results of a pilot clinical trial to explore whether physical exercise reduces the rate of beta cell loss in people newly diagnosed with T1D (58). Whilst immune changes were not reported as part of this trial, the results do suggest that exercise has the potential to protect beta cells in new-onset patients. The trial needs to be taken forward to a formal and adequately powered clinical trial for beta cell preservation. However, the innate immune cell changes we report here do provide mechanistic support for physical exercise modulating immune cell behaviour in T1D. Second, innate immune cells play an important role in infection and increasing the responsiveness of these cells is one approach to control this (32). People with T1D are at significantly increased risk of hospitalised infection (14) and the ability of exercise to mobilise

innate immune cells may be beneficial in the surveillance and management of this risk. In summary therefore, this study provides important immune based evidence to support the encouragement and provision of exercise to people with T1D. However, whilst our findings do demonstrate that vigorous exercise increases the mobilisation of innate immune cells in T1D, the mobilisation is not as significant as in people without diabetes. It may be therefore that exercise will need to be of higher intensity, longer duration or of a different form to achieve the mobilisation observed in non-T1D. Although this is the first study of its kind, it is important to note that participant numbers in our study were relatively low. This has largely contributed to the lack of statistical significance between groups. However, this study offers insight into exercise related changes in T1D compared to healthy participants. It provides a basis for future investigation into the effects of exercise on NK cells in T1D and this is imperative in moving research in this field forward.

In conclusion, mobilisation of NK cell subsets is observed in control and T1D groups following vigorous intensity exercise. However, $CD56^{dim}CD16^{bright}$ NK cells display a blunted increase following vigorous intensity exercise in T1D and this was driven by impaired mobilisation of an early mature KIR^{+} subset. This may be due to differences in lymphocyte beta-adrenergic receptor density in T1D. Our findings have implications for immune surveillance in T1D and supports the need for studies to explore whether vigorous intensity exercise can modulate the autoimmune response in T1D.

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Disclosures

Michelle Curran is now an AstraZeneca employee and is part of the AstraZeneca postdoc program.

Abbreviations

BMI	Body Mass Index
BP	Blood Pressure
CHF	Congestive Heart Failure
DC	Dendritic Cell
ddH2O	Double-distilled water
EDTA	Ethylenediaminetetraacetic Acid
EMRA	Effector Memory re-expressing CD45RA
EXTOD	Exercise for Type One Diabetes
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FMO	Fluorescence Minus One
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
HbA1c	Haemoglobin A1c
HSPC	Hematopoietic Stem and Progenitor Cells
KIR	Killer Immunoglobulin Receptor
KLRG1	Killer cell Lectin-like Receptor subfamily G member 1
mAb	Monoclonal antibody
mDC	Myeloid DC
NK	Natural Killer
NKG2A	Inhibitory C-type lectin receptor A
NOD	Non-Obese Diabetic
O2	Oxygen
PBS	Phosphate Buffer Saline
pDC	Plasmacytoid DC
REC	Research Ethics Committee
SSC-A	Side Scatter- Area
SSC-H	Side Scatter- Height
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Th1	Type 1 helper
Th2	Type 2 helper
TLR	Toll-like Receptor
tSNE	t-Distributed Stochastic Neighbor Embedding
UHBFT	University Hospitals Birmingham NHS Foundation Trust
WTCRF	Wellcome Trust Clinical Research Facility

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