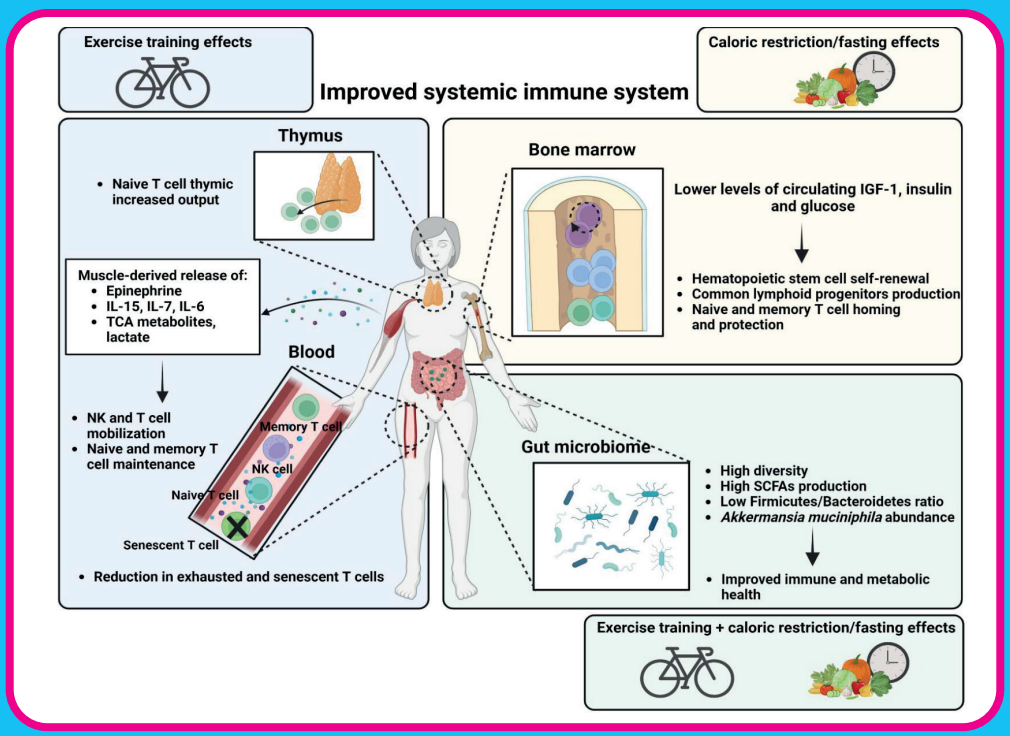
EXERCISE IMMUNOLOGY REVIEW



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Contents

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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Higher risk of upper respiratory tract infection post marathon running: when physical exercise becomes a threat to the immune system

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ABSTRACT

Background: Several studies have reported that marathon runners have a higher risk of upper respiratory tract infections (URTI) post marathon than non-exercising controls. However, other studies did not find a higher risk of URTI in the same participants before and after a marathon, precluding a conclusive consensus. Besides the between-subjects effects, another important confounding factor in these results is the different pre and post follow-up time to track URTI.

Objectives: Identify by meta-analysis whether a marathon running increases the risk of URTI, adjusting the follow-up time to track URTI.

Data sources: We searched for articles using MEDLINE (PubMed), Embase, Scopus, Web of Science, the Cochrane Library, and EBSCOhost, combining the marathon and respiratory infection descriptor synonyms, on 1st December 2022.

Eligibility criteria: The PICOS framework included human population, comparison between pre and post marathon running, of URTI symptoms (assessed from one to 4 weeks), in non-controlled intervention studies.

Data Synthesis: Because follow-up was longer before the marathon in many studies, we adjusted the number of subjects with infections before marathon to the equivalent post-marathon follow-up duration. There was 18% higher incidence of URTI post-marathon (OR 1.18 95%CI [1.05-1.33], p= 0.005) in a very consistent meta-analysis (I^2 = 0%, p = 0.69), with no risk of publication bias (Egger test p-value = 0.82) for the 7 studies included. The main issues in the quality of the studies were bias in measuring the outcome, bias in classification of intervention (participation in the marathon) and time-varying confounding (corrected for analysis), and therefore the quality of evidence was moderate (GRADE approach = 3).

Limitations: The need for follow-up time adjustment is a limitation, since the number of URTI recorded could be different if the original studies had used the same follow-up time pre and post marathon. The subjectivity of the URTI self-assessments is another limitation in this field.

Conclusions: There is an increased risk of URTI post marathon running and research on this topic to understand mechanisms might support runners to find efficient interventions to reduce this risk.

Protocol registration on in the International Prospective Register of Systematic Reviews (PROSPERO): CRD42022380991

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INTRODUCTION

There is no doubt that regular physical activity improves human health outcomes (1), including improved immune responses (2,3). Although there is no strong evidence of exercise training reducing risk of acquire upper respiratory infections, there is evidence that exercise training reduces the severity of upper respiratory tract infection (URTI) and the number of symptom days in adults (4,5). During a viral infection, moderate exercise training can reduce morbidity in animals (6), although exhaustive exercise in this condition trends towards an increase in mortality and severity of symptoms (6). The volume and intensity of exercise undertaken may therefore determine the impact on infections and the immune response to them.

Researchers have proposed that under healthy conditions, there is a potential for immunological suppression after an exhaustive physical exercise session, a phenomenon called "open window" (7). In support of the open window theory, there are animal studies showing that exhaustive exercise just preceding or immediately after virus injections leads to worse outcomes including death (8,9). In humans, immunological alterations observed after exhaustive exercise or after intensified periods of training with poor recovery have been interpreted as immunosuppressive. For example, very high exercise workloads are associated with transient immune impairment, inflammation, oxidative stress and muscle damage (7,10).

An extreme challenge type of exercise is the marathon. A Marathon is a road race of 42.195 km distance, and a more extreme event is the ultramarathon, that encompasses any distance above the marathon distance, such as the Comrades Ultramarathon in South Africa which is 88 km. During prolonged exercise we continuously increase the energetic demand for active muscles (11). There is a general negative association between exercise volume and intensity, to allow the body to meet its energetic needs, especially at high intensity (speed or pace in the case of the marathon) or volume (distance in the case of the marathon). Marathon runners cover 42.195 km at steady-state oxygen consumption, corresponding to 94% of their maximal capacity (12), making the marathon extremely challenging for the body. Thus, if the open window theory is true, the marathon will be a perfect combination of extremely high intensity and volume, to test whether the potential immunosuppression can be converted to a real increase in the risk of URTI.

In fact, after a marathon running, there is a reduction in the salivary immunoglobulin A levels (13,14), T cell proliferation (15), antigen presentation by macrophages (16), counts of natural killer and T cells (17), granulocyte oxidative burst (18), neutrophils (18) and changes in cytokines and stress hormones (18–20). Furthermore, the energy depletion during a marathon (11), could at least partially contribute to immune suppression considering the immune processes required to fight infections have a high energy cost (10,21).

Despite this literature there is no consensus whether excessive exercise, such as a marathon, could lead to an increased risk of URTI (10,22). Most of the evidence supporting the harmful effects of exercise in humans have been from studies in athletes from different modalities, and athletes undergo many other types of stress besides the exercise load (14,23,24). For example, they are often exposed to crowds or lower hygienic environments when accommodated with too many other team members, and therefore undergo higher exposure to pathogens, what is especially evident in a marathon run setting (23,25). Also, athletes can often be calorie restricted, sleep deprived, or subjected to high climate or altitude variations when traveling to competitions; and they undergo a very high psychological stress since they are always chasing perfection and improvement in performances (23).

URTI are very common in adults, occurring 1 to 3 times a year (26). The risk of URTI in marathon studies is usually assessed by common cold symptoms and it may result from viral infection of the upper respiratory tract, but could be also confounded with sinusitis, tonsillitis and laryngitis depending of the way it is assessed (26). Studies investigating the effects of marathon running have generally tested URTI by the incidence of self-reported symptoms or infectious episodes using a variety of criteria for the definition of URTI (13,27–30). Some of these studies have reported that marathon runners had higher frequency of symptoms of URTI than non-exercising controls (4,5) and that higher intensity running leads to even higher incidence of URTI (31). On the other hand, other studies have not found a higher risk of URTI comparing the same participants before and after a marathon, including para-athletes (13,27–30). It is not clear why these studies have divergent results, but besides the individual differences in studies comparing different groups of people for in each condition, another confounding factor might be the time of follow-up to assess URTI before and after a marathon in those studies.

To bring consensus to this conflicting literature, we carried out a meta-analysis with studies that compared the same subjects in longitudinal study designs, measuring the incidence of URTI before and after a marathon run with adjusted times of follow-up.

METHODS

Protocol and registration

Details of the systematic review can be found in the International Prospective Register of Systematic Reviews (PROSPERO, CRD42022380991).

Eligibility Criteria

The PICOS framework encompassed studies with humans of any sex, race, age or health condition (Population), running a marathon or ultramarathon (intervention/effect), with assessments before (comparator) the marathon and after the marathon run, to identify the number of individuals with URTI symptoms (outcome) in a certain period of follow-up in each time-point, in non-controlled intervention studies (study type).

Search and Study selection

We first did an exploratory comprehensive search encompassing any type of exercise protocol to conclude the URTI symptoms

were most frequent after a marathon run. Then, the new search focused only on this type of extreme exercise. The new search was conducted on PubMed/MEDLINE, Scopus, EMBASE, Web of Science, Cochrane, EBSCOhost for CINAHL and SPORTDiscus 1st December 2022, with no data or language restrictions. The search combined the marathon and the respiratory tract infection synonyms, including abbreviations on title, abstract and keywords. as detailed on PROSPERO (CRD42022380991). The retrieved studies were transferred to the Rayyan Systematic Reviews system (32), which automatically removes obvious duplicates. Two independent and experienced reviewers, then screened the articles using the Rayyan-Systematic Reviews system, excluding less obvious duplicates, non-original data studies (reviews, book chapters, conference papers, case study, protocols), cross-sectional studies, studies that did not assess URTI before the marathon and studies that did not assess number of participants with URTI. Next the two reviewers fully read the studies retrieved to confirm they had data to be extracted. Conflicts were solved by further discussion between the two reviewers and the opinion of the third reviewer was sought when defining the specific outcomes to answer the main question.

Data collection and items

Data was collected in duplicate and confirmed by automatic excel software check, as well as revised by both reviewers in case of conflict. We extracted basic characteristics of the studies such as age, place of marathon and supplementations tested if any. The main data collected for meta-analysis were the time of follow-up to assess URTI before and after the marathon, the total number of participants running the marathon and the number of participants with reported infection in each of the follow-up periods. Because a few studies tested the effect of supplementation on marathon-induced URTI, we extracted this information for analysis.

Risk of bias assessment

The Cochrane ROBINS-I-tool to assess risk of bias in non-randomized studies of interventions was selected here considering the Marathon running was an acute intervention with or without supplementation, where pre-post assessments were conducted without a control (non-marathon running group). The tool judged bias due to confounding, in selection of participants, in classification of interventions, due to deviations from intended interventions, due to missing data, in measurement of outcomes, and in selection of the reported result. For each of these items a series of questions needed to be answered to attribute a final score for each study (33).

Summary measures and Statistical analysis

The meta-analysis was performed using the software Comprehensive meta-analysis version 4.0. The outcome selected was the number of participants with URTI, excluding studies assessing for example number of symptom days or numbers of symptoms within the whole cohort. Because in many studies the URTI assessment period was longer before the marathon, we adjusted the number of subjects with URTI before marathon to the equivalent post-marathon follow-up duration (URTI pre/ [days pre/days post]). Next, the odds ratio (OR) and 95% confidence interval was calculated comparing the ratio of individuals reporting URTI before with the ratio of individuals reporting URTI after the marathon. Egger test was performed to identify risk of publication bias. In all tests we considered significant the $p \leq 0.05$. Additionally, we assessed heterogeneity by percentage of inconsistency between studies, in which we considered lower than 25% as low, between 25 and 75% as moderate, and above 75% as high (34).

Risk of bias across studies

The GRADE approach (35) was used to identify the quality of the evidence in which interventional studies start with maximal score (4 = high) and have one or two points removed if they show severe or very severe risk of publication bias, low quality of studies included, imprecision, inconsistency of results and indirect evidence.

Results

The new search was very specific to marathon running and thus only 132 were retrieved before we selected the final 7 studies. Three of these studies had 2 subgroups each, with a supplementation and placebo group which were included as an individual study for analysis, since they analysed different participants in each group.

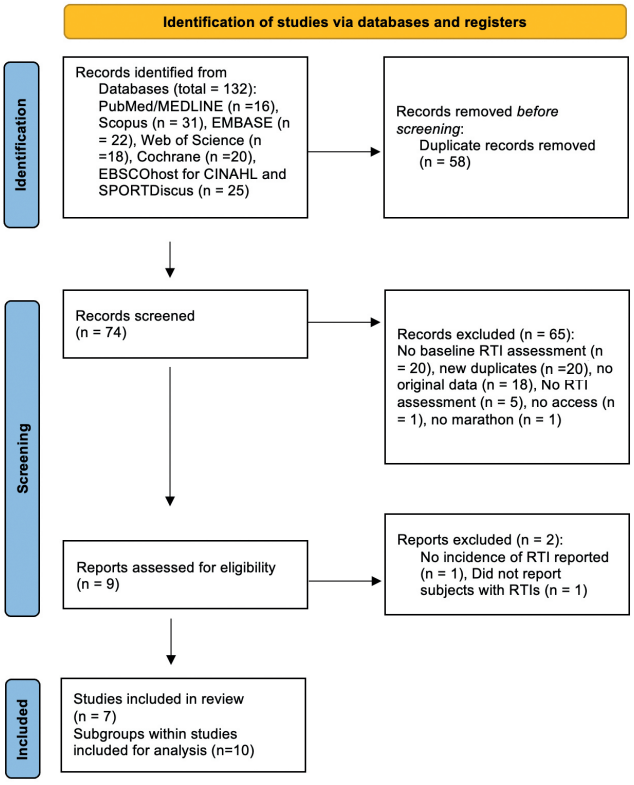

Figure 1. Flowchart of studies selection.
From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/.

STUDY CHARACTERISTICS

The main features of the analysed studies (13,27–29,36–38) are described on Table 1. The studies were performed in males and females, with a wide age range (20-69 years), with naturally acquired URTI. URTI was assessed by questionnaires, self-reported symptoms or self-reported infection episode, telephone interview and daily registration of symptoms in previously healthy individuals. In general, they did not define a criterion or how many symptoms would be enough to confirm a URTI and their results were presented as a binary variable (number of individuals with and without URTI in a given time-point). The comparator was the same individuals before and after the period in which the symptoms of infection was tracked. In general, there was longer follow-up before (15-84 days) than after the marathon run (7-21 days). ROBINS-I showed a few indications of low risk of bias (Supplementary Table 1), such as bias in measuring the outcome, bias in classification of intervention (participation in the marathon) and time-varying confounding, although this last one was corrected for analysis.

FIRST AUTHOR, YEAR	SAMPLE SIZE (SEX)	AGE	MARATHON	URTI ASSESSMENT
Ekblom, 2006	1694 (1354♂/340♀)	29-59y	2000 Stockholm Marathon (Sweden)	Self-reported infection episodes.
Furusawa, 2007	21 ♂	20-67y	1998 Oita International Wheelchair Marathon (Japan)	Self-reported any symptoms (sore throat, cough, fever, runny nose, sneezing) for more than 2 days and separated by at least 1 week from a previous episode.
Harden, 2004	L-methionine Supplement 11 (9♂/2♀) and Placebo 10 (8♂/2♀)	35- 36y	Comrades Ultramarathon (South Africa)	Minimum of 3 symptoms for cold (cough, sore throat, running nose, sneezing) or influenza (fever, aches and pains in joints or muscles, cough sore throat) such that they did not train, or they consulted a doctor for treatment.
Himmelstein, 1998	Vitamin C 30, Placebo 14, 44 total (33♂/11♀)	25-40y	1994 Duke City Marathon (USA)	Self-reported runny nose, cough, or sore throat.
Kekkonen, 2007	LGG Supplement 70 (62♂/8♀), Placebo 71 (63♂/8♀)	22-69y	2003 Helsinki City Marathon (Finland)	Any self-reported symptoms (fever, rhinitis, sore throat, cough, wheezing, earache) for at least 2 days in a row and if there were at least 3 days until the next symptoms appeared.
Nieman, 1990	(1702♂/300♀)	35-38y	1987 Los Angeles Marathon (USA)	Self-reported infectious episodes (yes or no).
Nieman, 2002	98 (86♂/12♀)	21-72y	1999 Charlotte Marathon (USA) and 2000 Grandfather Mountain Marathon (USA)	Self-report cold symptoms (runny, stuffy nose, sore throat, coughing, sneezing, coloured discharge) or flu symptoms (fever, headache, general aches and pains, fatigue and weakness, chest discomfort, cough) for at least 2 days in a row.

Legend: ♀: Women; ♂: Men; y: years old; LGG: Lactobacillus rhamnosus GG.

Table 1. Characteristics of the selected studies.

EVIDENCE SYNTHESIS

Figure 1 reveals a higher incidence of URTI post-marathon (OR 1.18 95%CI [1.05-1.33], p= 0.005) in a very consistent meta-analysis (I^2 = 0%, p = 0.69), with no risk of publication bias (Egger test p-value = 0.82) for the 7 studies included. The GRADE approach led to moderate quality of evidence (score 3) with the removal of one point due to low quality of the studies; while no point was removed from risk of publication bias, inconsistency, indirect evidence, or imprecision.

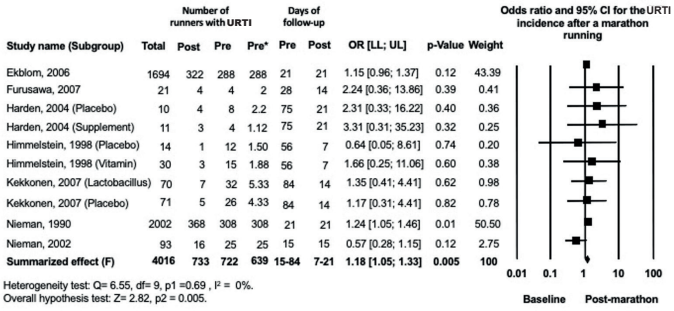

Figure 2. Forest plot for URTI incidence (odds ratio) before and after marathon running.
OR: Odds ratio; F: fixed effect; LL: Lower Limit; UL: Upper limit; CI: confidence interval; Q: Q-value for heterogeneity test (observed heterogeneity); df: degrees of freedom (expected heterogeneity); p1: p-value for heterogeneity test; I^2: inconsistency between studies; Z: Z-value for hypothesis test; p2: p-value for hypothesis test (difference of URTI incidence before and after marathon); URTI: Upper Respiratory Tract Infections; *adjusted to equivalent post-marathon follow-up duration.

Because there was no significant heterogeneity (p=0.69, I^2=0%) and most papers did not assess the major outcome (URTI) separately by confounding factors (e.g.: age, sex, training intensity, training load, marathon performance and supplementation), we were not able to run subgroup analysis to explore the mediators of outcome heterogeneity.

Discussion

We confirmed that a marathon can increase the risk of URTI, however the different magnitude of effects across studies suggests it deserves further investigation. Therefore, in the next paragraphs we are going to discuss the possible influence of the following confounding factors: climate conditions, age, sex, training load and carbohydrate supplementation. We will also consider the potential physiological mechanisms underpinning our results.

Climate condition

Periods of low temperatures coincide with epidemics of many respiratory viruses and it is known that URTI are more prevalent during the autumn and winter (~70%) than spring and summer (~30%) (39,40), which could influence the results. Nevertheless, a majority of the marathons in this review were performed in the summer and spring seasons and with temperatures varying from 50 to 77°F, whether they were in South Africa (36), Japan (27), Finland (37), Sweden (29), or in USA (13,28,38). In addition, since our control was the risk of URTI in the same individuals before the marathon, the possible differences in temperatures in different marathons within the studies will be controlled for by this internal comparison. Unfortunately, there was no control of the individuals that could be traveling from regions with different climate conditions just for the marathon.

Age

With regard to age, we observed there was a higher risk of URTI post marathon in younger participants in two studies with very large sample sizes of 2002 (38), and 1694 (29). This is despite the fact that pre-marathon there is a higher risk of URTI in older marathon runners (28), suggesting the older marathon runners might have the same impaired immune system seen in older adults in general, as we have shown previously (2,41). Indeed, Nieman, (38) showed age did not influence post marathon URTI for individuals who had a URTI before the marathon, while young male and female individuals (<30 years) had significantly higher post marathon URTI when compared between individuals who had no URTI before marathon. Another possibility is that older marathon runners are usually more experienced with marathon running, its training progression, diet etc compared to the younger ones who may not be as well prepared.

Sex

Only two studies reported separate results for men and women, limiting the investigation of this confounding factor in our analysis. At baseline there was a higher odds ratio of URTI in female marathon runners than males (OR 3.059, 95% CI 1.0 - 9.6, p-value 0.05)(28). However, one large study, n=1694, reported that the marathon did not increase the risk of URTI more in women than men (29).

Training load

Higher training speed and volume has been associated with higher risk of URTI post-marathon (27,28), which could be related to higher energetic depletion with higher exercise loads, or norepinephrine release that is commonly beneficial for immunosurveillance in shorter exercise bouts, but in prolonged exposure may lead to suppression of effector functions (42). During exercise, there is also an increase in cortisol, and its anti-inflammatory action can jeopardize the immune response after long exposure (43). In fact, the higher volume of the only study testing the ultramarathon (36), showed a much higher OR for URTI than marathon studies. However, the low sample size generated very high imprecision in this study, preventing a fair comparison with other studies and reducing the weight of this study in the overall meta-analysis results. In contrast, two studies identified that lower training volume was associated with higher URTI risk post-marathon (28,38), but the same studies suggested this contradictory finding might be explained by the opposing cause-effect, in which the previous URTI influenced the reduction of training volume.

Carbohydrate supplementation

There is considerable evidence showing that carbohydrate supplementation before, during and after exercise reduces inflammation, neutrophilia and monocytosis (22), and prevents decreases in granulocyte and monocyte phagocytosis, and cytokines in the circulation (44–49). The intake of food or beverages restores glucose levels, leading to reestablishment of normal stress hormone levels (epinephrine and cortisol) that are known to regulate immune function and also support the immune cell metabolic capacity (10,22,50), which ultimately regulate

immune cells function (21,51). Specifically after a high intensity run, carbohydrate supplementation attenuates leucocytosis and influences neutrophil and monocyte numbers (52,53). In contrast, exercising under glycogen-depleted conditions has been shown to amplify exercise-induced immune alterations, which might, in some cases, be detrimental to training adaptations (54,55). Here, just one of the studies included, tested carbohydrate supplementation, showing that within the 16 runners reporting URTI after a marathon, ten had consumed placebo and six had consumed carbohydrate (13), which did not led to a conclusive finding. Other supplements tested in the included studies were not meant to restore energetic depletion, and they did not affect URTI incidence in those studies (28,36,37), in agreement with previous reviews showing lack of evidence to support recommendation of other supplements (7,45).

Physiological mechanisms underpinning the effect of marathon running on URTI

The higher risk of URTI observed post-marathon, could be affected by the reduction in several types of lymphocytes in the circulation (naïve and memory CD4 helper T cells, activated CD8 cytotoxic T cells, NK, NKT, and B1 cells), reduction in delayed-type hypersensitivity response, salivary immunoglobulin A, T cell proliferation, antigen presentation by macrophages (by suppression of MHC II expression), natural killer cell activity, granulocyte oxidative burst, higher neutrophil/lymphocyte ratio and changes in cytokines and stress hormones that are known to change after this type of strenuous exercise (15,17,22). It is debatable to what extent these changes increase susceptibility to infection, but exhaustive exercise reduces cytokine production in response to antigen stimulation and increases mortality in animals with ongoing infection (6,56).

There is increasing evidence to suggest that the main trigger of immunosuppression with strenuous exercise is the lack of energy for the immune cells to exert their functions. For example, strenuous exercise has been shown to lead to reduced proliferative capacity (57), migration (58), and cytotoxicity in T lymphocytes and other immune cells (44), which are highly energy consuming functions. CD4+ T cells, important against respiratory infections, undergo metabolic stress during strenuous exercise (which does not happen with moderate intensity exercise) which influences their ability to metabolize ATP into adenosine leading to an immunosuppressive phenotype (59). A reduction in T cell proliferation in response to Concanavalin A (a T cell mitogen that can activate immune responses) also occurs after exercise (57,60) and there is a greater reduction when exercise lasts longer than one hour, regardless of exercise intensity (57) highlighting the influence of energy expenditure in this response. One review has detailed the evidence for exercise modulating peripheral lymphocytes metabolism and how it can be jeopardized with strenuous exercise (51).

Another mechanism that deserves more investigation is the effect of exercise induced endotoxemia during a marathon (61), that could be caused by an increase in gut permeability by the common use of Iboprufen by marathon runners, or by direct effects of high intensity exercise (62). However, this is more likely to lead to more severe types of infections rather than URTI.

Limitations

The need for follow-up time adjustment is a limitation in our study, since the number of URTI participants could be different if the original studies had used the same URTI assessment time pre and post marathon. Another limitation in URTI studies is the subjectivity of the assessments that do not absolutely define URTI, for example using a nasal swab and tests for an infection. Specifically, the studies with higher weight in our analysis, including the ones with higher significant increase in URTI, were the ones allowing the participants to define their infection episode without specifying how many symptoms, or days of symptoms, for example. Nevertheless, since we only included studies testing URTI in the same participants before and after a marathon and the main effect is based on each study difference between time those points, we had this confounding factor controlled in our analysis. Next studies need to repeat those experiments with high sample size and rigorous methodological control, and controlling variables such as sleep quality, stress and nutrition.

CONCLUSIONS

Our analysis shows that a marathon run increases the risk of a URTI by 18% over a period of 7-21 days post-marathon. The physiological mechanisms by which this type of exercise would increase susceptibility to respiratory tract infections is unclear and more research is needed to identify mechanisms to target to reduce this risk for runners.

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Investigating the impact of exercise on T and NK cells in skin cancer: a systematic review

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ABSTRACT

Skin cancer has the highest incidence of all cancers, and their incidence are increasing in both melanoma and non-melanoma skin cancers. Alternative adjuvant treatment strategies appropriate for their management are needed. Modifiable lifestyle factors influence disease outcomes, either improving or worsening outcomes. Exercise is an example of a modifiable lifestyle factor, and can be prescribed as an adjuvant therapy in other cancer types to improve immune function and overall clinical outcomes.

The initial aim of the review was to investigate the T-cell specific mechanisms of exercise which affect clinical/disease outcomes in skin cancer. Study quality was assessed by a modified Covidence quality assessment template with animal-model study specific criteria. A total of 10 articles were included; all articles were murine model studies investigating melanoma. Eight studies (n=8) employed a randomised controlled trial design, with two bio-informatics studies, and one study using human data which could solidify a link to human health.

While the review focussed initially on T-cells, many studies reported significant changes in NK cells, and as they share the same haematopoietic lineage/ common lymphoid progenitor as T cells, the data was included in the analyses. Most studies indicated that exercise reduced melanoma tumour burden. Exercising prior to melanoma inoculation was most effective for delaying carcinogenesis and reducing tumour burden. Synergism was a topic identified in studies; PD-1/PD-L1 treatment, and exercise were not synergistic. Conversely, exercise and mental stimulation were synergistic, and the temperature at which exercise was conducted significantly reduced tumour burden.

Several murine studies reported that exercise improved clinical outcomes in melanoma, and that long-term exercise was more effective in reducing tumour burden. Further studies are required to investigate this relationship in humans, and in other types of skin cancer.

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INTRODUCTION

Of all cancers, skin cancer has the highest incidence in jurisdictions with mostly white populations. Skin cancer, including both melanoma and non-melanoma skin cancer (NMSC), is one of the most prevalent and burdensome diseases globally [52, 62]. NMSC is an umbrella term describing all other cutaneous malignancies outside of melanoma, and the most common NMSCs are known as keratinocyte cancers (KC), forming around 97% of NMSC presentations [50]. Worldwide, 90% of all skin cancer cases are a KC, and their global absolute mortality rate surpasses melanoma [16, 50, 62].

Immune function is important to both the development and treatment of skin cancer. T cells are an integral immune cell involved in skin health and can originate and/or act dermally or systemically. Resident and circulating T cells have separate roles, but work and influence each other synergistically [32]. T cell composition appears to be different in the skin compared to the systemic circulation, and ~90% of dermal resident T cells are memory T cells (Tm) cells [32]. These cells are associated with immunosurveillance; however, it is unclear how their number and function change in the context of cutaneous malignancies [31]. There is a three times greater incidence of CD4+ Tm cells than CD8+Tm cells within the epidermis. The CD4+Tm population increases with age, indicating a pro-inflammatory phenotype, but it is unclear how this change may be influential in the development of KC, that are often seen in older adult, or in other NMSCs cases [33, 54].

When immune function is compromised, individuals are more likely to develop cutaneous malignancies, including the KC sub-types of basal cell carcinoma (BCC), and cutaneous squamous cell carcinoma (cSCC) [38, 50]. Individuals can become immunocompromised via UV radiation, viral illness, age-associated immunosenescence, and immunosuppressive drugs especially in the context of solid organ transplants [30, 38]. In the general population, the ratio of BCC cases to cSCC cases is 4:1; this ratio is reversed in immunocompromised cohorts [49]. Individuals are up to 100 times more likely, depending on the transplanted organ, to develop invasive cSCC [30, 49]. Immunosuppression is an established prognostic factor for determining the invasiveness of disease. Metastatic

risk for cSCC can be as low as 0.1% but as high as 13.7% in immunocompromised individuals [25].

There are many under-recognised burdens associated with a KC diagnosis that impact health and quality of life [19]. Only limited systemic treatment options and highly invasive surgery are typically offered as a treatment solution for complex, high-risk and/or re-occurring lesions [16, 22]. Surgical operations might cause lifelong structural and functional alterations, especially in the head and neck impacting the patients' quality of life [22]. Individual burden is further compounded by the fact that almost 75% of individuals diagnosed with a KC will have multiple primary carcinomas [8, 34]. Disability-adjusted life years (DALYs) data clearly highlight the significant global burden of KC [52]. KCs have a higher DALY rate across all global regions and age ranges than melanoma [52]. The limited treatment options have resulted in a gradual shift in the classification of KCs to a chronic illness, which acknowledges their severity and burden. Further research into appropriate treatment strategies, and development of adjuvant therapies and rehabilitation programs for individuals living with KC is required [18, 48] .

Modifiable risk factors including lifestyle choices and habits such as alcohol consumption and tobacco use, poor diet and physical inactivity can influence initiation, incidence and outcomes of various diseases including cancer. In the United States, ~42% of all cancer cases are associated with a modifiable risk factor [9]. Sun exposure is one of the better-described modifiable risk factors for development of skin cancer [23]. Other studies investigating modifiable factors such as diet, obesity and exercise have informed the Centre for Diseases Control and Prevention (CDC) and World Health Organisation (WHO) guidelines for general cancer prevention. Their exercise recommendation for cancer prevention is a combination of resistance exercises focusing on all major muscle groups, in conjunction with either 75-150 min of vigorous intensity aerobic exercise or 150-300 min of moderate-intensity aerobic exercise per week [7, 13]. There is a perception that exercise may be skin cancer-provoking, but this arises from studies that either did not conduct exercise in sun-safe manners or indoors, and/or failed to examine the direct effect of exercise or physical activity. Several studies only investigated body mass index (BMI) and prematurely concluded that exercise increased the risk of skin cancer [57, 59]. Investigating the direct effect of exercise on underlying biological mechanisms such as the immunological response to exercise would better assess whether exercise is an appropriate and effective adjuvant therapy for skin cancer.

Exercise improves a multitude of cancer and cancer treatment-related symptoms such as fatigue, more effectively than some pharmaceutical interventions [20]. Progressive increases in the intensity of resistance exercise can reduce lymphedema flares in breast cancer [24, 44, 45]. Exercise can also improve psychological parameters in cancer, reducing depression and anxiety to improve the overall quality of life [5, 7, 11]. Exercise has been recommended across the entire cancer care continuum to improve cancer outcomes, in both haematological and solid malignancies [7, 21, 58].

Studies indicate that exercise can modulate the immune system. Exercise and physical activity are recognised as adjuvant therapy strategies for a variety of cancers, including chronic lymphocytic leukemia and non-Hodgkins lymphoma [36, 61]. An acute 45-60 minute bout of treadmill exercise, at an intensity equivalent to 70% of heart rate reserve can modulate T-cell frequencies, T regulatory (Treg) count, while CD4+, CD25+ and FOXP3+ cells are decreased in number , and T helper 17 (Th17) , Interleukin 6 (IL6) and transforming growth factor beta increased after exercise [36]. In individuals with non-Hodgkins lymphoma, a single acute bout of cycle ergometry exercise at a moderate intensity for 30 minutes, increases the concentration of IL-6 and macrophage migration inhibitory factor, and induces epigenetic modification of natural killer (NK) cells [61].

Improving immune function through immunotherapies has been successful in skin cancers as a whole, likely as a function of these malignancies being characteristically 'immunogenic' [56]. In addition to immunotherapies, exercise is an adjuvant therapy and can potentially enhance immune function, including immunosurveillance and T cell function in cancer [15]. While exercise has been used as an adjuvant therapy in other cancers, there is no clinical evidence to support its use in skin cancer thus far. Preliminary evidence has been complicated by the confounding effects of sun exposure and body mass index [57, 59]. Characterising immune cells involved in skin cancer is needed to identify relationships between exercise, immune function, and skin cancer. Investigating the direct effect of exercise on the immunological response to exercise would better assess whether exercise is an appropriate and effective adjuvant therapy for individuals with locally advanced or metastatic skin cancer. The aim of this review was to investigate the effect of exercise on NK and T-cell specific mechanisms to describe disease outcomes in all types of skin cancer.

METHODS

Search strategy

Four databases were searched: Science Direct, Web of Science, Medline via EBSCO host and Scopus. The search string/terms used in the search were as follows: (Physical activity OR sport OR exercise), (melanoma OR non-melanoma skin cancer OR skin cancer) and (immune OR "T cell" OR CD8 OR CD4 OR CD25 OR CD3 OR CD45 OR lymphocyte OR PD-1). Articles were searched by title, abstract and keyword textual contents where available. The searches were finalised in September 2023.

Eligibility Criteria and study selection

The protocol for this systematic review was registered with PROSPERO (PROSPERO registration: CRD42023397176). All skin cancer types including melanoma and non-melanoma skin cancers, of all stages of disease; pre-malignant non-melanoma skin cancers, melanoma in-situ, early and late stage skin cancer were included. In addition, all stages of treatment were included; pre, during and post treatment. Studies had to have a focus on exercise or physical activity and measure T cells or PD-1. Initial searches indicated very limited research in humans, and consequently we included both human and animal model studies, from any publication date and any

experimental research article type; bioinformatics/in-silico, animals and human models. The only caveats were that articles had to be published in English, and that other review articles; systematic reviews, narrative reviews, study protocols, case studies or articles submitted in other formats such as posters, presentations, commentaries, editorials, opinion articles or abstract-only journal entries were excluded.

We used the Covidence platform (https://www.covidence.org/) which adheres to the PRISMA checklist and tracks study selection to automatically populate a PRISMA flowchart. Figure 1 presents a graphically altered PRISMA flowchart that includes all relevant information of study selection in a simple visual form. Articles were screened independently by (HB) and (CG) with any conflicts resolved by discussion, or involvement of a third assessor (DP).

Data extraction

ExtrExtraction was completed using the Covidence extraction template 2.0 [10]. Multiple measures were extracted from the eligible studies and separated into 3 main categories: general article information, characteristics included in the study, and outcomes. The measures extracted in the general article information were; study ID/ DOI, title, lead authors contact details, the country in which the study was conducted, and other study notes. The information extracted from the 'characteristics included in the study' category included: the aim of the study, study design, source of funding, research methods, mice type, cancer type, inclusion criteria of participants, exclusion criteria, the total number of participants, intervention and comparisons/ groups and controls, exercise type, other environment condition information, immune cells analysed, and other measures. Lastly, the outcomes extracted included changes in immune cell NK and T cell percentage, counts, frequencies, or involvement of immune related genes or their expressions and non-immune-related measures. A simplified representation of this data is presented in table 1

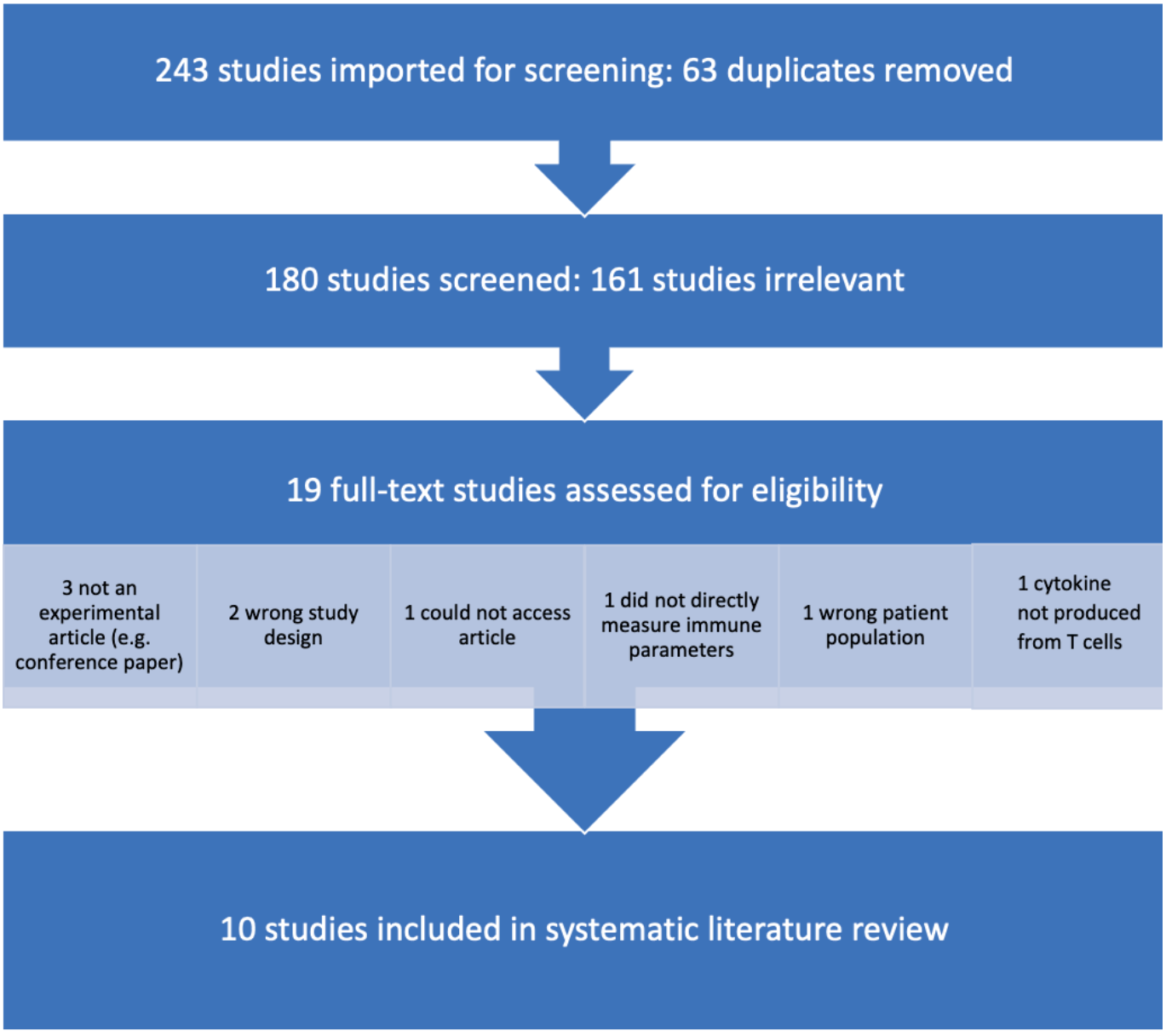

Figure 1. PRISMA flowchart for effects of exercise on immune parameters in animal models of skin cancer.

Table 1: Extracted study outcomes investigating general measures and changes in immune cells after exercise in murine models of skin cancer.

Title	Changes in immune cells			Other disease measures
	No change/other results	**Decrease/Downregulated**	**Increase/Upregulated**	
[29] (Lee, 2019)	•CD4+T cell no. and differentiation of CD4+T cells		• CD8+ T cells producing IFNγ by 1.5x in circulation •IFNγ+ CD8+ T cells frequency by 3x in spleen of thermoneutral mice •CD8+ T cells, effector memory CD8+T cells, γδT cells, NKT and NK cells sig. in thermoneutral group.	• Min. tumour growth, ↓ tumour mass in thermoneutral group vs. body temperature and control groups
[35] (Pedersen, 2016)			•NK, dendritic, NK1.1+, CD4+, CD8+ and CD3+ T (+gamma delta and NKT cells) after exercise •NK frequency, cell cytotoxicity/function and promoted epinephrine-dependant mobilisation of IL-6 sensitive NK cells to tumours. •NK cell no. and infiltration was inversely correlated with tumour burden	• Pre-exercise mice had greatest ↓ in tumour growth (61%) and tumour volume (67%), followed by the exercise group. • All exercising groups significantly ↓ no. of metastases • 66% ↓ in tumour burden in T cell-depleted mice, indicating NK cells activity • Tumour growth was ↑ in NK-depleted athymic mice.
[60] (Zhu, 2021)	•3 major pathways; NF-kappa B, chemokine signalling and immune responses • 6 hub genes; genes relevant to T cell functioning included : WDFY4 (CD8+ T cells, alpha beta T cell activation) and ITGAM (macrophages). These genes were weakly associated with overall and disease-free survival. • Exercise related hub genes were dysregulated in melanoma, and verified using melanoma and healthy tissue data from the TCGA database.	•Itgam expression in human melanoma, stages 1-4, differed dependent on disease stage showing stage 2 had the lowest Itgam expression. •Wdfy4 expression in human melanoma stages 1-4, didn't differ depended on disease stage		• 1627 DEGs were identified; 1285 upregulated genes and 342 downregulated genes in response to voluntary exercise

Title	Changes in immune cells			Other disease measures
	No change/other results	Decrease/Downregulated	Increase/Upregulated	
[55] (Xia, 2020)	•3 main group of DEG functionality identified s; molecular function (MF), cellular component (CC) and biological process (BP). BP group associated with immune and inflammatory responses, MF group prominently enriched in chemokine and cytokine activity.	• 5 BP genes were downregulated and 3 MF genes were downregulated. • Immune-related hub genes were: C3, Fgg, Pf4, Orm1 all involved in ↓ immune function	• 209 BP related genes were upregulated and 42 MF genes were upregulated.	• 315 DEGs were identified, 294 upregulated genes, and 21 downregulated genes.
[42] (MomessodosSantos, 2019)	• IL-4, IL-10, IL-17 and IL-6 secretions unchanged in mice with/without melanoma on any treatment.	• IL-2, IFN-gamma and TFN alpha in high fat+ exercising cohorts	• Lymphocyte proliferation in mice with melanoma +exercise vs. mice without melanoma or sedentary groups on any diet	• High fat diet ↑ tumour growth • High fat diet + exercise had slower tumour growth
[2] (Bay, 2020)			•Spleen weight after PD-1 treatment, indicating immune cell expansion, associated with ↑ PBMC killing capacity: 17% in sedentary mice, 23% mice of PD-1 treatment, 15% exercised mice, 17% exercise and PD-1 treatment.	•Wheel running sig. ↓ tumour growth (72%) •Wheel running+PD-L1 treatment reduced tumour size (83%). •Treatments separately were effective •No synergistic effect
[3] (Buss, 2021)	• T cell, CD3+ T cells and NK infiltration in any group	•CD8 T cell no. in exercise +antiPD-1 •CD8+T cell % in CD3+T cell population, indicating immunosuppressive phenotype shift	•NK cell no. in mice with antiPD-1 •FoxP3+ T cells no. in mice with antiPD-1 but not sig. •FoxP3+CD3+ cell % in tumour immune cell hotspots	•Exercise had no effect on tumour growth rate, tumour cell proliferation or mice survival •Exercise did not affect perfusion of vessels, vessel density or hypoxic state of tumour

Title	Changes in immune cells			Other disease measures
	No change/other results	**Decrease/Downregulated**	**Increase/Upregulated**	
[4] (Cao, 2010)	• In tumour burden in running only mice, but had an enhanced immune response • Improved clinical outcomes achieved in enriched in environment indicated synergism between mental and physical stimulation	• IGF-1 in running only mice, similar to enriched mice	•NK cytotoxicity and CD8 T cell functionality in enriched environment (started prior to melanoma inoculation)	•Delayed carcinogenesis: 17% of enriched environment mice did not develop macroscopically visible tumours. All control mice developed tumours. •Enriched environment mice had 77.2% lower tumour burden vs. control. They had ↓ tumour cell proliferation
[14] (Fei, 2020)			•NK cell tumour infiltration in all swimming treatments. Effect increased with increasing intensity •NK cells no. in the pre-free swimming group	• Continuous swimming ↓ tumour mass, detraining ↑ tumour growth •Thymus weight ↑ in pre-free swimming
[43] (Savage, 2023)	• CD4+ T, CD3+ or FoxP3 T reg cells in YUMMER • FoxP3 T cells in B16F10 • CD8+ T cell no. or presentation of CD69 activation marker and PD1 expression in B16F10 • Efficacy of anti-PD1 treatment in YUMMER	•CD3+ and CD4+ T cells no in B16F10	•VCAM1 expression in tumour vessels, but unchanged in normal vessels indicating exercise differentially affecting cancer/normal tissue •CD8+ T cell abundance and no. presenting CD69 activation marker and PD1 expression in YUMMER • CD8+ T cell mobilisation to tumour	•Exercise sig. ↓ tumour size in YUMMER but not B16F10 tumours •Exercise significantly improved vasculature in both tumour models •Exercise ↓ hypoxia in YUMMER cells only.

Quality assessment

The Covidence quality assessment template was modified as it was clear at the point of extraction all included articles were conducted in animals. Therefore, the quality assessment needed to be appropriate and relevant for this type of study. The Covidence quality assessment template was modified using animal study-specific criteria [28]. An explanation of the criteria and assessment strategy are detailed in Table 2 using the descriptors of 'high', 'low', or 'unsure'.

RESULTS

One article was identified and added to the Covidence platform through manual searching [29]. This article was deemed to be appropriate and suitable for inclusion in this systematic review. In total, 10 articles were extracted. All articles had a focus on melanoma, and no studies investigated the immune-modulating effects of exercise in animals with any other skin cancer types. Despite this investigation being open to both human and animal studies, only animal studies met the specified inclusion criteria.

General information on each study is presented in Table

Table 2: Quality assessment characteristics and descriptions used in assessing all studies

Quality assessment characteristic	Description
Treatment allocation/randomization	Did this study describe how humans/mice were randomized to treatment type? If so were treatments allocated as the method indicated? High= the study detailed that treatment allocation was randomized
Blinding of personnel	Did the researchers/ assistance know which mice/humans were on certain treatments? High= high quality and adequately blinded
Sample size	Does the paper clearly indicate the sample size, and was the sample size adequate? High=article clearly indicated what the resulting sample size was, and the sample size appeared to be adequate
Explanation of statistical analysis/what models were used	Did the paper indicate what statistical models were used/ why they were used? High= high quality explanation/explanation was present
Whether all animals were accounted for/ incomplete outcomes	Were all animals accounted for throughout the study and at the conclusion of the study/ in the results? High= yes all animals were accounted for.
Descriptions of animals used	Study had clear descriptive of animals e.g. mouse type, age. High= Detailed descriptions of animals were included
Statement of compliance with any regulatory requirements	Have the authors clearly stated that the study was conducted in compliance with any regulatory requirements High=yes, article clearly stated the study was conducted in accordance with regulatory requirements, and referred to any codes/ registrations
Conflicts of interest reported	All conflicts of interest were acknowledged and reported High=yes
Other sources of bias	Were other sources of bias acknowledged within the article/ discussion? High=yes
Selective reporting	Does the article discuss all results or only pay attention to one aspect of the study? High=no selective reporting

Studies were marked as 'unsure 'when the article was ambiguous, or when some elements were included but did not meet include the entire criteria to be assessed as 'high' or 'low'.

Table 3: Simplified table of extracted article information and study characteristics

Title	Study design	Population description and cancer type	N=	Intervention and Comparisons/ groups and controls:	Exercise type And other environment conditions	Immune cells analysed	Measures
[29] (Lee, 2019)	Randomised controlled trial	C57BL/6 (B6) mice, 7 weeks old B16F10 murine melanoma cells	n=27 (n=9 mice per group)	Housed/control: no exercise TT: Thermoneutral temperature 29°C BT: Body temperature 36°C	Swimming in either 29°C water OR 36°C for 30 minutes, 6 days a week., for 3 weeks.	NK cells, γδT cells, NKT cells, and cytotoxic CD8+ T cells	Rectal body temp. Tumour volume Conc. of immune cells Detection of soluble γc receptor
[35] (Pedersen, 2016)	Randomised controlled trial	C57BL/6 mice, female mice. 3 months old and 18 month old. Tg(Grm1)EPv transgenic male mice B16F10 murine melanoma cells	Unclear but n ≈11 for the separate groups in each study	PBS: sedentary EX: control exercising aPD-L1: aPD-L1 sedentary aPD- L1 þ EX: aPD-L1 treated exercising	Voluntary wheel running for 4-6 weeks. Average distance per mouse was 4.1km/day.	NK cells, T cells, cytokines: IL1 alpha, iNOS, dendritic cells, NKT cells gamma delta T cells, B cells	Tumour volume Lung tumours (mets.) Microarray analysis on B16 tumours Immune cell frequencies NK cell cytotoxicity
[55] (Xia, 2020)	Bio-informatics study	Data from Pedersen 2016	Data from Pedersen 2016	Data from Pedersen 2016	Data from Pedersen 2016	Genes associated with immune function: C3, Fgg (T-cell associated), Pf4 (T cell function), Orm1 (associated with decreased immune function)	Identifying hub genes Signalling pathways/ pathway analysis Gene expression levels Identification of DEG's Functional enrichment of DEG's (to investigate their biological functioning)
[60] (Zhu, 2021)	Bioinformatics study	Data from Pedersen 2016	Data from Pedersen 2016	Data from Pedersen 2016	Data from Pedersen 2016	Genes associated with the functioning of: WDFY4 (CD8+ T cells, alpha beta T cell activation) ITGAM (macrophages). NK cells.	Measures/ type of analysis: Gene Ontology (GO) analysis. Pathway enrichment analysis Identification of DEG's

Title	Study design	Population description and cancer type	N=	Intervention and Comparisons/ groups and controls:	Exercise type And other environment conditions	Immune cells analysed	Measures
[42] (Momessod osSantos, 2019)	Randomised controlled trial	C57BL/6 mice, female 6 weeks old B16F10 murine melanoma cells	N=80	1) normolipidic (N) control 2) N + melanoma (NM) 3) high-fat (H) control 4) H + melanoma HM) 5) N control + physical exercise (NE) 6) N melanoma + physical exercise (NEM) 7) H + physical exercise (HE), and 8) H melanoma + physical exercise (HEM).	Moderate treadmill running 10 weeks, 3 times a week, 10 minutes each day Normolipidic diet: diet composed of 10% of energy from fat/lipids sources High fat diet: 60% energy from lipid fat sources.	T-helper 1 cells, M1 macrophages T-reg cells. Th-17 cells	Serum leptin levels Lymphocyte concentration Treg and Th17 cell counts. Cytokine production by stimulation of lymphocytes. •Lymphocyte proliferation. •Melanoma growth
[2] (Bay, 2020)	Randomised controlled trial	C57Bl/6NTac or NMRI-Foxn1nu mice, female, 8-16 weeks old Murine B16F10 melanoma cells	n=14 (however, 6 mice were sacrificed prematurely)	PBS: sedentary EX: control exercising aPD-L1: aPD-L1 sedentary aPD- L1 þ EX: aPD-L1 treated exercising	Voluntary running wheel exercise (5 weeks prior to melanoma inoculation) Half the participants were treated with aPD-L1	PD-L1, PBMC's	Tumour weight Tumour volume Spleen size Spleen volume
[3] (Buss, 2021)	Randomised controlled trial	C57BL/6 mice Female mice. Aged 6-10 weeks B16F10 murine melanoma cells	n=24 Some were prematurely euthanised due to ulceration of tumours (n=4)	Mice were randomly assigned to either the exercise OR no exercise group	Voluntary wheel running for 2-5 weeks (this was dependant on tumour size). Mice ran on average 8km/day	NK cells, T cells: Treg, cytotoxic T cells	Tumour volume Conc. of intratumoural T cells, NK cells and infiltrating NK cells. Spatial infiltration of T cells within tumour

Title	Study design	Population description and cancer type	N=	Intervention and Comparisons/ groups and controls:	Exercise type And other environment conditions	Immune cells analysed	Measures
[4] (Cao, 2010)	Randomised controlled trial	C57BL/6 mice, 3 week old. Males B16 melanoma cells	n=18-20	Control/grouped housing, EE (enriched environment), grouped housing, and a sub study with voluntary running mice only	EE had free access to running wheel and other stimuli. Running mice had free access to running wheel . EE group ran an average of 0.64km/day and running mice ran 2km average per day	Splenic lymphocytes NK cells CD 8 T cell IGF-1	Tumour size Tumour volume Cellular proliferation Apoptosis.
[14] (Fei, 2020)	Randomised controlled trial	C57BL/6 mice Male mice. Aged 6-8 weeks B16F10 murine melanoma cells	n=50	Free swim (FS) Exhausted swim (ES) T con: 4 wks rest, melanoma injection, after 2 wks. F group: FS 4 wks, melanoma injection, FS 2 wks, analysed. E group: 4 wks ES, melanoma injection, 2 wks ES, analysed. F pre: 4 wks FS, melanoma injection, 2 wks rest, analysed E pre: 4 wks ES, melanoma injection, 2 wks rest, analysed.	6 week intervention. Free swimming, without load and exhausted swimming, mice are load-bearing.	T lymphocytes, NK cells	Tumour weight Thymus and spleen weight Proliferation of splenic T lymphocytes NK cell tumour infiltration
[43] (Savage, 2023)	Randomised controlled trial	C57Bl/6 WT male mice, 8-12 weeks old for most of the study (mixed gender for ERK5 S496A knock-out study) YUMMER 1.7 and B16F10 murine melanoma cells	N=52?	Control: sedentary mice Exercised mice with YUMMER cells (inoculated 5-6 days prior to exercise) Exercised mice with B16F10 cells (inoculated 7 days prior to exercise)	Aerobic exercise (treadmill exercise) 45 minutes per/day, 12-14 consecutive days	CD8+ T, CD3+ T cells, CD4+ T cells and FoxP3+ regulatory T cells, myeloid-derived suppressor cells (MDSC), myeloid cells (TAM1, TAM2, and Mono DCs), and lymphocytes (NK, CD4T, CD8, and Cycling CD8T	Tumor vessel hyperpermeability and perfusion assays, VCAM1 expression in tumor vasculature

Table 4: Assessment of study quality

Study ID	Treatment allocation	Blinding of personnel	Sample size	Explanation of statistical analysis/ models used	All animals accounted for/ incomplete outcomes	Details of animals used	Statement of regulatory compliance	Conflicts of interest reported	Other sources of bias	Selective reporting	Overall quality
[29] Lee 2019	Unsure	Unsure	Low	Low	High	High	High	High	Unsure	High	Moderate
[35] Pedersen 2016	High	Unsure	Low	High	Low	High	High	High	Unsure	High	High
[60] Zhu 2021	High	Low	High	High	Unsure	Low	Low	High	Unsure	High	Moderate
[55] Xia 2020	High	Unsure	High	High	High	High	Low	Low	High	High	High
[42] MomessodosSantos 2019	Low	Unsure	Unsure	High	High	High	High	High	Unsure	High	High
[2] Bay 2020	High	Unsure	Low	High	Low	High	High	High	Unsure	High	High
[3] Buss 2021	High	Unsure	High	High	High	High	High	Low	Unsure	High	High
[4] Cao 2010	High	Unsure	Low	Low	Low	High	High	Unsure	Unsure	High	Low
[14] Fei 2020	High	Unsure	High	Low	High	High	High	High	Unsure	High	High
[43] Savage, 2023	Low	Low	Low	High	Low	High	High	High	High	High	High

3. All but two studies employed a randomised controlled trial design, with the remaining two studies evaluating omics, mostly genomics data from animal-based studies. Most studies used the same cancer type - B16/B16F10 murine melanoma cells to inoculate mice, but one study used another murine melanoma cancer cell line, YUMMER 1.7, which produced markedly different results [43]. Most studies used the same murine model - C57BL/6 mice - with the exception of one study [35]. However, there was apparent variation in mouse characteristics, including gender and age, which fluctuated between the studies.

The studies were of moderate to high quality (Table 4), but there were categories in which most studies scored either 'low' or 'unsure'. These categories were blinding of personnel and a discussion or statement explaining any other sources of bias. Overall article quality was determined by counting the number of 'high' scores received compared to number of 'low' or 'unsure' scores. If the number of 'high' scores, were less than the number of 'low'/'unsure' then the article was deemed as a low-quality article. If they were equal, then the article was of moderate quality and if there were more 'high' scores then the articles was rated as high quality.

Substantial heterogeneity in experimental design and selection of immune measures between the studies was identified, so a meta-analysis was deemed not appropriate. Moreover, an array of exercise prescription and types of exercise employed in the studies for the mice was found, varying from wheel running, treadmill running to swimming. Nevertheless, a key experimental aspect these studies shared was they all examples evaluated the effects of aerobic exercise.

While the effect of exercise on T cells was a priority, many of the included studies also investigated the effect of exercise on natural killer (NK) cells. We decided to include NK cells as they share the same haematopoietic lineage/ common lymphoid progenitor as T cells. Additionally, one study [43] explored myeloid cells. This information was beyond the scope of this review, so the myeloid-specific results were not reported.

Describing the effect of exercise was investigated on T cell subsets and NK cells provided additional context to disease outcome measures. CD4+ T cells, FoxP3+Treg cells and CD8+ T cells were the most notable T cell subsets identified. Mixed results were observed in the effect of exercise on CD4+ T cells, where one 3-week swimming study observed no change [29]. Another shorter 12-14 day treadmill intervention observed a decrease in CD 4+ T cell numbers [43]. However, the longest of all studies that recorded CD4+ T cell changes observed an increase in their numbers in their 4-6 week wheel running intervention [35].

FoxP3 Treg cell results were also inconclusive and only explored in two studies; one study observed a non-significant increase in FoxP3 T cell numbers in their exercise and anti-PD1 treatment group [3]. The same study also observed that FoxP3+CD3+T cell percentage was increased in immune cell 'hot-spots' within tumours [3]. The second study found no change in FoxP3+T cell numbers in either their YUMMER or B16F10 melanoma model [43]. These studies were 2-5 weeks and 12-14 days in length respectively [3, 43].

CD8+T cell results number and function were investigated in more studies. Of the five studies that documented change in CD8 T cells, 4 out of 5 studies reported an increase in their number, abundance, function [4, 29, 35, 43]. The only caveat was that one of these studies completed the same experiments in another melanoma model (B16F10, as opposed to YUMMER) and reported no change in CD8+ T cells numbers nor presentation of CD69 activation marker and PD1 expression in the B16F10 model [43]. The final opposing study reported a decrease in the percentage of CD3+CD8+T cell population in the exercise and anti-PD-1 treatment group[3].

A more conclusive effect was also observed in NK cells, potentially due to the larger number of studies exploring them. A majority of studies (4/5) studies that documented NK cells reported that exercise increased the number, frequency, cytotoxicity and infiltration of cells into tumours [4, 14, 29, 35]. Exercise appeared to have the greatest effect on NK cell number, function, and mobilisation to tumours, to potentially elicit the observed disease outcomes such as reduced tumour burden. This effect was evident in athymic mice, where a significant reduction in tumour burden was still achieved through exercise, mediated by NK cells [35]. The one study that opposed these results reported no change in NK infiltration in tumours, but observed an increase in NK numbers in the exercise and anti-PD-1 treatment group [3].

Many studies tested various factors, varying from diet to temperature, and synergism between other treatments, while other treatments, yielded a wide variety of changes in immune parameters and animal well-being. Nevertheless, exercise generally improved cancer outcomes. Diet was explored in a moderate intensity treadmill intervention. The diet comprised of 60% of the dietary energy sourced from lipids, and the prescribed exercise regimen was 10 minutes per day, 3 time a week over 10 weeks. Mice with melanoma on a high fat diet had tumour volumes of approximately 2000mm3 compared to 800mm3 for mice on the same melanoma on a high fat diet [42].

Several studies investigated the importance and effectiveness of exercise before melanoma development, and four studies investigated exercise in combination with other factors such as PD-1 immunotherapy [2, 3, 43], mental stimulation [4], and ambient temperature [29]. Three studies investigated the effect of PD-1 treatment in combination with exercise. Studies came to the same conclusion that exercise and PD-1 immunotherapy worked well independently of each other, but not synergistically [2, 3, 43]. A modest increase in CD8+T cell abundance [43] was not observed in the other studies [2, 3]. The incongruence in outcomes may relate to starting the immunotherapy at different times during the exercise intervention. However, these studies indicate that exercise and anti-PD1 therapy in a murine melanoma model done concurrently may not be synergistic, and other exercise/ immunotherapy regimens may elicit different results.

In contrast, the combination of mental stimulation and exercise appeared to have a synergistic effect in an animal model. The effect may have been mediated through improvements in CD8+ T cell and NK cell function [4]. Ambient temperature in which the mice swam was also identified as an important factor

in immune function and skin cancer. Swimming in thermoneutral water (29°C) or colder water, compared to body temperature water (36°C), increased the number of effector CD8+ T cells, and improved their function with a 1.5-fold increase in CD8+ T cells producing IFNy compared to other groups [29]. Temperature may be a factor that works synergistically with exercise to modulate immune cell frequencies and function to reduce tumour burden in melanoma [29].

Two omics studies were also identified [55, 60]that used the same main data set [35]. One of the studies also used data from The Cancer Genome Atlas to investigate whether their findings from animal data had biological relevance to human melanoma [60]. Despite using the same main dataset, the studies reported different outcomes; the earlier study investigated biological functioning, and the functional enrichment of differentially expressed genes (DEG's) to identify 3 main groups: molecular function , cellular component and biological process [55]. The biological processes group exhibited enriched immune and inflammatory responses, and genes in this category were specific to immune system function, inflammation, and immune response. The molecular function group was prominently enriched in chemokine and cytokine activity.10 top nodes/ hub genes were reported, C3, Fgg, Pf4, and Orm1 were immune-related genes, and generally are associated with decreased immune function (Xia et al., 2020). Conversely the later study identified six different hub genes, with two specific to immune/ T cell function, Wdfy4 (CD8+ T cells, alpha-beta T cell activation) and Itgam (macrophages). The exercise-related genes identified from the animal data were dysfunctional in human melanoma samples, and only weakly associated with disease-free and overall survival [60]. Wdfy4 had decreased expression in melanoma stages 1-4, but did not differ depending on the stage of the disease. Decreased expression of Wdfy4 was not seen in normal tissue [60]. Itgam expression was also decreased in melanoma, stages 1-4, and differed depending on the stage, showing that stage 2 was the most decreased. Moreover, Itgam was not observed to be downregulated in normal tissue [60].

In general, exercise reduced the number of metastases/ tumour burden and delayed carcinogenesis, through different immunological mechanisms. The improved outcomes may have been mediated by the identified exercise-induced T-cell and NK cell modulations. However, results were magnified with increased exercise intensity [14] and in a continuous exercise regimen commenced 3-6 weeks prior to melanoma inoculation. Starting exercise prior to melanoma inoculation delayed carcinogenesis [4] and reduced tumour burden [14, 35]. This effect was mediated by improving the function of CD8+T cells [4] and NK cells. NK cell function was improved characterised by increased cytotoxicity [4] and their ability to mobilise and infiltrate tumours [14, 35]. Collectively these responses would likely to prepare and bolster the immune system against melanoma carcinogenesis [4, 14, 35].

DISCUSSION

The studies evaluated in this review were limited to murine models of melanoma skin cancer. Studies were relatively heterogenous in design, methods and immune measures, as different types of exercise were investigated. Synergism was also investigated various studies involving PD-1 immunotherapy [2, 3, 43], mental stimulation [4], and/or temperature [29]. Overall, it appears that exercise increased immune cell numbers in a melanoma murine model, in particular T cells and NK cells. Exercise was shown to improve immune cellular function with the effects magnified when exercise was started prior to melanoma inoculation.

When exercise was started 3-6 weeks prior to melanoma inoculation, CD8+T cell [4] and NK cell function was improved [4]. While other immune cell changes were within systemic circulation and may potentiate anti-cancer effects and tumour infiltrating potential downstream, cells CD 8+ T cells and in particular NK cells, were observed to migrate and infiltrate tumours. This sequence of events exemplifies a tumour-specific targeted response to exercise [14, 35, 43]. Longer adherence to these exercise regimens, or exercising prior to development of a melanoma appeared to elicit better clinical outcomes in experimental settings. The results are congruent with recommendations made by the Centre for Disease Control and Prevention (CDC) and the World Health Organisation (WHO), who recommend exercise for the prevention of cancer [7, 13]. Exercise may 'prime' the immune system, improving immune function and allowing the immune system and its surveillance capabilities to better combat melanoma development [4, 35].

Unlike the recommendations made by WHO and CDC, these studies did not incorporate or investigate anaerobic/strength training type exercises. All studies were representations of aerobic exercise primarily treadmill, wheel running and swimming. Most authors did not investigate whether aerobic exercise had any effective, causative or even direct or indirect association (or causation) with the outcome that may lead to further discussion or future investigations. The exception was in a study of soleus muscle citrate synthase activity, which identified that moderate aerobic exercise increased citrate synthase activity and mitochondrial activity [42]. Taken together these results indicate that moderate physical activity is a positive stressor on cells/ cellular function, preserves mitochondrial function and integrity, and combats a range of dysfunctions that may lead to cancer development. However, to contrast the effects of aerobic exercise with strength (resistance) training, or high intensity interval training, would provide a better understanding of how the type of exercise affects outcomes. Comparative studies would also improve mechanistic knowledge, as they may highlight cellular or molecular pathways that differ between exercise types and may help identify a causative effect. This approach would bring a deeper understanding of associations between exercise, immune function, disease progression and clinical outcomes, and downstream guide what types of exercise might be most beneficial in human participants. On the basis of the initial studies evaluated here that the duration of animal exercise studies that was most effective was 3-6 weeks, preferably begun prior to melanoma inoculation. The general consensus was that moderate intensity exercise was effective [42] but there were opposing opinions and results [14,55].

It appears that exercise may have had the greatest impact on CD8+ T cells and NK cells in murine melanoma models, by improving their function to potentially improve disease outcome measures. This outcome was not unique to this study; NK cells in particular respond well to both acute and chronic exercise interventions, and their functions are improved in the context of cancer [27, 46]. NK cells are a topic of interest in cancer therapy research, as improving NK cell function is understood to improve the effectiveness of immunotherapies [6, 46]. Therefore, an intervention or activity to improve their function, like exercise, would be an ideal adjuvant therapy, potentially for use in locally advanced or metastatic KC's.

The effect of exercise on T cells have been widely studied in the context of healthy adults, showing phenotypic T-cell changes, resulting in longer-lasting improved outcomes associated with immunosenescence and functional changes resulting in increased cytotoxicity [46, 51, 53]. More recently, the effects of exercise on CD8+T cells in the context of cancer are now being explored, highlighting that exercise plays an immunomodulatory role towards CD8+T cells to enhance disease outcomes [17, 27].

The results for CD4+ T cells and FoxP3+T cells evaluated in animal studies, were uncertain, possibly a consequence of disparities in the duration of exercise interventions. FoxP3 T cell results were inconclusive in interventions that were comparatively shorter, and a longer intervention may uncover the effect of exercise on Fox3+ T cells in melanoma models. A similar discrepancy was seen with the results of the CD4+ T cells. No clear outcome could be concluded as the results were highly varied with exercise intervention durations ranging from 3-6 weeks.

Another point of methodological variation between studies was the use of different murine melanoma models. In the slowly developing mouse model Tg(Grm1)Epv, exercise was equally effective in reducing tumour burden regardless whether commenced before or after melanoma inoculation [35]. Furthermore, exercise differentially effected the outcome in YUMMER 1.7 murine melanoma model, which exhibited an increase in CD8+ T cells presenting with their activation marker CD69 - these cells also expressed PD1. The increase in CD8+T cell abundance relates to mobilisation through the increased Vascular Cell Adhesion Molecule 1 expression, and decreased levels of tumour hypoxia seen only in the YUMMER model. These processes in turn increase the effectiveness of immune cell function and reduced tumour size in the YUMMER model, and not in the B16F10 cells [43]. Exercise may differentially impact various cancer types, such as the case for the slower growing Tg(Grm1)Epv, which may be likened to other slower growing skin cancers such as KCs. Acute exercise may promote immune function in other skin cancers underpinning its utility as a clinical/lifestyle intervention for skin cancer management. These lifestyle factors and clinical interventions are important to investigate in both isolation and combination as they may be associated with developing a cancer, and more reflective of an accurate 'picture' of the behaviours seen in humans [42].

Diet also appears to play in role in cancer management, in particular the effects of a high-fat diet on melanoma in mice who exercise [42]. Leptin may increase pro-inflammatory and potentially pro-carcinogenic factors in T-helper 1 cells and M1 macrophages. Reduced serum leptin levels, and decreased production of pro-inflammatory cytokines, are associated with chronic sustained low-grade inflammation that accompanies both obesity and carcinogenesis [42]. Several studies investigating the relationship between diet and skin cancer in humans have been conducted [41, 47]. It would be beneficial to extend this knowledge as this high-fat content study has done and identify how exercise and nutrition are best managed in clinical and community settings. Studies also investigated synergism of therapeutic and lifestyle approaches in skin cancer management in a murine model. Whether exercise and anti-PD1 therapy is synergistic or not in cSCC is unclear, as cSCC has an even better response rate to immunotherapies than melanoma [56]. Further studies investigating synergism between exercise and immunotherapies are indicated as improving the response rate and reducing drug tolerance/resistance is a common goal in immunotherapy research [1]. Improving the clinical and mechanistic understanding of immune function is required to make advancements in immunotherapy.

Mental stimulation used in combination with exercise was also investigated. The objective of investigating mental stimulation or cerebral health in relation to its synergism with exercise in combating cancer development was to investigate how the macroenvironment interacts with cancer development [4]. Only a limited number of studies in humans that test the effects of both cognitive training and exercise in cancer patients. Exercise in many cancer types improves cognition and other mental symptoms such as depression and brain fog [26, 39]. A single study has investigated whether there was a synergistic effect between cognitive and physical training in humans [37]. This study found no synergistic effect, and did not investigate whether there was any modulation in underlying immunological mechanisms, cells or function. This is a promising area to expand research efforts.

Swimming water temperature was another identified potential synergistic factor with exercise. Swimming in different temperatures is an already established and utilised rehabilitation strategy, but there is limited understanding its effects on the immune system. Identifying temperature-induced modifications that enhance effectiveness of immune regulation may be of great benefit. Cold swimming water improved CD8+ T cell number and function to reduce tumour burden [14]. However, these outcomes contrasts with the area of hyperthermia medicine; stemming from Dr. William Coley's observation made over 100 years ago, of patients with cancers who experienced high fevers were more likely to experience remission [40]. Heat or fevers may enhance immune function to improve clinical outcomes associated with cancer through changing the tumour microenvironment by a multitude of mechanisms; heat increases CD8+ T cell trafficking into immune organs and to the tumour, and can improve macrophage and dendritic cell function and NK cell cytotoxicity [12, 40]. Exercising temperature is a factor that should be investigated further, as this could improve clinical outcomes in combination with skin cancer immunotherapy.

While the results identified in this review were based in murine melanoma models, genomic sequencing/ omics studies take a whole genome approach that allows investigators to

expand research efforts into areas which warrant further study, especially when coupled with human data. One of these omics studies used The Cancer Genome Atlas to identify prognostic genes relevant to human melanoma [60]. The study utilised this resource and additional data, to contextualise the results for the human health 'picture'. However, the value of animal models should not be discounted, as they are the foundation of the drug discovery and development pipeline, and lead to solutions relevant to human health. Outcomes of animal studies provide a mechanistic framework for evaluating the effects of exercise on skin cancer in humans as a potential lifestyle intervention in a broader cancer management strategy. A combination of experimental studies in both human and animal models coupled with omics studies utilising human data is recommended. Further studies of how T cells, NK cells, and other immune parameters are implicated in the management of keratinocyte cancer, using exercise alone or in combination with therapeutic approaches, are warranted.

CONCLUSION

We identified multiple potential multiple immunomodulatory mechanisms of aerobic exercise specific to T and NK cells in relation for improving disease outcomes in murine models. CD 8+ T cells and NK cells can directly migrate to and infiltrate tumours to reduce tumour burden, particularly when exercise was begun prior to melanoma inoculation and continued for3-6 weeks Other factors that improved clinical outcomes and worked synergistically with exercise were lower swimming water temperature and increased mental stimulation. More foundational animal models and bioinformatics studies will inform the planning and execution of future mechanistic or bio-informatics research utilising human data. Studies are needed to determine whether the results from animal studies are observed in humans in relation to both keratinocyte cancer and melanoma. In conjunction with human interventions, these results have the potential to uncover the value of exercise as an adjuvant treatment for humans with skin cancer, a cancer with a high prevalence and burden in the community.

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Unleashing anti-tumour immunity: dietary restriction and exercise interventions adjunct to chemotherapy for cancer patients

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ABSTRACT

Conventional chemotherapies can stimulate the immune system by increasing tumour antigenicity (e.g., neoantigen exposure to immune cells) and altering adjuvanticity in the tumour (e.g., danger associated molecular patterns and cytokines). These molecules promote the recruitment, activation, and maturation of dendritic cells, which in turn, prime and activate cytotoxic T cells against tumour cells. However, several factors can decrease the immunostimulatory efficacy of chemotherapeutic agents. These include reduced tumour cell antigenicity and adjuvanticity and compromised immune function at a local and systemic level. Findings from preclinical studies show that dietary restriction and exercise promote systemic changes that may help to restore immune system function through several mechanisms, including an enhanced infiltration and function of antitumoral immune cells and a decrease in immunosuppressive cells, leading to a reduction in tumour volume. In addition, dietary restriction and exercise training in mice have been shown to enhance the efficacy of chemotherapy. In human studies there is also emerging evidence that dietary restriction and exercise can impact the immune system towards a more antitumoral profile. In this review, we discuss the immunostimulatory effects of dietary restriction (caloric restriction and fasting) and exercise training in preclinical cancer models, and potential synergies with chemotherapy. We then review clinical studies assessing the effects of these interventions on immune-related endpoints and tumour responses. Finally, we propose that combining dietary restriction with exercise could be a promising strategy to increase chemotherapy efficacy.

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INTRODUCTION

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 alone (189). Treatment often includes combinations of surgery, radiotherapy, and/or systemic therapy (chemotherapy, hormonal treatments, targeted biological therapies, immunotherapy) (189). Chemotherapy is a systemic cancer treatment that often targets cancer cells during division, leading to cell death and tumour shrinkage (190). It can be given as a definitive primary treatment to destroy all tumour cells, or as neoadjuvant or adjuvant therapy administered prior to or after locoregional treatments to increase their effectiveness. If cure is not possible, chemotherapy may be used as a palliative treatment to relieve disease symptoms or to temporarily arrest disease progression (190).

It is widely established that anti-tumour immune responses contribute to the success of chemotherapy agents (61, 185). Supporting this concept, there is a link between tumour lymphocytic infiltrates and improved patient prognosis (58, 144) and with higher responses to neoadjuvant chemotherapy in breast cancer (42, 85). In mouse models, the efficacy of conventional chemotherapies is much higher in immunocompetent animals than their immunodeficient counterparts (61, 95, 105, 185). This provides the rationale for the combination of chemotherapy and immune checkpoint inhibitor-based immunotherapy in cancer treatment, as certain chemotherapeutics are hypothesized to convert "cold" tumours into "hot" tumours and thereby sensitize cancers to immune checkpoint inhibitors. Consequently, this combination is currently used in patients with certain types of cancer (61, 95). Nevertheless, in some cases, combining chemotherapy and immunotherapy may increase treatment side effects such as acute kidney injury (65). An alternative approach to enhance the efficacy of chemotherapeutics could be the addition of dietary manipulation (e.g. caloric restriction, fasting or fasting mimicking diets) and exercise programs, as these interventions have been demonstrated to independently improve several aspects of immune function and to suppress tumour growth in preclinical studies (40, 93, 94, 120), as well as improve patient-related outcomes in clinical studies, with few or no side effects (30, 102, 175). Our focus in this review is to present the scientific premise by which dietary restriction and exercise have the potential to restore and potentiate antitumoral

immunological responses in the setting of chemotherapy. To this end, we will first highlight the importance of the host immune system to support chemotherapy efficacy. We will then review preclinical and human studies reporting how dietary restriction and exercise interventions exert immune mediated antitumoral effects, modulate the microbiome and synergize with chemotherapy. Finally, we will review studies combining dietary restriction with exercise, and summarise how these combinations may augment antitumoral-immunity and thus culminate in enhanced clinical responses to chemotherapy.

IMPORTANCE OF THE IMMUNE SYSTEM FOR CHEMOTHERAPY SUCCESS

Chemotherapeutic agents can promote the activation of an antitumoral immune response by inducing crosstalk between tumour cells and elements of the immune system through cellular stress-related processes termed immunogenic cell death (ICD) and immunogenic modulation (53, 61, 95). Chemotherapy-driven ICD is mediated by two processes that determine the immunogenicity of the tumour cells: antigenicity and adjuvanticity (53, 61). Antigenicity is the presentation of antigens via MHC-I on tumour cells; these antigens can be expressed only by tumour cells, and are termed tumour-specific antigens or neoantigens, or they can be overexpressed in tumour cells but also expressed on healthy cells and are termed tumour-associated antigens (53, 166). Chemotherapy-induced cell stress promotes the expression and presentation of tumour-specific and tumour-associated antigens and MHC-I on tumour cells (61, 80, 95, 106). Adjuvanticity refers to the cell surface exposure and release of immunostimulatory molecules from dying tumour cells named danger associated molecular patterns (DAMPs), of which the best studied are calreticulin, ATP, high mobility group box 1 and type-I IFNs (53, 61, 95). Cognate receptor binding of DAMPs on antigen-presenting cells such as dendritic cells, promotes their recruitment and phagocytosis of antigens released by dead tumour cells, which leads to their activation and maturation (53, 61, 95). Dendritic cells can cross-present the exogenous engulfed tumour antigens onto MHC-I molecules to prime naive CD8 T cells in the lymph nodes and provide co-stimulatory molecules and cytokines for full T cell activation, hence stimulating an adaptive anticancer response (61, 95) . Moreover, dendritic cells also present antigens on MHC-II molecules to CD4+T cells and induce their activation and differentiation into different subsets (104). Although they are usually less considered in the context of antitumoral immunity, CD4+ T cells have important antitumoral roles, including the provision of help to CD8+ T cells, the support for antigen-presenting cells, and the activation of B cells for the secretion of antibodies against tumour antigens (8, 158). Importantly, tumour cells are also able to express MHC-II molecules, and CD4+ T cells can have direct cytotoxic activity against tumour cells under certain conditions by recognizing the antigens presented in these MHC-II molecules (8, 158).

Chemotherapy can also render cancer cells more susceptible to immune-mediated killing without inducing classic ICD through a process called immunogenic modulation (53). Immunogenic modulation can occur when chemotherapy-induced cell damage does not result in cell death but there are alterations in the biology of tumour cells that make them more susceptible to killing by innate and adaptive immune effectors (53). These alterations include changes in surface marker expression that sensitizes tumour cells to CD8+ T cells and NK cells killing, and downregulation of anti-apoptotic and/or pro-survival genes (53, 67).

Chemotherapy efficacy can be lost or reduced in several ways (61). First, antigenicity of tumour cells may be reduced by losing tumour antigens through selection pressures that facilitate the expansion of cell clones that do not express antigens recognized by the immune system (61, 63), or by downregulation of MHC-I (61, 166, 186). Second, tumours can lose adjuvanticity due to alterations in the secretion or exposure of DAMPs or activation of mechanisms that antagonize DAMPs (61, 186). Finally, the efficacy of chemotherapy can be reduced by any microenvironmental or systemic immune defect (61, 185). Microenvironmental immune defects include low infiltration of immune effectors into the tumour microenvironment (TME) (the heterogenous milieu of molecules, blood vessels, and tumour, immune and stromal cells), exhaustion or anergy of CD8+ T cells or suppression of their activity by tumour cells, the infiltration of immunosuppressive cells (cells that inhibit the antitumoral response, promoting tumour immune escape) into the TME such as regulatory T cells (Tregs), M2-subtype macrophages, N2-subtype neutrophils, and myeloid-derived suppressor cells (MDSCs), or by immunosuppressive environmental conditions such as hypoxia and low pH (61, 100, 166, 185). At the systemic level, immunosuppressive alterations of the gut microbiota may also compromise the efficacy of chemotherapy (61, 71). There is growing evidence regarding the interplay between the gut microbiota and host immune system (84, 182), which can impact cancer immune surveillance (182, 184). Of note, the use of antibiotics in mice or using germ-free mice has been shown to reduce immune responses and the efficacy of different ICD inducers and immunotherapy (84). Therefore, improving the immunological competence of the patient and the tumour microenvironment is of paramount importance to increase chemotherapeutic efficacy.

EFFECTS OF DIETARY RESTRICTIONS

Dietary restrictions include caloric restriction and fasting regimens. Caloric restriction is defined as a reduction in total daily energy intake by 15-30%, without changing the macronutrient ratio (159). Fasting includes various eating regimens: water-only fasting or a restriction of over 50% of the usual calorie intake, lasting between 24 hours and several days; time-restricted feeding where eating is limited to a 6-to-12-hour window; and fasting-mimicking diets (FMD) where eating is limited to low calorie, low protein, low sugar, plant-based foods (19, 165). When caloric intake is reduced through dietary restriction starvation is induced which leads to systemic changes in the levels of circulating hormones and metabolites,

including low levels of glucose, insulin, insulin-like growth factor 1 (IGF-1) and leptin, high levels of glucagon, ketone bodies and adiponectin. These changes result in an increase in glycogenolysis, lipolysis, hepatic gluconeogenesis, and protein catabolism and a decrease in muscle uptake of glucose (41, 120). At the cellular level, these changes modulate key molecular cascades to counteract the metabolic stress (41, 120). In the context of cancer, caloric restriction and fasting regimens have been shown to have beneficial effects on tumor incidence, progression, metastasis, and survival in various animal models with normal weight (13, 44, 60, 101, 120, 178). Moreover, population-based studies in humans indicate that caloric restriction may reduce cancer incidence and cancer mortality rates (26, 81). Such anticancer effects are thought to be mediated through a reduction of circulating glucose, insulin, and IGF-1, which affects the ability of malignant cells to survive or adapt and induce an alteration of the systemic immune state to promote an antitumoral immune response (40, 41, 120, 124, 159).

Immune mediated antitumoral effects of dietary restriction

Caloric restriction and fasting regimens have been shown to influence the immune system (41, 120, 132) and synergize with immunotherapy in preclinical models (2), confirming these immunomodulatory effects. Caloric restriction and fasting regimens may boost anti-tumour immune responses by improving antitumour immunity effectors and decreasing local immunosuppression (40). Moreover, these interventions have beneficial effects on the gut microbiome, which may in turn promote a more appropriately responsive immune system (132).

I. Improved antitumoral immunity.

Evidence for a beneficial effect of caloric restriction or fasting regimens on the regeneration of progenitor immune cells and maintenance of memory T cell subsets with an enhanced antitumoral function has been obtained from preclinical studies. The reduction of IGF-1 and protein kinase A activity induced by several cycles of short-term fasting in mice led to signal transduction changes in long-term hematopoietic stem cells, increasing hematopoietic stem cell protection, self-renewal, and regeneration (28). Importantly, several cycles of short-term fasting also diminished immunosuppression and mortality caused by chemotherapy (28). Similarly, an FMD increased the number of CD8+ circulating lymphocytes by a third and the number of common lymphoid progenitor cells by two-fold in the bone marrow (44). Short-term fasting (155) and caloric restriction (34) have also been reported to increase the number of naïve CD4+ and CD8+ T cells (155) and memory CD4+ and CD8+ T cells (34) in the bone marrow of mice, due to an increased migration from secondary lymphoid organs and blood, which is thought to aid survival and maintain T cell functionality under unfavourable conditions such as during nutritional restriction (34, 155). Importantly, the caloric restriction-induced memory T cell homing to the bone marrow was associated with an increased antitumoral effect against melanoma tumours in vivo (34) (Table 1). The level of glycolytic activity in T cells also influences the maintenance of memory CD8+ T cells, the differentiation of effector CD8 T cells is impacted (153). Indeed, effector T cells rely on glycolysis rather than oxidative phosphorylation to facilitate faster proliferation (110). Consequently, effector T cells may have difficulty competing with tumour cells for glucose under fasting conditions (40). However, glycolysis may reduce longevity of T cells (91, 153). Inducing a high glycolytic activity in CD8+ T cells compromises the generation of long-lived memory cells and, conversely, limiting glycolysis with the glucose analogue 2-deoxyglucose in CD8+ T cells ex vivo triggers the activation of starvation signalling, favouring memory versus effector differentiation (153). Notably, when CD8+ T cells primed in the presence of 2-deoxyglucose were adoptively transferred into mice bearing melanoma tumours, they had higher antitumor capacity compared to T cells cultured without 2-deoxyglucose, prolonging survival in mice (153). Therefore, limiting glucose availability through caloric restriction or fasting regimens may promote similar effects. In a recent preclinical study, short-term fasting of 48 hours in mice bearing lung tumors improved the efficacy of anti-PD1 and anti-PD-L1 treatments, increasing survival (2). Importantly, 50% of the mice treated with anti PD-1 therapy and short-term fasting had a complete response, and were resistant to a tumor rechallenge, implying that they had developed immune memory against this type of tumor (2). Furthermore, the combination treatment increased the tumor infiltration of NK and CD8+ T cells and decreased the number of Tregs, whereas depletion of CD8+ T cells abrogated the antitumor efficacy of short-term fasting and anti PD-1 treatment (2). The efficacy of the treatment was also dependent on the reduction of IGF-1 levels and autophagy in tumor cells induced by short-term fasting (2) (Table 1). Taken together, this demonstrates that fasting induced reduction of IGF-1 levels may help to restore the antitumoral immunity through a mechanism that involves increased autophagy in tumor cells, synergizing with anti PD-1/PD-L1 immunotherapy.

II. Decreased local immunosuppression.

It has been reported from preclinical studies that caloric restriction and fasting regimens reduce the number of immunosuppressive cells in the tumour microenvironment. Both a daily 20% caloric reduction compared to ad libitum mice and an FMD significantly reduced the infiltration of MDSCs within tumours and retarded tumour growth (133) (Table 1). Similarly, two short-term fasting periods of 48 hours in mice decreased the infiltration of MDSCs in the spleen, suppressing tumour growth as efficiently as chemotherapy (60) (Table 1). Alternate day water-only fasting has also been shown to reduce the number of M2-subtype macrophages through the induction of autophagy in tumour cells (154). Autophagy is a lysosome-mediated process by which cells eliminate damaged or unnecessary proteins and organelles and excised genomic fragments (81, 124) and is highly induced under fasting conditions (81, 130). Culturing colon tumour cells in fasting conditions increases autophagy and reduces the expression of CD73, an ecto-enzyme that converts extracellular AMP into immunosuppressive adenosine, hence reducing adenosine levels and decreasing the polarization of macrophages into the immunosuppressive subtype M2 (154). In vivo, short-term fasting leads to reduced levels of M2 macrophages and reduced tumour growth, demonstrating that fasting has antitumoral effects through autophagy-induced reduction in M2 macrophages (154) (Table 1). Autophagy-defective tumour cells upregulate ecto-ATPase CD39 that converts extracellular ATP into AMP and ADP, promoting the formation of adenosine and attracting immunosuppressive Tregs expressing adenosine receptors into the tumour bed (130, 131). Hydroxycitrate, a caloric restriction mimetic that induces autophagy and mimics the metabolic effects of fasting, caused Treg depletion in a Kras-

induced lung cancer model in mice and reduced tumour growth (131). However, in transgenic mice overexpressing CD39 in the tumours, this failed to reduce tumour growth, underscoring the importance of inducing autophagy in tumour cells for Treg depletion, which in turn improves immunosurveillance (Table 1).

Fasting-induced changes in tumour cell metabolism may also decrease the excess lactate they produce, and the consequent low pH, which are strong immunosuppressants (110) . Tumour cells are characterized by high glucose uptake and a high glycolytic rate rather than using oxidative phosphorylation, regardless of oxygen concentration, resulting in high lactate production, a phenomenon known as the "Warburg effect" (38, 40). Colon carcinoma cells cultured in conditions mimicking starvation (low glucose and low serum) for 48 h, showed a metabolic shift, down-regulating glycolysis while up-regulating oxidative phosphorylation, promoting an "anti-Warburg effect" and decreasing extracellular lactate concentration (15). In vivo, short-term fasting had a transient effect on tumour growth, slowing it during the fasting but not during the post-fasting period (15). Notably, short-term fasting had an additive effect when combined with oxaliplatin, demonstrating a higher reduction in tumour growth than oxaliplatin alone (15).

III. Modulation of the microbiota

Both caloric restriction and fasting regimens have been shown to modify the types and abundance of gut bacteria in preclinical models and in humans (92, 132, 135). In rodents, caloric restriction can increase the abundance of probiotic bacteria (e.g., Bifidobacterium and Lactobacillus spp.) (132). In human studies, time-restricted feeding, and short-term fasting regimens, such as Ramadan and Buchinger fasting, promote the enrichment of Faecalibacterium, which produces short-chain fatty acids (SCFAs) from dietary fibre (57) . SCFAs modulate the interaction between the gut microbiota and the immune system and induce a wide range of beneficial effects (132). Notably, the SCFA butyrate has been associated with cancer prevention and enhanced treatment efficacy (132). Moreover, caloric restriction reduces the ratio of Firmicutes/Bacteroidetes (184). These are two primary phyla in the gut, and a high ratio has been associated with various conditions, such as obesity and cardiovascular disease (157), and to a decreased response to different anti-cancer therapies (chemotherapy or a combination of chemo- and immunotherapy) (78). Ramadan and Buchinger fasting regimens have also been demonstrated to increase the abundance of *Akkermansia muciniphila* in humans (57, 151, 152), which has been linked to cardiometabolic health in mice (22). Interestingly, intravenous injection of *Akkermansia muciniphil*a-derived extracellular vesicles in immune-competent mice reduced the tumour growth of prostate cancer and increased the proportion of CD8+ T cells with an activated profile and the recruitment and polarization of macrophages into a M1 profile (107). In the context of cancer therapy, its presence is associated with a better response to anti PD-1 immunotherapy and increased infiltration of immune cells in the TME (78, 180). Therefore, it may well be that the antitumoral immune system may be enhanced by altering the gut microbiota through fasting. Nevertheless, correlation does not imply causality, and prospective studies should validate if these potentially beneficial shifts in the gut microbiome induced by dietary restriction cause an increased response to chemotherapy or immunotherapy.

Synergy between dietary restriction and chemotherapy

The therapeutic synergy of caloric restriction or fasting regimens with chemotherapy has higher antitumoral efficacy than either intervention alone, extending survival in different murine cancer models (18, 41, 101, 120). When combining caloric restriction or fasting regimens with chemotherapy, a differential stress response between cancer and normal cells is observed, whereby normal cells become protected against toxins (differential stress resistance, DSR) and cancer cells are sensitized to toxins (differential stress sensitization, DSS), leading to a major delay in cancer progression (38, 101).

Importantly, the synergy between fasting regimens and chemotherapy seems to depend on the antitumor immune system, as the ability of fasting to synergize with chemotherapy is abolished in athymic mice or mice depleted of CD8+ T cells, or when non-immunogenic chemotherapeutics such as cisplatin are used (44, 130, 131). Thus, Di Biase et al. showed that in a breast cancer model, FMD cycles combined with ICD inducers (doxorubicin and cyclophosphamide) increased circulating CD8+ T cells and delayed tumour progression, an effect lost upon CD8+ T cell depletion (44). Microarray analysis showed that in tumor cells, compared to normal cells, FMD induced the downregulation of Heme oxygenase (HO-1) expression, which protects against oxidative damage and apoptosis (44). The researchers demonstrated that reduced tumour cell HO-1 mediates, at least in part, the anticancer effect of FMD by increasing CD8+ T cells in tumours and their expression of granzyme-B, and by decreasing Treg infiltration (44) (Table 1).

Autophagy in tumor cells is also involved in the immune-mediated synergy between caloric restriction or fasting regimens and ICD inducers (130). A proficient autophagy response in tumour cells is needed for cross- presentation of tumor derived antigens by dendritic cells that prime CD8+ T cells against tumor cells (62). This effect may be mediated through increased adjuvanticity of dying tumor cells due to the autophagy-induced delivery of some DAMPs such as ATP and HMGB1, which attract dendritic cells into the tumour bed (62, 95). Indeed, autophagy is a causative factor for ATP secretion during chemotherapy-induced ICD (95), and autophagy-deficient cancer cells fail to induce an anti-tumour immune response in immunocompetent hosts due to an impaired capacity to release ATP in response to chemotherapy (62, 131). Furthermore, autophagy can enhance tumor cell antigenicity, as autophagy-dependent degradation of tumour antigens increases MHC-I presentation in dying tumor cells, which leads to greater CD8+ T cell responsivity and decreased tumor growth in vivo (27). Interestingly, markers of autophagy are correlated with increased CD8+ T cell/Treg cell ratios and good prognosis in cohorts of triple negative breast cancer (95). The increased stimulation of autophagy by fasting regimens may boost the therapeutic efficacy of ICD inducers by an improved antitumor immunity (24, 62). Castoldi et al. (24) reported that short-term fasting improved the capacity of mice to mount an antitumor immune response against fibrosarcoma cancer cells injected 7 days after being vaccinated with the same cell line treated in vitro with mitoxantrone (MTX) to induce ICD. Notably, when fasted mice were treated with dimethyl α-ketoglutarate, an inhibitor of starvation-induced autophagy, the percentage of tumour-free mice decreased by 55%, indicating that starvation has immunostimulatory effects

when combined with ICD-inducing chemotherapy through an enhanced autophagy in tumour cells (24). They further showed that the efficacy of MTX was independent of the autophagy competence of the host (24). In another fibrosarcoma preclinical study, hydroxycitrate (a caloric restriction mimetic that induces autophagy and mimics the metabolic effects of fasting) or short-term fasting combined with ICD-inducing chemotherapies enhanced tumour growth control as compared to either treatment alone, an effect depending on CD8+ T cells (131). They also demonstrated that low levels of IGF-1 induced by hydroxycitrate (or short-term fasting) combined with ICD-inducers can trigger an autophagy-dependent anticancer immune response that relies on extracellular ATP and low levels of Tregs in the tumour (131).

Nevertheless, the role of autophagy in the context of cancer is controversial. It is tumour suppressive since defects in autophagy can drive DNA damage, genomic instability, mitochondrial defects, and tumour growth in preclinical models. Conversely, it can be pro-tumoral, as established tumours can utilize autophagy to cope with stressors such as hypoxia, damaging stimuli, and nutrient deprivation (5, 62, 124). Therefore, additional work is required to understand how and when to inhibit or activate autophagy to develop therapeutic strategies to increase the effects of chemotherapy and improve clinical outcomes in cancer patients.

Fasting regimens in clinical trials in cancer patients

Clinical studies of short-term fasting or FMD in patients undergoing chemotherapy support their feasibility and overall safety, with no serious fasting-side effects and no significant reductions in body weight observed (9, 12, 39, 55, 77, 138, 164, 167, 187). Moreover, short-term fasting and FMD reduced side effects such as nausea, vomiting, fatigue and DNA damage in peripheral blood mononuclear cells and improved quality of life in patients undergoing chemotherapy (12, 48, 138, 164, 187). Furthermore, there is some evidence that FMD can improve antitumoral immune responses (Table 2). Thus, in a single-arm trial in patients with different neoplasms, an FMD in combination with standard antitumor therapies downregulated circulating immunosuppressive myeloid cell subsets and increased cytolytic NK cells and CD8+ T lymphocytes with an activated/memory phenotype (167). In a subset of breast cancer patients from the ongoing DigesT trial (NCT03454282) adhering to an FMD before surgery, the surgical tumour sample showed a significant decline in IGF-1R expression and increased CD8+ T cell infiltration compared to the paired pre-FMD biopsy analyses. Additionally, RNA-seq analyses revealed upregulation of immune signatures previously associated with good prognosis and/or better response to therapies in patients with cancer, and an increase in NKT cells (a subpopulation of T cells that express a limited repertoire of T cell receptors (TCRs) and a number of cell surface molecules in common with NK cells), activated dendritic cells and memory CD4+ and CD8+ T cells (167). In peripheral blood, FMD increased dendritic cells, NK cells, B cells and several subsets of memory T cells, and reduced exhausted T cells, Tregs, and MDSCs(167). Notably, short-term fasting and FMD have also been shown to reduce chemotherapy induced damage in T cells (37, 39, 48) (Table 2). Although very limited, the available clinical evidence related to cancer-related outcomes is promising. In a feasibility trial of an FMD in patients with breast cancer undergoing active treatment, patients receiving endocrine therapy showed longer progression free survival than the average in those settings (164). In a similar fashion, De Groot et al. showed in a phase II randomized trial that a radiologically complete response and a 90–100% tumour-cell loss in intention-to-treat and per protocol analyses, respectively, was more likely to occur in patients adhering to a 4-day FMD during neoadjuvant chemotherapy cycles compared to patients consuming their regular diet (37) (Table 2). Of note, only a third of patients in this study were able to complete at least 4 FMD cycles. The study used a standardised kit of foods consisting of soups, broths, teas and snacks, and the main reason for the low compliance was the dislike of some of the food items provided. Additional challenges to compliance in the clinical setting may include the impact of these interventions in the patients' daily routines and social interactions. To optimize compliance, future clinical studies in the cancer population could incorporate a higher variety of options, explore other less restrictive interventions such as time-restricted eating, and having support from dieticians to tailor the diet plan to address clinical needs (e.g., patients with low body mass index).

EFFECTS OF EXERCISE TRAINING

It has been extensively reported from preclinical studies that exercise training can reduce tumour incidence, tumour growth, and metastasis in different transplantable, genetic, and chemical-induced tumour models (30, 83, 178). Observational findings in patients with cancer include an inverse association of physical activity levels and all-cause and cancer-specific mortality in those with a diagnosis of breast, colorectal, or prostate cancer, with relative risk reductions up to 40% and 60% for disease progression and risk of disease-related death, respectively (86, 113, 143). It is hypothesized that exercise controls tumour progression through effects on tumor intrinsic factors (cell growth rate, metastasis) and via modulation of systemic factors that can influence several cancer hallmarks in the TME (tumor cell metabolism, angiogenic signaling pathways, hypoxia, immune modulation) (6, 83, 89).

Immune-mediated antitumoral effects of exercise

Physical exercise augments the anti-tumour immune response, modulating the tumour microenvironment by acting on the innate and adaptive immune systems (30, 83, 89, 93). Notably, epidemiology studies show that the benefit of physical activity occurs in cancers with higher numbers of mutations, which determine the immunogenicity of tumour cells, as somatic mutations generate neoantigens that will be recognized by CD8+T cells (51). Conversely, physical activity shows less benefit in cancers with lower numbers of mutations (51). This implies that the immune system is also a substantial contributor to the anti-cancer effects of physical activity in humans (51). Exercise-induced reductions in tumour growth have been shown to be dependent on CD8+ T and NK cells in several preclinical studies (51, 70, 74, 128, 137, 171) (Table 3), and it is proposed that this effect is mediated via different mechanisms that involve improvement of the general immune status of the blood, mobilization of immune cells and infiltration within the tumour

bed, and a shift towards a less immunosuppressive TME (51, 73, 82, 181). In addition, exercise may help to maintain a healthy immune system though its effects on the gut microbiome (25).

I. Improvement of general immune status.
Several forms of T cell dysfunction—anergy, exhaustion, and senescence—have all been reported in the cancer microenvironment, and it is a hallmark of inadequate anti-tumor immune responses (43). Regular exercise appears to reduce T cell dysfunction and to preserve or increase the frequency of naïve T cell populations (73).

Anergic T cells arise due to the absence of a costimulatory signal from dendritic cells because of the competitive binding of CTLA-4 expressed on Tregs to CD80 and CD86 on dendritic cells (36, 43). In animal studies, exercise interventions have been shown to reduce the tumour tissue expression of the Treg marker foxp3 (74, 111) (Table 3). In humans, observational studies show lower frequencies of Tregs in physically active people compared to people who were not involved in regular exercise (49, 76). Hence, decreasing the number of Tregs in the TME by exercise may reduce in turn the number of anergic T cells.

Senescent T cells appear with aging, due in part to thymic atrophy that reduces naïve T cell output, and to the exposure to various pathogens during life (21, 49, 129, 148). Based on a recent systematic review, Donovan et al. (47) concluded that acute exercise induces the mobilization of senescent CD8+ T cells into peripheral blood, and a tendency towards a decreased production/accumulation of senescent CD8+ T cells induced by increased cardiorespiratory fitness (47). Moreover, in sedentary adults over 65 years old resistance training interventions also appear to reduce the number of senescent CD8+ T cells (47), and those who are physically active have lower number of senescent CD4+ T cells than their inactive counterparts (72). Nevertheless, it should be noted that this review by Donovan et al. (47) only included studies in healthy populations which had several limitations such as small sample size and differences in immunophenotyping strategies. As such, the effects of exercise training on features of immunosenescence, remains unclear, as summarised recently elsewhere (51). Moreover, a key limitation of studies in the field is that T cells previously considered to be senescent (e.g., via CD27-, CD28-, CD57+, KLRG1+) expression may in fact be highly differentiated T cells that retain proliferative and effector functionality. Indeed, effector memory T cells with a highly differentiated, late/terminally differentiated phenotype share several features with senescent cells, such as shorter telomers, accumulated DNA damage and metabolic changes, but they retain proliferation capacities and effector functions under certain circumstances, as opposed to senescent T cells (127). Therefore, further studies are needed to elucidate the effects of regular exercise on truly senescent cells by combining several markers of T cell senescence and markers of cellular senescence (127).

Exhausted T cells, which upregulate PD-1 and other co-inhibitory receptors such as CTLA-4 (46), appear due to the action of inflammatory cytokines and to a prolonged stimulation of T cells, which progressively inhibits T cell effector functions (46, 47). Of note, this dysfunctional state is not irreversible, as blocking PD-1 or CTLA-4 with immunotherapy can restore CD8+ T cell-mediated immunity directly, and via depletion of CTLA-4 expressing regulatory T cells in the case of anti-CTLA-4 drugs (134, 173) . In healthy adults, acute exercise has been shown to induce the mobilization of PD-1+ exhausted CD8+ T cells into the peripheral blood compartment, however, acute exercise did not change the levels of CTLA-4+ CD8+ T cells (47). Conversely, having a sedentary lifestyle may increase the frequency of exhausted CD8+ T cells compared to physically active people (47). In early prostate cancer, a recent study showed that an acute bout of exercise on a cycling ergometer preferentially mobilized CD8+ T cells with the inhibitory receptor TIGIT, associated with T cell exhaustion introducing the concept that immunotherapy could synergize with exercise by reactivating mobilized but exhausted T-cells (141). Nevertheless, whether such changes correspond to an altered immunophenotype in the tumour remains unknown. We also note that PD-1 is transiently expressed upon T-cell receptor-mediated activation, and thus the sole expression of this inhibitory receptor may be insufficient to differentiate between exhausted and activated T cells (146, 173). Therefore, future studies should include a more comprehensive panel of markers to evaluate if exercise can mobilize bona fide exhausted T cells and prevent their accumulation, and moreover, understand whether exercise can alter immunophenotypes in the tumour microenvironment.

Mobilized T cells extravasate from the blood to peripheral and / or inflamed tissues after exercise, and it is thought that T cells are then exposed to a variety of pro-apoptotic stimuli (e.g., reactive oxygen species, glucocorticoids, cytokines) that may cause apoptosis of these cells (114, 147). If senescent and exhausted T cells are preferentially mobilized during exercise, they would be more exposed to apoptosis (47, 129, 147).. It is hypothesized that the removal of lymphocytes, and specifically senescent T cells, would create “immune space” to produce or maintain naïve T cells (47, 147), which would ensure an adequate immune response to detect newly encountered pathogens and novel cancer neoantigens (172). In a study by Mooren & Krüger (115), the injection of apoptotic CD3+ cells or the supernatant from these cells in untrained mice led to the mobilization of hematopoietic progenitor cells into the blood, which can differentiate into lymphocytes, which provides some support for parts of the immune space hypothesis. Two cross-sectional studies reported an increased frequency of naïve T cells, with higher number of both naïve CD8+T and CD4+ T cells in highly physically active adults (males cycling 100 km and females 60 km at least twice in the 3 weeks prior to testing) compared to non-active people of the same age group (49), and higher number of naïve CD8+T cells in subjects with higher aerobic fitness, indicated by estimated VO2max, as compared to less aerobically fit people (148). Similarly, both a concentric and eccentric endurance intervention (uphill and downhill walking) increased the frequency of naïve CD8+ T cells in pre-diabetic subjects (129). Nevertheless, a recent RCT in middle aged/older women at high risk of breast cancer showed that the number of CD4+ naïve T-cells and CD4+ recent thymic emigrants (the youngest subset of naïve T cells) decreased after a 12-week high intensity interval exercise program, whereas in patients who followed a moderate intensity interval exercise program there was no statistically significant difference in the frequency of these cells (123). Of note, some limitations to the immune space hypothesis have been proposed, including the proposal that there is a fixed

number of T cells, whether truly senescent/exhausted T cells versus highly differentiated T cells are eliminated by apoptosis, and whether it is advantageous depleting these cells instead of restoring their functions (51, 163). Indeed, highly differentiated T cells, among which some may be senescent and exhausted T cells, are necessary to control persistent virus (163). However, the removal of senescent cells that accumulate in tissues and organs due to aging can delay age-related pathologies (10) and, therefore, future studies analysing the effects of exercise and able to distinguish senescent and highly differentiated T cells, should evaluate if removing senescent T cells has benefits in the cancer setting.

Higher frequencies of naïve T cells found in physically active people may also be due to IL-7 and IL-15, which are both highly secreted by exercising muscles (49, 51, 73). These cytokines maintain naïve T-cell populations by promoting their survival and expansion in the periphery (73, 169). In addition, IL-7 and IL-15 sustain the survival of memory T cells (73, 150) which provide a better antitumor protection than late differentiated effector T cells (109). Furthermore, they promote the induction and expansion of stem cell memory T cells (51, 73, 108), which are more persistent and effective against tumours than central memory T cells (69). IL-7 treatment prevents CD27 and CD28 loss, which is a marker of T cell senescence, and maintains proliferative capacity and IL-2 production in human T cells co-cultured with tumor cells (73). IL-15 also promotes the differentiation and proliferation of NK cells (150) and the differentiation of T cells into resident memory T cells, which are thought to protect against tumour relapse and enhance therapeutic outcomes (110). In an orthotopic pancreatic ductal adenocarcinoma model, low-intensity treadmill-running has been reported to inhibit tumour growth by enhancing the infiltration of CD8+ T cells, while blocking IL-15 signalling reversed the exercise-mediated tumour protection and the influx of IL15Rα+ CD8 T cells into the tumour (99). Therefore, higher levels of IL-7 and IL-15 induced by exercise may promote the generation or maintenance of different subsets of T cells and NK cells, improving the host immune antitumoral response.

II. Mobilization and infiltration of immune cells within the tumour.

An acute bout of exercise leads to a rapid increase of immune cells in the blood, which is dependent on the haemodynamic shear stress and epinephrine-mediated stimulation of beta-2-adrenergic receptors on the surface of lymphocytes (6, 93). It is hypothesized that their distribution into peripheral tissues, especially NK cells and CD8+ T cells with a more potent effector phenotype, contribute to an enhanced immune surveillance (17, 21, 97, 181).

Exercise induces normalization of the tumour vasculature (6, 14, 112), which together with release of IL-6 from exercising muscles, promotes the upregulation of adhesion molecules on tumour vascular endothelium, facilitating the infiltration of immune cells into the tumour (6, 181). In healthy humans, acute exercise mobilizes mature and cytotoxic NK cells (17, 20) and enhances their cytolytic activity (136). In mice, Pedersen et al. showed that exercise decreased the incidence and tumor growth in a transplantable mouse model of melanoma and increased the number of NK cells infiltrated in the tumors as compared to non-exercised mice (128) (Table 3). Moreover, this effect was dependent on the release of epinephrine and IL-6 during exercise (128). Interestingly, tumors were smaller in wild type exercised mice than in athymic exercised ones, implying that T cells also mediated the exercise-induced antitumoral effect, and that the lack of T cells failed to control the growth of small tumours (128). They also showed that exercised mice had higher infiltration of dendritic cells in the tumours than control mice (128), which are necessary for the priming and activation of CD8+T cells (96). Other preclinical training studies have reported that exercise augments dendritic cell numbers and promotes their maturation and function (16, 29) (Table 3). Therefore, exercise may also promote an adaptive immune antitumoral response via increased number and function of dendritic cells.

Several studies in mice have confirmed the involvement of CD8+T cells in the antitumoral effects of exercise (70, 74, 99, 137) (Table 3). Thus, exercise modulates their mobilization and tumour infiltration through different mechanisms, such as maintenance of the gradient of the lipid mediator sphingosine 1-phosphate between blood, lymph, and tissues (99). This gradient is necessary for the trafficking of lymphocytes and for promoting an appropriate population of circulating lymphocytes (160) Another mechanism by which exercise induces the mobilization of CD8+ T cells is the loss of surface expression homing markers caused by increases in of tricarboxylic acid cycle metabolites and lactate in blood and secondary lymphoid organs (137). Finally, exercise can increase their recruitment into tumours via chemokine signalling the tumours (70).

III. Decreased local immunosuppression.

Exercise can ameliorate immunosuppressive conditions in the TME such as hypoxia, high lactate concentrations with consequent acidity, and increased inflammatory mediators thereby promoting antitumor immunity (6, 51, 93, 181). Exercise increases tumoral blood flow by improving tumour microvessel density and maturity (6, 14, 112), which reduces tumor hypoxia (6, 14, 112). Moreover, in breast cancer bearing mice, endurance training decreased tumour volume and modulated the expression of enzymes involved in lactate metabolism, resulting in decreased lactate in the tumour, which may reduce acidification of the TME (7). Reducing acidity in the TME would contribute to an antitumoral immunity, as an acidic tumour microenvironment impairs T cell and NK cell function (6, 110, 181). Inflammatory inhibitors of T cells such as IL-10 and TGF-beta are released by tumour cells and immunosuppressive cells (100). Exercise in mice has been reported to reduce tumor growth together with a reduced infiltration of innate and adaptive immune cells associated with an immunosuppressive microenvironment, such as macrophages (174, 183), neutrophils (99, 174, 183), MDSCs (66, 99, 171) and Tregs (74, 111) (Table 3). Furthermore, there is some preclinical evidence that exercise can promote polarization towards an anti-tumoral M1 subtype in macrophages isolated from the peritoneum (1, 119), and decrease the pro-tumoral M2 subtype polarization amongst tumour associated macrophages (90, 111) (Table 3). However, the effects of exercise on the polarization of neutrophils have not been assessed (149).

Exercise-induced reductions in hypoxia and acidity, a reduction to immunosuppressive cells and a higher infiltration and competency of antitumour T cells could theoretically increase the number of dead tumor cells, in turn reducing the

immunosuppressive conditions in the TME (51, 83, 178, 181). Preclinical studies have assessed gene expression profiling within the TME, showing that exercise interventions in different cancer models induce a more pronounced antitumoral immune environment, with upregulation of oxidative metabolism (70), increases in NK cell (128) and CD8+ T cell (88) markers, and a downregulation of several pathways involved with immunosuppression (70) (Table 3).

IV. Modulation of the microbiome

In murine models, running has been reported to induce changes in the composition and function of the microbiota, with an increased alpha diversity, a measure of microbial diversity, within a sample, and associated with a higher response to cancer treatments (78), more butyrate-producing taxa and an increase in the production of SCFAs (4, 32, 168). Some cross-sectional studies in athletes have also reported that exercise influences the microbiome, increasing microbial diversity and the number of butyrate producing species and species associated with improved metabolic health (31, 88). Moreover, cardiorespiratory fitness is a predictor of microbial diversity and production of butyrate in healthy humans (32, 52). Regarding longitudinal exercise training effects, in sedentary subjects with insulin resistance, both sprint interval running, and moderate-intensity continuous training modified microbiota profile by increasing the Bacteroidetes phylum and decreasing the Firmicutes/Bacteroidetes ratio, and also decreasing systemic and intestinal inflammatory biomarkers (117). Similarly, in unfit volunteers, exercise interventions have been reported to significantly increase microbial diversity (11), Akkermansia abundance (118), and butyrate-producing taxa and SCFAs (4). Conducting faecal transplants from a breast cancer survivor who underwent an exercise intervention into germ-free cancer-bearing mice that did not undergo any exercise training resulted in smaller tumour volume in those mice who received post exercise faecal samples compared to those receiving pre-exercise samples, demonstrating that exercise may have antitumor activity through an effect only mediated by the gut microbiome (139). Nevertheless, in cancer patients, the effects of exercise on gut microbiome remains unexplored, especially in terms of treatment efficacy (168). An ongoing RCT in patients with prostate cancer receiving androgen deprivation therapy will assess the impact of a supervised exercise program on gut microbiota (121).

Synergy between exercise and chemotherapy

Exercise can synergize with chemotherapeutics in several ways (6, 30, 179). First, exercise may limit chemotherapy toxicity in highly perfused organs such as brain, bone marrow, heart, lungs and kidneys by increasing the blood perfusion in muscles, and hence, the volume for chemotherapy distribution. (30). Second, exercise-induced increase of blood irrigation helps to deliver drugs to tumours (6). Indeed, Betof et al. demonstrated that exercise improved tumour perfusion, reduced hypoxia, and also increased the effectiveness of cyclophosphamide chemotherapy, delaying tumour growth in two orthotopic models of murine breast cancer (14). Similarly, in murine models of pancreatic ductal adenocarcinoma (56, 140), melanoma (140) and Ewing sarcoma (116), gemcitabine and doxorubicin were significantly more efficacious at reducing cancer burden in exercised mice due to the exercised-induced normalization of tumour vasculature. Interestingly, cyclophosphamide, gemcitabine and doxorubicin are considered ICD inducers. Although the antitumoral effects of exercise through immune mechanisms were not assessed, it may be the case that ICD was enhanced by chemotherapy in these studies. Considering hypoxia, this reduces the expression of MHC- I in tumour cells (6, 145), which would impede the ability of CD8+T cells to recognize tumour cells (6, 61, 145). Moreover, hypoxia inhibits antigen uptake by dendritic cells (50) and limits their maturation and activity, preventing them from priming naïve T cells (170). Therefore, by relieving hypoxic conditions with exercise, the antigenicity of tumor cells succumbing to chemotherapy-induced ICD and the activity of dendritic cells may be enhanced, enabling CD8+ T cell function. Furthermore, because the adaptive immune response triggered by ICD can only be executed if the TME conditions are favorable for the infiltration and function of T cells (63, 95), exercise may have a synergistic effect by increasing the infiltration of T cells and reducing immunosuppressive conditions in the TME. It should be noted, however, that some of the mentioned studies used nude mice (56, 116), which lack T cells. Nevertheless, they have an innate system and so exercise-induced increased infiltration of NK cells may synergize with ICD inducers as they can modify the tumor surface phenotype and sensitize them to NK cells-mediated killing (53).

Exercise interventions in clinical trials in cancer patients

The safety and feasibility of exercise during and after anti-cancer treatment across several cancers, disease stages and treatment regimens has been established. Additionally, beneficial effects on improvement in body composition, cardiovascular fitness, muscle strength, physical function, and psychosocial outcomes, and reduced treatment-related adverse effects are well documented (30, 89, 102, 175). However, little is currently known about the effects of exercise on immune parameters in cancer patients (Table 4). In a recently published clinical trial in patients with oesophageal cancer undergoing neoadjuvant chemotherapy, the exercise group (combined aerobic and strength training) had higher rates of tumour regression compared to the control group (75% vs 36.8%, p=0.025), and differentially regulated immunity and inflammatory markers, with higher counts of CD3+ and CD8+ T cells (188). Similarly, a pre-surgical aerobic and strength training intervention in breast cancer patients resulted in upregulation of pathways related to immune cell function and inflammatory signaling and a trend towards a decreased expression of Foxp3 within the TME although patient outcomes have not been reported (103).

Recently, patients with pancreatic adenocarcinoma who underwent exercise concurrent with neoadjuvant chemotherapy or chemoradiation prior to surgical resection in a prospective clinical trial, had a higher number of infiltrating CD8+ T cells and a trend toward higher expression of granzyme B compared with matched historical controls (99). Notably, patients who had a higher number of CD8+ T cells or granzyme B in the tumour had higher median overall survivals (p=0.01 and 0.04 respectively) (99). In a 12-week intervention of supervised aerobic training (cycle ergometry) in patients with eight different solid tumours receiving cytotoxic therapy and synthetic erythropoietin, those exercising showed a trend towards an increased number of total circulating CD8+ T cells and CD8+CD45RA+ T cells, compared to patients receiving usual care, together with decreases to pro-inflammatory cytokines and angiogenic factors in their blood (68).

Moreover, there is evidence of enhanced NK cells activity after exercise interventions (54, 98, 126, 141, 161). Thus, an increment has been reported after a single bout of acute exercise in prostate cancer patients scheduled to undergo prostatectomy (141), after a 15-week aerobic exercise intervention in post-menopausal breast cancer survivors (54), and after 9-12 weeks of aerobic and resistance training during (neo)adjuvant chemotherapy in breast and colon cancer patients (161). Moreover, exercise modified the expression of NK receptors, with a decreased expression of the inhibiting receptor KIR2DL1 (126) and an increased expression of the activating receptors NKG2D (126) and NKp46 (161), suggesting a higher cytotoxic potential (126, 161). Regarding the infiltration of NK cells in cancer patients, two recent randomized controlled trial in men with localized prostate cancer have been conducted. Schenk et al., showed that after an acute bout of aerobic exercise on the day before surgery, there were not differences in NK cell infiltrates in the tumor tissue between the exercise and the control group (142). The other RCT used a preoperative aerobic high intensity interval training four-times per week from time of inclusion until scheduled surgery. It was reported that there was a within-group increase in tumour NK cells in the exercise group, and a between-group difference in healthy prostatic tissue, though NK cell frequency was low (45, 64). Nevertheless, the study did not show a difference in the number of infiltrating NK cells in the tumours from baseline to follow-up between groups, neither with the intention-to-treat nor the per-protocol analysis.

Regarding disease outcomes in observational studies, a recent prospective cohort study of 1,340 breast cancer patients concluded that meeting the minimum guidelines for physical activity, defined as the MET hour equivalent of 150 minutes of moderate-intensity regular physical activity per week, before and after chemotherapy treatment significantly reduced the risk of cancer recurrence and mortality (23). Moreover, a systematic review and meta-analysis found improved survival with higher prediagnosis or postdiagnosis levels of physical activity for 11 cancer types (59). Similarly, overall mortality was significantly reduced by a higher level of physical activity in the 8 non-randomised trials in patients with advanced cancer (breast, colorectal, lung and brain) included in a systematic review and meta-analyses (156). Regarding intervention studies, a recent systematic review of exercise interventions during neoadjuvant, primary, and adjuvant therapy reported an enhancement of the efficacy of cancer therapies although the studies included were not designed for this purpose (179). Three ongoing large multicenter randomized trials are evaluating if targeted exercise improves clinical outcomes including progression free and overall survival in colonic, prostate and hematological malignancies (35, 122, 176).

COMBINING FASTING AND EXERCISE INTERVENTIONS

Combining fasting or caloric restriction with endurance training has been reported to have synergistic effects in a non-cancer population, with attenuation of muscle autophagy and increase in muscle repair, as well as metabolic changes (87). In the cancer setting, work to date has focused on targeting single molecules within an individual regulatory network; however, complementary strategies able to act on multiple higher order networks can be a more effective therapeutic approach (94) . Similarly, immune-oncology has aimed mainly to act on the TME, yet immunity is coordinated across multiple tissues (79). Thus, therapeutic interventions that include both fasting and exercise interventions, which are able to target multiple systemic responses, may be a useful strategy to supplement chemotherapy with synergistic or additive effects.

Although scarce, some preclinical studies have shown positive results in cancer models (Table 5). Using a skin tumor-sensitive murine model for enhanced susceptibility to TPA-promoted skin carcinogenesis, Xie et al. demonstrated that a 20% reduction in calories was sufficient to decrease PI3K and Ras pathways, but only when combined with treadmill exercise training was there a significant increase in caspase-3-like proteolytic activity in tumours, suggesting an apoptosis-mediated mechanism (177). In a metastatic model of breast cancer, voluntary wheel running combined with a 10% reduction in calories compared to control mice was shown to delay tumour growth, metastatic progression, and improve survival, as compared to either intervention alone (162). Additionally, the combination reduced the expression of metastatic and immunosuppressive genes and increased the CD8+ T cell/MDSC ratio in the tumour (162). Furthermore, adding caloric restriction to wheel running in mice further protected from the cardiotoxicity induced by doxorubicin, a chemotherapeutic used for the treatment of a variety of cancers (75).

Regarding human studies, there are a few promising reports regarding feasibility, patient-related and therapeutic outcomes (Table 5). One proof-of-concept case study utilized a fasting and exercise intervention in a woman with recurrent stage III ovarian cancer, during a 3-day period once a month over the course of 3 consecutive months (3). There were improvements in physical and psychological symptoms, and the participant adopted positive lifestyle modifications. A randomized controlled trial compared intermittent energy restriction vs continuous energy restriction for weight control throughout the 4.5–6-month course of adjuvant/neoadjuvant chemotherapy in early breast cancer patients. The intermittent energy restricted diet consisted of two consecutive low-energy, low-carbohydrate days, which provided 650–1000 kcal, immediately prior to chemotherapy infusion; for the other five days of the week patients were recommended to follow a Mediterranean diet, which was tailored so that overall weekly intake matched their energy requirements for weight loss or maintenance. The continuous energy restricted diet was a Mediterranean diet every day of the week tailored to their energy requirements for weight loss or maintenance. This trial also included exercise recommendations for both intervention arms (77). Exercise levels did not increase in either group as compared to baseline levels, but it was maintained at 3 weeks post chemotherapy, as opposed to the previously reported 20% decrease in physical activity alongside chemotherapy in these patients (77). A prospective, non-randomized, controlled trial designed to decrease fat gain during the 4 weeks of induction chemotherapy in adolescent patients with acute lymphoblastic leukaemia using a caloric deficit of ≥10% and a home-based exercise intervention combining aerobic + resistance training,

significantly reduced minimal residual burden risk compared to historical controls (125). Although adherence to the prescribed exercise was only 31%, lean mass loss was similar to that found in historical controls, indicating that caloric restriction did not worsen the loss of lean tissue (125).

LIMITATIONS AND CHALLENGES OF DIETARY RESTRICTION AND EXERCISE INTERVENTIONS

Most of our knowledge of the effects of dietary restriction and exercise arise from preclinical data. However, the physiological, metabolic, immunological, and genetic differences between rodent models and humans, and the high diversity of cancers, treatments, and comorbidities in humans, challenges the translation of animal studies to humans. For example, the fasting timeframe shown to be beneficial in animal studies cannot be translated to humans, as mice have a much higher metabolic rate. Specifically, the equivalent of a 24h water only fasting in rodents would be equivalent to 5 days in humans (33). Similarly, there are knowledge gaps related to the role of the intensity, dose, and mode of exercise on the immunomodulatory aspects in preclinical studies, which makes it difficult to develop generalized exercise guidelines as cancer treatment. For example, different durations and intensity may impact the release of different myokines and promote differential shifts on the immune cell's phenotype. Therefore, while animal models provide valuable insights into biological mechanisms, the translation of findings to human populations requires careful consideration of the limitations mentioned above.

CONCLUSION AND FUTURE DIRECTIONS

The efficacy of conventional chemotherapeutics greatly depends on the immunological competence of the host. Chemotherapy agents increase the immunogenicity of tumour cells by increasing the presentation of antigens on MHC-1 molecules and by inducing the release of molecules from dying tumour cells that unleash an adaptive immune response in the host against the tumour. This increased immunogenicity must be perceived by the immune system. However, because tumours evolve to evade recognition by immune cells, the functions of the antitumoral immune system are often compromised, reducing the efficacy of chemotherapy. We propose that interventions including both dietary restriction and exercise could be used as adjunct non-pharmacological approaches in clinical trials in cancer patients during chemotherapy treatment, as they could increase tumour response, while having no serious adverse events for cancer patients. Dietary restriction and exercise have the potential to each improve different subsets of immune populations at the systemic level and ameliorate the conditions within the TME towards a more tumour-suppressive and less immunosuppressive one, through overlapping but also through different pathways, eventually leading to enhanced therapeutic response.

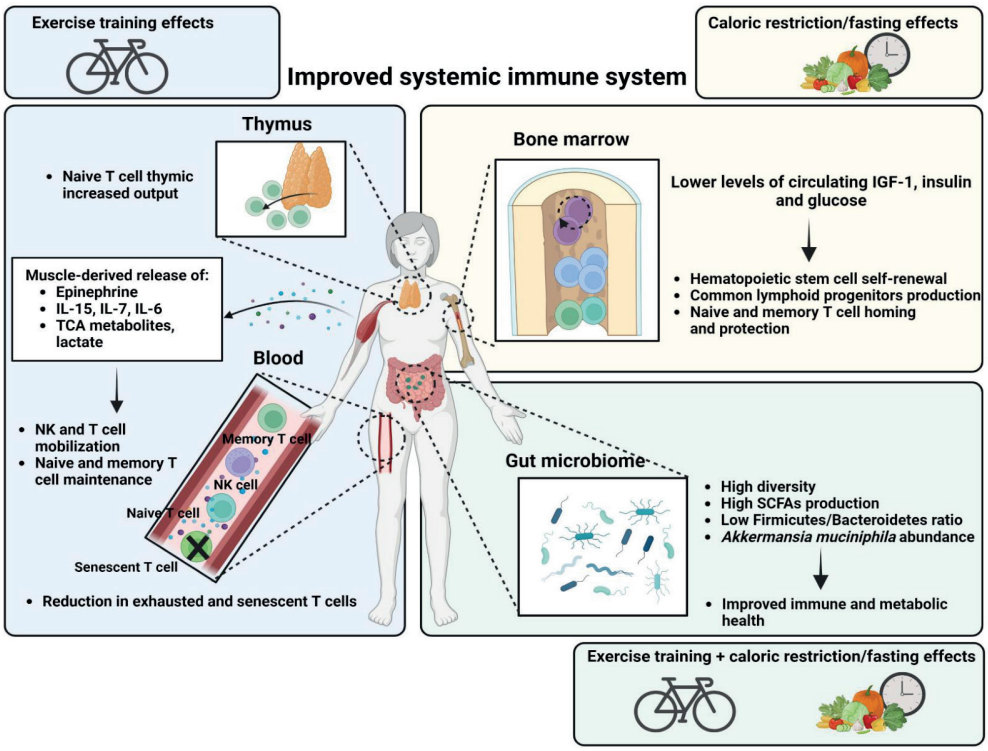

Figure 1. A synthesis of the systemic changes induced by caloric restriction/fasting and exercise. Caloric restriction/fasting decrease the availability of nutrients and growth factors, and this reduction leads to the protection of progenitors, naïve and memory T cells in the bone marrow. Conversely, exercise may decrease the number of circulating senescent and exhausted T cells, and in turn, increase the output of naïve T cells. Moreover, exercise can induce the maintenance and mobilization of antitumoral immune cells, increasing their ability to patrol peripheral tissues. Finally, caloric restriction, fasting and exercise may induce modifications in the gut microbiome that are related to improved metabolic and immune health.

At the systemic level, fasting regimens may improve the antitumoral immunity by enhancing hematopoietic stem cell regeneration, the production of common lymphoid progenitors in the bone marrow and by maintaining naïve and memory T cell populations. Similarly, exercise can enhance systemic immunity by decreasing dysfunctional T cells such as exhausted and senescent cells, while increasing the output of naïve T cells from the thymus, and the maintenance of naïve and memory T cells. Moreover, exercise also mobilizes T cells and NK cells, increasing their surveillance capability of peripheral tissues. Finally, both interventions may influence systemic innate and adaptive immune components by promoting beneficial shifts in the gut microbiota (Figure 1). At the local level, the immunogenicity (antigenicity and adjuvanticity) of tumour cells succumbing to ICD may be increased by both fasting-induced autophagy and exercise-induced reduced hypoxia. The increased immunogenicity of the dying tumour cells will increase their susceptibility to an immune attack, and this process starts with antigen uptake and maturation of dendritic cells. Exercise could support this part of the process by increasing the recruitment, function, and maturation of dendritic cells, which are then able to prime and activate naïve CD8+ T cells against tumour cells. Finally, dietary restriction and exercise interventions can help increase the infiltration of CD8+ T and NK cells within the TME and maintain their effector functions through the reduction of immunosuppressive conditions by decreasing the number of MDSCs, T regs and M2 macrophages, and reducing hypoxia and lactate concentration. A shift in the TME towards a more tumour-suppressive one would, in turn, increase the number of dying tumour cells, which would decrease the immunosuppressive factors released to the TME, further enabling immune cells competency against the tumour (Figure 2).

Supervised dietary restriction and exercise interventions could be implemented as part of the supportive care guidelines for cancer patients under chemotherapy treatment, as they are inexpensive, have low risks, and can decrease drug toxicity, improving quality of life. Although preclinical findings are that dietary restriction and exercise can increase the efficacy of chemotherapy, this remains unknown in humans. Adequately powered and carefully planned clinical trials are required to study the safety and efficacy of interventions using dietary restriction and exercise training during chemotherapy treatment, measuring response rates and survival outcomes. Furthermore, clinical trials should be designed to identify the most beneficial form of dietary restriction and exercise type and dosage to define standards of care. Finally, the biological mechanisms behind the effects of these interventions should also be further studied by including biological samples (e.g., tissue, serum or plasma, peripheral blood mononuclear cells) to address research gaps such as the impact on the release and exposure of DAMPs in tumour cells, composition and functions of the different circulating and tumour-infiltrating innate and adaptive immune cells, composition of the gut microbiota, and the genetic and epigenetic landscape of the tumours. Further, these phenomena should be tested for association with objective response measures (e.g., tumour size, pathological and radiological response in the neoadjuvant setting) and time to event outcomes (i.e., in the adjuvant setting).

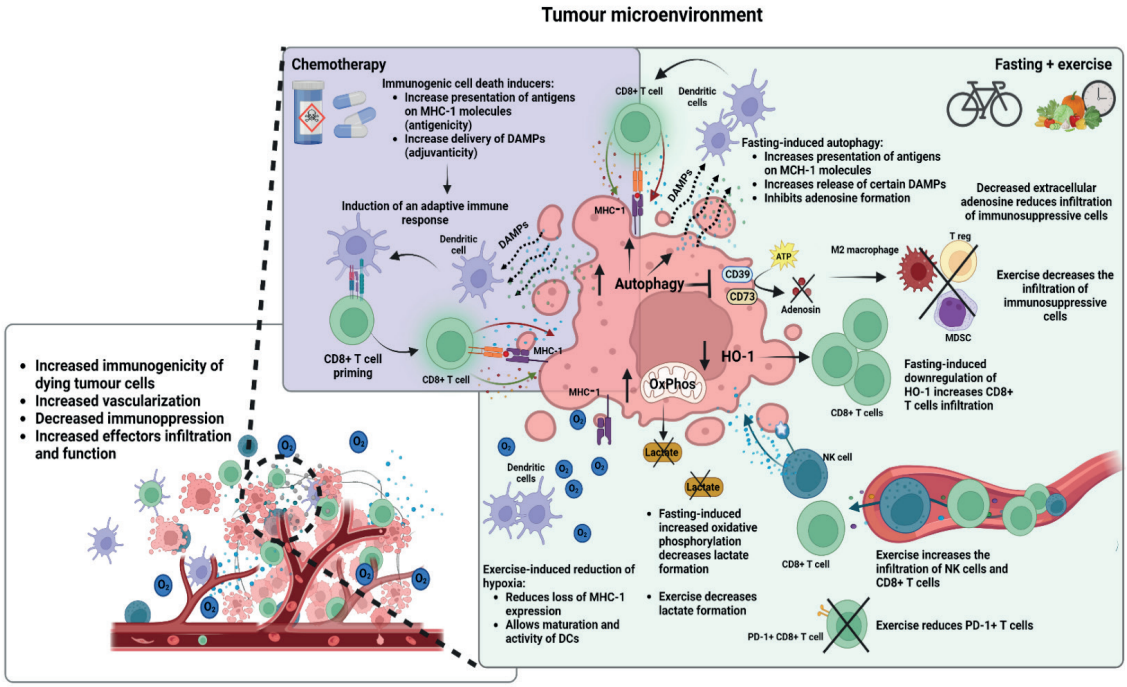

Figure 2. A synthesis of local changes promoted by caloric restriction, fasting and exercise. Tumour cells dying due to ICD inducers increase their antigenicity and their adjuvanticity, inducing an adaptive immune response against the tumour. Fasting and exercise may support antigenicity and adjuvanticity of tumour cells by promoting autophagy and relieving hypoxia, respectively. Fasting and exercise interventions may also promote a shift in the TME conditions into a more antitumoral and less immunosuppressive environment, reinforcing tumour cell death induced by chemotherapy. Thus, fasting may lead to an increased infiltration of CD+ T cells by downregulating the expression of HO-1, and to a decreased accumulation of immunosuppressive cells by decreasing adenosine production. Moreover, fasting increases oxidative phosphorylation in tumour cells, which decreases the production of lactate. Conversely, exercise increases the infiltration of CD8+ T cells and NK cells and it may reduce the number of exhausted T cells (PD-1+). Additionally, exercise can decrease immunosuppressive conditions by reducing the infiltration of immunosuppressive cells, decreasing hypoxia, and lowering lactate production by tumour cells.

Table 1: Overview of in vivo cancer models reporting immunomodulatory effects of caloric restriction/fasting

Table 1. Overview of *in vivo* cancer models reporting immunomodulatory effects of caloric restriction/fasting.			
Ref	**Model**	**Intervention**	**Outcomes**
Di Biase et al., 2016. Ref (44)	• BALB/c (wild type) and BALB/c-nude mice C57BL/6 mice • 4T1 murine breast cancer and B16 murine melanoma cells, implanted subcutaneously	• 4 days FMD. One FMD cycle every 2 weeks, after tumour implantation. • 4 days FMD+ DXR or CP, 2 or 3 cycles every 2 weeks, after tumour implantation	• FMD alone retarded tumour growth and reduced IGF-1. Increased in CD8+ T cells in blood and common lymphoid progenitor cells in the bone marrow. • FMD+ DXR or CP had the highest antitumoral effect. • FMD + DXR increased CD8+ T cells and decreased Tregs in tumours. Required downregulation of HO-1 in tumour cells.
Pietrocola et al., 2016. Ref. (131)	• C57Bl/6, BALB/c, and nude athymic (nu/nu) mice, LSL-K-rasG12D; Atg5fl/fl; CD39+ mice • MCA205 murine fibrosarcoma, TC-1 non-small cell lung, or CT26 colorectal cancer cells implanted subcutaneously	• 48 h STF, 1 cycle, + MTX or OX, after tumours became palpable. • Caloric restriction mimetic: daily administration of hydroxycitrate (HC)	• STS + MTX or OX reduced tumour growth. Required immunocompetency and autophagy. • HC+ MTX or OX reduced tumour growth. Required CD8+ T cells, autophagy, and extracellular ATP. Mediated by T regs depletion. • HC induced autophagy in KRas-induced lung adenocarcinomas, and decreased tumour growth and the infiltration of Tregs. No effect of HC in autophagy deficient, T and B cells deficient or CD39 overexpressing transgenic mice.
Sun et al., 2017. Ref. (154)	• BALB/c mice • CT26 colorectal cancer cells implanted subcutaneously. • CT26 and RAW264.7 cells, in vitro	• Alternate day 24 h STF, 5-7 days after tumour implantation, for 2 weeks	• Suppression of tumour growth and M2 subtype polarization of macrophages. • Mechanisms in vitro: suppression of M2 polarization, increased expression of autophagy genes, decreased expression of CD73 and decreased generation of adenosine.
Collins et al., 2019. Ref. (34)	• C57BL/6 mice • Virally induced transgenic CD8+ pmel-1 cells against the melanoma epitope gp-100. • B16 melanoma cells, implanted subcutaneously	• Daily 50 % CR for 3 weeks, before tumour implantation	• Memory T cells reversely accumulated within the bone marrow. • Enhanced protective function of memory T cells against melanoma tumours, increasing mice survival.

	• B16 melanoma cells, implanted subcutaneously		
Ajona et al., 2020. Ref (2)	• Sv/129 and C57BL/6J mice • 393P or LLC lung tumour cells implanted subcutaneously	• 48 h STF, 3 cycles + anti-PD-1 therapy, 6 days after tumour implantation	• Inhibition of cancer progression and increased survival. • Increased tumour infiltration of NK and CD8+ T cells and decreased number of Tregs. • Decreased IGF-1–IGF-1R signalling in tumour cells.
Pomatto -Watson et al., 2021. Ref. (133)	• BALB/c mice • 4T1 murine breast cancer cells implanted subcutaneously	• 4:10 cycles: 4 days FMD (50% reduced on first day, 70% reduced on days 2-4) or 4 days of CR diet (50% reduced on first day, 70% reduced on days 2-4), followed by 10 days *ad libitum*, 7 days after tumour implantation (Post) or 21 days before tumour implantation (Pre). • Daily 20% CR, 7 days after tumour implantation (Post) or 21 days before tumour implantation (Pre).	• All interventions delayed tumour growth compared to ad libitum mice. • Higher effect of daily CR (pre ad post) over 4:10 cycles diets. • Daily CR reduced metastasis in lungs. • Reduced number of Tregs in blood in daily CR and 4:10 cycles FMD and CR, compared to *ad libitum* mice. • Reduced number of MDSCs in spleen in daily CR mice, and reduction in tumours in FMD and daily CR mice. • Increased number of effector CD8+ and CD4+ cells in blood and spleens in daily CR mice. • Reduction in total MDSCs in tumours in FMD and daily CR mice.
Fu et al., 2021. Ref. (60)	• BALB/c mice • 4T1 and 4T07 murine breast cancer cells implanted subcutaneously	• 48 h STF, 2 cycles, +/-DOC, 14 days after tumour implantation.	• STS suppressed of tumour growth as efficiently as DOC. • STS inhibited splenic accumulation of CD205+ G-MDSCs by attenuating cell trafficking and inducing apoptosis trough inhibition of glucose metabolism.

[1] STF: short-term fasting. FMD: fasting mimicking diet. CR: caloric restriction. DXR: Doxorubicin. CP: cyclophosphamide. DOC: docetaxel. MTX: Methotrexate: MTX. OX: Oxaliplatin. HO-1: heme oxygenase-1. IGF-1: insulin growth factor 1. MDSCs: myeloid-derived suppressor cells. Tregs: regulatory T cells.

Table 2: Overview of clinical studies reporting immunomodulatory effects of fasting in in cancer patients

Ref	Cancer type	N	Intervention	Outcomes
De Groot et al., 2015. Ref. (39)	Breast cancer	13	• STF (0 kcal) 24 h before and 24 h after CT. 6 cycles. • Control arm: regular diet	• Decreased IGF-1. • Higher erythrocyte and thrombocyte numbers. • Reduced DNA damage in PBMCs.
Dorff et al., 2016. Ref. (48)	Distinct cancers	20	• STF (<200 kcal per day) 24 h, 48 h prior CT or 72 h (divided to 48 h prior to and 24 h post chemotherapy). 2 cycles maximum.	• Decreased IGF-1 by 30, 33 and 8 % in the 24, 48 and 72 h fasting cohorts, respectively. • Reduced DNA damage in PBMCs when fasting ≥48 h.
De Groot et al., 2020. Ref. (37)	Breast cancer	129	• FMD, 5 days, 3 days prior and on same day of NACT. 8 cycles. • Control arm: regular diet	• FMD arm did not receive dexamethasone. • Increased rate of radiologically response and pathological response. in ITT and PP analysis, respectively • Reduced DNA damage in PBMCs.
Vernieri et al., 2022. Ref. (167)	Distinct cancers	95	• FMD, 5 days, + standard antitumor therapies. 8 cycles. • Pre versus post intervention.	• Reduced glucose, insulin, and IGF-1. • Decreased immunosuppressive myeloid cells and Treg cells. • Increase of NK and CD8+T cells with an activated phenotype.
Vernieri et al., 2022. Ref. (167)	Invasive breast cancer	100	• FMD, 5 days, 1 cycle, 7-10 days before surgery, without treatments. • Pre versus post intervention	Preliminary data in breast cancer patients: -<u>In tumours</u> • Decreased IGF-1R and p-IGF-1R. • Increased CD8+ T cells infiltration and CD8/CD68+ ratio. • Increased expression markers of NKT, activated dendritic cells, central and effector memory CD4+ and CD8+ T cells and M1 subtype macrophages, and no change in M2 subtype. -<u>In PBMCs:</u> • Increase of DCs, NK cells, B cells and several subsets of memory T cells and reduction of exhausted and hyperexhausted T cells, Tregs, and MDSCs.

[2] STF: short-term fasting. FMD: fasting mimicking diet. CT: chemotherapy. NACT: neoadjuvant chemotherapy. PBMCs: peripheral blood mononuclear cells. DCs: dendritic cells. NKT cells: natural killer T cells. MDSCs: myeloid derived suppressor cells. Tregs: regulatory T cells. ITT: intention to treat. PP: per protocol. IGF-1: insulin growth factor 1. IGF-1R: insulin growth factor 1 receptor. p-IGF-1R: phosphorylated insulin growth factor 1 receptor.

Table 3: Overview of in vivo cancer models reporting immunomodulatory effects of exercise

Ref	Model	Intervention	Outcomes
Zielinski et al., 2004. Ref. (183)	• BALB/cByJ mice • EL-4 lymphoma cells implanted subcutaneously.	Treadmill running, high intensity, 3 h/day or until volitional fatigue, daily. Starting immediately before tumour inoculation.	• Delayed tumour growth. • Reduced macrophages and neutrophils infiltration (determined by hematoxylin-eosin histological analysis).
Murphy et al., 2004. Ref. (119)	• C57BL/6 mice • B16 melanoma cells implanted intravenously.	Treadmill running, 1 h/day, for 6 days. With or without oat fibre beta-glucan. Tumour inoculation 30 min after the last day of exercise.	• Decreased number of tumour foci at lungs in all groups compared to non-exercised non-beta glucan group. • Increased macrophages cytotoxicity against B16 cells *in vitro* compared to non-exercised non-beta glucan group.
William et al., 2009. Ref. (174)	• Swiss mice • Ehrlich tumour cells implanted subcutaneously.	Swimming, at 50% or 80% of maximal weight, 1 h/day, 5 days/week for 6 weeks. Starting 4 weeks before tumour inoculation.	• Reduced tumour growth at 50% of maximal workload. • Reduced macrophages and neutrophils infiltration (determined by N-acetylglucosaminidase and myeloperoxidase activity, respectively).
McClellan et al., 2014. Ref. (111)	C57BL/6 ApcMin/+ mice	Treadmill running. 1 h/day, 6 days /week from 4 to 16 weeks of age.	• No effect of exercise on polyp's number. • Reduction in the number of large polyps. • In mucosal tissue, decreased expression of general macrophage marker, M2 macrophage markers and Foxp3, increased expression of CD8.
Abdalla et al., 2014. Ref. (1)	• Balb/c mice • Breast tumours induced by7,12-dimethylbenzanthracene.	Swimming, 5 days/week for 8 weeks.	• M1 profile of peritoneal isolated macrophages (higher secretion of IFN-γ, TNF-α and IL-12).
Pedersen et al., 2016. Ref. (128)	• C57BL/6 mice • B16F10 melanoma or • Lewis's lung cells implanted subcutaneously or intravenously. • DEN-induced liver tumours • Transgenic (Grm1) EPv transgenic mice as melanoma model	Voluntary wheel running. 4 weeks before and/or after tumour inoculation.	• Reduced tumour growth/incidence and reduced lung metastases. In Tg(Grm1)EPv transgenic a trend to delayed tumour formation. • Upregulation of immune-related pathways in B16 tumours. • Increased NK, CD3+ and dendritic cells in B16 tumours. • Antitumor effects of exercise in B16 model suppressed by blockade of b-adrenergic signalling and anti-IL-6 antibody.

Bianco et al., 2017. Ref. (16)	• Balb/c mice • 4T1 murine breast cancer cell line implanted orthotopically.	Swimming, 5 days/week for 4 weeks, progressing from 30 to 45 min /day. Starting with one week of adaptation (15 min) when tumour inoculation.	• Reduced tumour growth. • Trend to increased infiltration of CD8+ T cells. • Increased number of differentiated bone marrow-derived DCs and expression of DC costimulatory CD80 and CD86 in tumours.
Hagar et al., 2019. Ref. (74)	• BALB/c wild type and nude (athymic) • 4T1 murine breast cancer cell line, implanted subcutaneously.	Treadmill running, increasing velocity up to 26 min/day, 5 days/week, for 8 weeks. Tumour inoculation 72 h after exercise intervention	• Reduced tumour growth and increased survival. • Increased total white blood cells. • Reduced FoxP3+Tregs in tumours. • No effect of exercise on tumour growth in athymic mice.
Rundqvist et al., 2020. Ref. (137)	• FVB mice • I3TC murine breast cancer cell line, implanted subcutaneously.	Voluntary wheel-running, 14 days prior to and after tumour inoculation, until end of experiment.	• Reduced tumour growth and increased survival. • Increased infiltration of CD8+ T cells in tumours, spleens, and tumour-draining lymph nodes. • Increased lactate and TCA cycle metabolites in plasma and secondary lymphoid organs. • Lactate and TCA cycle metabolites induced loss of CD62L in CD8+T cells *in vitro.* • Lactate increased Granzyme B expression in CD8+ T cells and cytotoxicity *in vitro.* • CD8+ T cells from trained mice transferred to tumour-bearing untrained animals increased survival and reduced rate of tumour growth, when compared to animals receiving CD8+ T cells from non-trained animals.
Wennerberg et al., 2020. Ref. (171)	• BALB/c mice • 4T1 murine breast cancer cell line, implanted subcutaneously.	Treadmill running, 30 min/day, 5 days/week, 8 days after tumour inoculation, until end of experiment.	• Reduced tumour growth. • Reduced number of MDSCs in spleens and tumours. • Increased Ki-67 and CD69 expression in NK cells in spleens. • Increased CD69 expression in CD8+T cells in tumours.
Garritsoni et al., 2020. Ref. (66)	• BALB/c mice • 4T1 murine breast cancer cell line implanted orthotopically.	Voluntary wheel running. Starting 6 weeks before tumour inoculation, and continued 6,20,24, and 28 days post-tumour injection.	• Reduced number of MDSCs in spleen, blood and tumour and reduced immunosuppressive effect of MDSCs over CD3+CD4+T cell proliferation. • Non-significant reduction in metastatic lung nodules.
Kim et al., 2020. Ref. (90)	• BALB/c mice • 4T1 murine breast cancer cell line	Treadmill running, low (10 m/min) or moderate intensity (15 m/min on a	• Reduced breast tumour latency and growth in low-intensity group. • No effect on cell proliferation.

	implanted orthotopically.	slope of 2.5°), for 5 days/week, 13 weeks. Started before tumour inoculation and continued after it. Mice were fed a high fat diet.	• Increased apoptosis in tumours in low-intensity group. • Decreased number of M2 macrophages in both exercising groups, no effect on M1 macrophages. • Myostatin inhibited M2 polarization *in vitro.*
Gomes-santos et al., 2021. Ref. (70)	• C57BL/6 wild-type and Cxcr3−/−, FVB and Balb/c mice • E0771, EMT6, MMTV-PyMT, MCa-M3C murine breast cancer cell lines, implanted orthotopically.	Treadmill running, progressing from 30 min to 45 min, daily, moderate-to-vigorous intensity, starting when tumours reached ~20 or 100 mm3, until end of experiment	• Reduced tumour growth and metastatic burden. • Tumour vessels' maturation and decreased hypoxia. • Increased CD8+ T cells in tumours, chemokines mediated. • Gene expression: reduced glucose uptake/insulin pathways, upregulation of oxidative metabolism and cytokine activation and downregulation of pathways involved with. immunosuppression in breast cancer.
Kurz et al., 2022. Ref. (99)	• Pancreatic cancer genetic model • p48Cre/+; LSL-KRasG12D/+ C57BL/6 mice p53R172H/+; KRASG12D/+; pdx-1Cre/+ (KPC) cells implanted orthotopically.	Treadmill running, low-intensity, 30 mins/day, 5 days/week. Starting 1 or 12 days after tumour inoculation	• Reduced tumour initiation and progression. • Antitumoral effects of exercise dependent on: CD8+T cells and their IL-15/IL-15Rα axis, beta-adrenergic signalling, and lymphocyte egress of from blood and secondary lymphoid organs.

[3] TCA: tricarboxylic acid. MDSCs: myeloid-derived suppressor cells. DCs: dendritic cells. Tregs: regulatory T cells. IFN γ: interferon gamma. TNF α: tumour necrosis factor alpha. IL 12: interleukin 12. IL-15: interleukin 15. IL-15Rα: Interleukin 15 receptor alpha.

Table 4: Overview of clinical studies reporting immunomodulatory effects of exercise in cancer patients

Ref	Cancer type	N	Intervention	Outcomes
Zylstra et al., 2022. Ref. (188)	Oesophageal adenocarcino-ma	40	• AE+RT, moderate intensity, supervised, during NACT. • Control arm: no restrictions on physical activity.	• Higher rates of tumour regression (Mandard scoring system). • Higher circulating CD3+ and CD8+ lymphocytes.
Ligibel et al., 2019. Ref.(103)	Breast cancer	49	• 60-90 min supervised: 20 minutes of RT + 30-45 minutes of moderate-intensity AE/ session, 2 sessions/week, + up to 180 min of unsupervised, moderate-intensity, aerobic exercise. For 3-6 weeks, pre surgery. • Control arm: book + relaxation audio guide	• Upregulation of pathways implicated in immunity and inflammation. • Trend towards a decrease in T regs cells in tumours.
Kurz et al., 2022. Ref. (99)	Pancreatic ductal adenocarcino-ma	70	• 60 min AE + 60 min RT/ week, unsupervised, during NACT or chemoradiation. • Controls: historical data.	• Increased infiltration of CD8+ T cells. • Trend toward higher expression of granzyme B in tumours. • Increase in the median overall survival of patients with high levels of CD8 or granzyme B.
Glass et al., 2015. Ref. (68)	Solid tumours	44	• 20-45 min AE, 3 sessions/ week, for 12 weeks, + cytotoxic therapy and synthetic erythropoietin. • Control arm: usual care.	• Trend toward higher circulating CD8+T cells and CD8+CD45RA+ T cells. • Decreased in pro-inflammatory cytokine and angiogenic factors.
Pal et al., 2021. Ref. (126)	Breast and prostate cancer	21	• 6–52 weeks after the end of primary therapy. • Acute exercise with cycle ergometer (cardiopulmonary exercise tests). • Chronic exercise with cycle ergometer (12 weeks): -Standard endurance: 30 min at vigorous intensity, 2 sessions/week -Polarized endurance: HIIT 4 times 4 min at 85–95% HR peak and 3 times 3 min recovery at 70% HR peak, 1 session/week + moderate-intensity continuous training at the first lactate threshold, 1 session/ week.	• Lower expression of inhibiting NK cell receptor KIR2DL1 after acute exercise. • Increased expression of the activating NK cell receptor NKG2D in the polarized group compared to the endurance standard group after 12 weeks of intervention.

Toffoli. et al., 2021. Ref. (161)	Colon and breast cancer	14	• 30 min AE + 20 min RT, 2 sessions/week, moderate-high intensity, supervised. During first 9– 12 weeks of (neo)adjuvant chemotherapy. • Control arm: no exercise but offered the intervention during the second half of chemotherapy.	• Preserved NK cell degranulation after chemotherapy. • Higher expression of the activating receptor NKp46 on CD56dimCD16+ NK cells. • Trend towards increased cytotoxicity. • Trend towards increased IL-6 levels.
Fairey et al., 2005. Ref. (54)	Breast cancer survivors	53	• 3 AE sessions/week. Progressive, 15 min for weeks 1–3 and then systematically increased by 5 min every 3 weeks to 35 min for weeks 13–15. Intensity 70–75% of peak oxygen consumption. Supervised. • Control arm: no Exercise.	• Increased NK cells cytotoxic activity. • Increased unstimulated peripheral lymphocytes proliferation.
Schauer et al., 2022. Ref. (141)	Prostate cancer	20	• One acute bout of AE in a cycle ergometer: watt-max test followed by four high-intensity intervals of 1 min, interspersed by 3 min of recovery at 30% of watt-max. Before radical prostatectomy. • Pre versus post intervention.	• Increased blood concentration of monocytes, neutrophils, and lymphocytes. • Preferential mobilization and egress of CD56dim over CD56bright NK cells, and CD8 T cells with more pronounced mature and cytotoxic phenotype, potentially accompanied by cell exhaustion (CD8+ CD57+ NKG2C+ Granzyme-B+ Perforin bright TIGIT+). • Increased NK cytotoxic activity against prostate cancer cell lines K562 and LNCaP but not PC-3. Decreased NK cytotoxic activity per-cell during exercise but improved 1-h post-exercise against all three cell lines.

AE: aerobic exercise. RT: resistance training. NACT: neoadjuvant chemotherapy. HIIT: high intensity interval training.

Table 5: Overview of preclinical and clinical studies using dietary restriction + exercise in cancer.

Preclinical studies using caloric restriction and exercise in cancer models.			
Ref	**Model**	**Intervention**	**Outcomes**
Xie et al., 2007. Ref. (177)	SENCAR mice (enhanced susceptibility to TPA-promoted skin carcinogenesis)	Experimental groups • AL sedentary (Control) • AL + Exe • Pair-fed + Exe (paired-fed at the same amount as the control group) • 20% CR • Exe + 20% CR CR: 20% less total calories from carbohydrates and fat for 12 weeks Exe: treadmill exercise 60 min/day, 5 day/week for 10 weeks.	• Reduced IGF-1 levels in CR and Exe + CR groups. • CR abrogated both Ras and PI3K signalling. • Increase in caspase-3-like proteolytic activity in Exe +CR compared to control.
Turbitt et al., 2019. Ref. (162)	BALB/c mice 4T1 murine breast cancer cell line implanted orthotopically.	Experimental groups • AL, sedentary (Control) • Exe • 10% CR • Exe + 10% CR. CR: 20% less total calories Exe: voluntary wheel running. Starting 8 weeks before tumour inoculation and continued after.	Exe + CR: • Delayed tumour growth, reduced metastatic burden, and improved survival. • Increased CD8+T cells and decreased MDSCs infiltration. • Reduced the expression level of metastatic and immunosuppressive genes.

Clinical studies using dietary restriction + exercise in cancer patients.				
Ref	**Cancer type**	**N**	**Intervention**	**Outcomes**
Albrecht et al.,2012. Ref. (3)	Advanced ovarian cancer	1	• Fasting of 18 h + 3.33 g flaxseed oil/kg body weight + two 200 mg dose of caffeine+ at least 90 minutes of exercise in treadmill. During 3 consecutive days for 3 consecutive months.	• CA-125 levels increased, but no signs of disease progression on CT scan. • Positive lifestyle modifications. • Improvements in anxiety, perceived stress, emotional functioning, and physical symptoms of peripheral neuropathy.

Harvie et al., 2021. Ref. (77)	Breast cancer	172	• Intermittent fasting group (IER): before CT, 2 days of 650-1000 kcal, 50 g carbohydrate, 50–70 g protein, 30–40 g fat). • Continued caloric restriction group (CER): Mediterranean diet, 30% energy from fat, 25% energy from protein and 45% from low glycaemic load carbohydrates, 5 portions of vegetables and 2 portions of fruit per day, alcohol to <10 U/week). Tailored to their energy requirements for weight loss (up to 25% energy restriction) or maintenance, depending on baseline BMI. • PA recommendation: 5 × 30 minutes of moderate intensity physical activity/ week & 2–3 sessions resistance exercise/week During adjuvant or neoadjuvant chemotherapy.	• Feasible. • Reported baseline PA was maintained at 3 weeks post chemotherapy in both groups.
Orgel et al., 2021. Ref. (125)	Acute lymphoblastic leukemia (10 to 21 years of age)	40	Caloric deficit of ≥10% and a home-based exercise intervention combining aerobic +resistance training, 15- to 30-min/day, daily (200 min/week). During the 4 weeks of induction chemotherapy.	• Feasible. • Reduced minimal residual burden risk. • Decreased fat gain in overweight and obese patients.

AL: ad libitum. Exe: exercise CR: caloric restriction. MDSCs: myeloid-derived suppressor cells.

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Involvement of neutrophils and macrophages in exhaustive exercise-induced liver, kidney, heart, and lung injuries

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ABSTRACT

Moderate exercise is effective for maintaining or improving overall health. However, excessive exercise that exhausts the adaptive reserve of the body or its ability to positively respond to training stimuli can induce tissue damage and dysfunction of multiple organs and systems. Tissue injury, inflammation, and oxidative stress are reportedly induced in the skeletal muscles, liver, and kidneys after exercise. However, the precise mechanisms underlying acute tissue injury after intense exercise have not yet been fully elucidated.

Studies using various experimental models of acute tissue injury, other than intense exercise, have demonstrated infiltration of inflammatory cells, including neutrophils and macrophages. These cells infiltrate injured tissues and induce inflammatory and oxidative stress responses by producing inflammatory cytokines and reactive oxygen species, thereby exacerbating tissue injury. In addition to the activation of blood neutrophils and increase in their levels during and/or after prolonged or intense exercise, chemokines that contribute to leukocyte migration are released, facilitating the migration of neutrophils and monocytes into tissues. Therefore, neutrophils and macrophages, activated by exhaustive exercise, may infiltrate tissues and contribute to exhaustive exercise-induced tissue injury. Recently, the contributions of neutrophils and macrophages to various tissue injuries caused by exhaustive exercise have been reported. In this review, we summarize the involvement of neutrophils and monocytes/macrophages in exhaustive exercise-induced non-skeletal muscle tissue injury. In addition, we present novel data demonstrating the contribution of neutrophils and macrophages to exhaustive exercise-induced cardiac and pulmonary injuries. Our study findings and the evidence presented in this review suggest that neutrophils and macrophages may play pivotal roles in exhaustive exercise-induced tissue injuries.

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INTRODUCTION

Moderate exercise is effective in maintaining and improving overall health, particularly in preventing metabolic syndrome, type 2 diabetes, and cardiovascular diseases [6, 80]. These effects arise from the stimulation of the internal environment of the body, triggering adaptive responses within the musculoskeletal, metabolic, digestive, cardiovascular, and respiratory systems. Specifically, endurance exercises cause an increase in mitochondrial content and function, resulting in enhanced aerobic power [10]. Consequently, various metabolic changes occur, including slower utilization of muscle glycogen and blood glucose, increased reliance on fat as an energy source, glycogen preservation, and lower production of lactate [23]. Furthermore, endurance exercises trigger cardiovascular adaptations, such as increased skeletal muscle capillary density, increased maximal cardiac output, and respiratory changes such as increased ventilation [3, 12, 20]. However, excessive exercises that exhaust the adaptive reserve of the body or its ability to positively respond to training stimuli can counteract the benefits of exercise [42, 62, 74]. Several conditions, including age, fatigue, disease state, and acute or chronic excessive exercise, can impair the adaptive reserve of the body. Recent studies suggest that the benefits of exercise may be decreased in individuals who frequently perform intense exercises, and a J- or U-shaped association has been reported between all-cause or cardiovascular mortality and running amount and frequency [42, 62, 74]. This phenomenon may be influenced by prolonged strenuous exercise-induced organ dysfunction and injury, as evidence suggests that injury occurs in tissues, such as the skeletal muscle, liver, and kidney, after prolonged endurance exercise in humans [78, 79]. Similarly, injury, inflammation, and oxidative stress have been reported in the skeletal muscle, liver, and kidney in animal models following exhaustive exercise using treadmill running and forced swimming methods [47, 52]. Therefore, exhaustion models have been widely utilized to reveal the mechanisms underlying tissue injuries caused by prolonged intense exercise and to establish preventive methods.

Recently, the contribution of neutrophils and macrophages, mediated by inflammation and oxidative stress, to exercise-induced tissue injuries has received increased attention. Prolonged strenuous

exercise increases the number of neutrophils in the blood circulation. This increase is bimodal, with an initial increase in the number of segmented neutrophils recruited from the marginated pool [82]. In contrast, neutrophils whose counts increase 2–3 h after prolonged strenuous exercise show a leftward shift of the nucleus [84] and high expression of CD62L, an adhesion molecule [39], indicating that these cells are derived from the bone marrow reserve. In addition, the plasma levels of neutrophil-derived granular enzymes increase with prolonged exercise [84], suggesting that prolonged and strenuous exercise activates neutrophils and causes degranulation. We have previously reported an increase in blood neutrophil counts after exhaustive exercise in a mouse model [61]. This increase may be attributed to the mobilization of neutrophils from the bone marrow reserve pool, as evidenced by the increased number of CD62L-expressing neutrophils and the decreased percentage of neutrophils in the bone marrow [59], supporting the results of previous studies [39, 84]. We have also noted increased CD11b and CD62L expression [61], suggesting that neutrophil migration is enhanced by exhaustive exercise. Furthermore, the number of monocytes in the blood increases, and these cells are activated after acute strenuous exercise [7]. Leukocyte infiltration into tissues is regulated by chemokines and adhesion molecules. Interleukin (IL)-8, which promotes neutrophil infiltration and activation, is released into the circulatory system during intense exercise [84, 85]. In addition, the level of monocyte chemotactic protein (MCP)-1, a chemokine that promotes monocyte and macrophage infiltration and activation, in the blood increases after prolonged strenuous exercise [84, 85]. Thus, after prolonged or intense exercise, neutrophils and monocytes are likely to migrate into the tissues. These cells have been reported to infiltrate tissues in various tissue injury models and cause inflammation and oxidative stress through the production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, and IL-1β, and reactive oxygen species (ROS), which are involved in tissue injury [15, 40, 68, 87, 92, 93]. In addition, these cells also contribute to exercise-induced muscle, liver, and kidney injuries [30, 31, 60, 61,102].

Exhaustive exercise can induce a systemic inflammatory response, resulting in tissue injury in the heart, lungs as well as in skeletal muscle, kidney and liver [44]. Notably, neutrophils and macrophages contribute to the onset and exacerbation of tissue injury in cardiac and pulmonary damage models, in which injuries have been induced using methods other than exercise [14, 21, 57, 58], suggesting that these cells may also contribute to exhaustive exercise-induced heart and lung injuries. Therefore, the present study aimed to test the hypothesis that increased infiltration of neutrophils and macrophages after exhausting exercise induces secondary cardiac and lung injuries. Although the involvement of neutrophils and macrophages in exercise-induced skeletal muscle injury has been systematically documented [83, 86], to the best of our knowledge, there are no reviews on the mechanisms of tissue injury other than skeletal muscle injuries. Therefore, we provide a comprehensive review that summarizes the involvement of neutrophils and macrophages in exhaustive exercise-induced liver, kidney, heart, and lung injuries.

Involvement of neutrophils and macrophages in exhaustive exercise-induced liver injuries

Prolonged exercise causes injuries to both the skeletal muscle and the liver. The susceptibility of the liver to injury from intensive exercise is attributed to sustained energy depletion and metabolic disturbances [25]. This susceptibility arises because the liver is a tissue that contributes to the neutralization of toxic substances, requiring substantial amounts of ATP [73], and becomes exposed to toxic metabolites when its production of ATP is suppressed by energy depletion and metabolic disturbances. In addition, the liver receives approximately 30% of the total cardiac output, 70% of which is via the portal venous system and 30% via the hepatic artery; this complex vascular supply renders hepatocytes susceptible to circulatory disturbances [4, 53]. After endurance exercise, the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), biomarkers of liver injury in the blood, increase in both animals and humans [72, 79]. In addition, we have previously observed histological changes, including hemorrhage, cytoplasmic vacuolation, inflammatory cell infiltration, impaired radial arrangement of hepatocytes, and cytoplasmic fragmentation, in the liver after exhaustive exercise [61].

Various experimental models of acute liver injury other than exhaustive exercise have demonstrated the infiltration of inflammatory cells, including neutrophils and macrophages [68, 91]. The role of neutrophils as major amplifiers of liver injury via the production of inflammatory cytokines and ROS has been previously discussed [5, 16]. neutrophil depletion using anti-GR1 antibodies ameliorates liver injury [55]. Blocking neutrophil infiltration in MMP-9-knockout mice or mice treated with MMP-9-inhibitory antibodies alleviates ischemia/reperfusion-induced liver injury and decreases the production of inflammatory cytokines [17]. Furthermore, alleviating oxidative stress via the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase alleviates neutrophil-induced liver injury after ischemia/reperfusion [18]. Recently, we have demonstrated that suppressing neutrophil infiltration into the liver using anti-neutrophil antibodies decreases the production of inflammatory cytokines and ROS in the liver after exhaustive exercise and alleviates exhaustive exercise-induced liver injury, assessed by blood ALT and AST activity and the liver stress score based on hematoxylin and eosin (HE) staining [61].

Macrophages are mobilized to the liver by chemokines, such as MCP-1, and play a role in acute liver injury [28]. They trigger an inflammatory response in the liver by activating the production of inflammatory cytokines and ROS, which exacerbate liver injury [26, 63]. Notably, Zhou et al. have recently reported increased macrophage infiltration in the injured liver after exhaustive exercise [102]. They reported that the administration of anti-inflammatory and antioxidant substances suppressed macrophage infiltration, inflammatory cytokine release, and liver injury, which was assessed by blood AST and ALT levels or histologically (HE staining), suggesting that macrophages are mediators of the inflammatory response in exhaustive exercise-induced liver injury. Furthermore, we have reported that the administration of anti-neutrophil antibodies suppresses macrophage infiltration and MCP-1 production [61]. As neutrophils release MCP-1, which modulates macrophage infiltration into local tissues and triggers an inflammatory response [77], neutrophils may serve as a source of chemokines in the injured liver, thereby contributing to liver injury via macrophage infiltration after exhaustive exercise.

Involvement of neutrophils and macrophages in exhaustive exercise-induced renal injuries

Strenuous exercise-induced tissue injury extends to the kidneys, with the incidence of acute kidney injury after endurance exercise events, such as marathon and triathlon races, reported to be 4%–86% [22, 29, 46, 54, 70]. A recent systematic review reported 27 cases of acute kidney injury requiring hospital treatment following an endurance event, as described in 11 case reports [13]. In addition, assessment of renal function before and after endurance exercise in 800 endurance event participants in 21 studies showed increased levels of serum creatinine, a marker of kidney injury, after endurance exercise. These findings were corroborated in an animal model in which exhaustive exercise increased plasma blood urea nitrogen and creatinine levels [45]. Moreover, histological changes, such as enlarged glomeruli, collapsed tubular epithelial cells, loss of brush border membranes in proximal epithelial cells, dilatation of tubules, and intratubular cast formation, have also been observed in animal models [45].

Exercise-induced acute kidney injury is attributed to several causes, including muscle injury, sympathetic tone, hypohydration, and ischemia/reperfusion. In particular, ischemia/reperfusion induces injury in various tissues, including the kidney. During exercise, blood flow to the kidneys is reduced by up to 25%; therefore, strenuous exercise can cause acute kidney injury [69], similar to that induced by ischemia/reperfusion. In ischemia/reperfusion-induced acute kidney injury, inflammatory cells, such as Neutrophils and macrophages, infiltrate and exacerbate tissue injury through the production of inflammatory cytokines and ROS. Neutrophils are among the early responders, entering the kidney within hours after ischemia/reperfusion-induced injury [43]. Notably, neutrophil depletion by anti-neutrophil serum administration ameliorates acute kidney injury induced by ischemia/reperfusion [19, 36]. Similar results have been reported with neutrophil depletion following the administration of antibodies against ICAM-1 or knockout of ICAM-1 [33, 34]. In addition. macrophage infiltration increases within 24 hours [99]. Studies on clodronate liposome-induced macrophage depletion have suggested that macrophages promote early injury after ischemia/reperfusion [11, 66]. Furthermore, Jo et al. reported that macrophage depletion with clodronate suppresses the production of inflammatory cytokines and chemokines after ischemia/reperfusion-induced acute kidney injury, indicating that macrophages are important mediators of this injury [27]. We tested our hypothesis that neutrophils and macrophages are also likely involved in exhaustive exercise-induced acute kidney injury. We found that exhaustive exercise induced marked histological changes, including congested and swollen glomeruli, tubular dilatation, and nuclear infiltration, as well as increased levels of kidney injury markers, apoptosis, and inflammatory responses in the kidneys. Notably, these exhaustive exercise-induced responses were suppressed by macrophage depletion [60]. Furthermore, we found that blocking neutrophil infiltration in the kidneys using anti-neutrophil antibody alleviates exhaustive exercise-induced renal injury (unpublished data).

Exhaustive exercise-induced injury to the heart, lungs, and pancreas

The cardiovascular response to exercise involves an increase in the heart rate and contractility due to increased sympathetic activity, resulting in increased cardiac output [41]. Exhaustive exercise induces adverse responses in the heart, such as impaired cardiomyocyte Ca2+ handling, mitochondrial dysfunction, and enhanced apoptotic signaling [48, 67]. Liao et al. reported increased levels of creatine kinase MB isozyme (CK-MB) and cardiac troponin I (cTnI), which are markers of myocardial injury [35], after exhaustive exercise [44]. Furthermore, focal necrosis, intravascular coagulation, scattered interstitial hemorrhage, and inflammatory cell infiltration have been observed in cardiac tissues [44]. Although exhaustive exercise-induced cardiac injury is generally attributed to the increased load on the heart resulting from increased cardiac output during exercise [48], in the present review, we focused on injuries secondary to neutrophils and macrophages. In ischemia/reperfusion-induced cardiac injury, neutrophils infiltrate into the heart where they are activated and produce ROS via NADPH oxidase and myeloperoxidase (MPO) [8, 21, 94]. Hiroi et al. reported that neutrophil accumulation via the activation of transient receptor potential melastatin-2, which is highly expressed in immune cells, promotes inflammatory responses and exacerbates cardiac injury [21]. Macrophages, which are mobilized during the initial inflammatory phase, also promote inflammation during cardiac injury through the production of TNF-α and IL-1β [64]. These reports suggest the possible involvement of neutrophils and macrophages in exhaustive exercise-induced cardiac injury.

In the lung tissue, histological changes, such as thickening of the alveolar septum, infiltration of inflammatory cells, and massive necrosis, occur after exhaustive exercise [44]. These changes may be caused by direct injury due to increased ventilation in response to intense exercise or ischemia/reperfusion-induced injury during exercise. Notably, both neutrophils and macrophages are involved in ischemia/reperfusion-induced lung injury [13, 15]. In particular, alveolar macrophages are important for initiating ischemia/reperfusion-induced lung injury [21]. TNF-α and IL-1β, produced by alveolar macrophages, are important mediators of intercellular signaling in the pathogenesis of lung injury [58]. Furthermore, neutrophil activation and infiltration are reportedly involved in ischemia/reperfusion-induced lung injury [58, 96]. However, it remains unclear whether neutrophils and macrophages contribute to exhaustive exercise-induced lung injury.

Despite limited studies, pancreatic tissue injury has also been reported [71]. In dogs, reduced blood flow to the pancreas during exercise has been reported [37], suggesting that ischemia/reperfusion may also be involved in exercise-induced pancreatic injury. Therefore, it is necessary to comprehensively examine the organs affected by exercise-induced tissue injury, warranting future investigations to reveal the mechanisms underlying tissue injuries.

METHODS

All animal experimental procedures for this study complied with the Guiding Principles for the Care and Use of Animals at Waseda University and were approved by the Institutional Animal Care and Use Committee of Waseda University (2013-A110). We investigated the effects of neutrophil and macrophage

depletion in male C57BL/6J mice. To investigate the effects of neutrophil depletion, male C57BL/6J mice were divided into four groups: sedentary with control antibody (n = 10), sedentary with anti-neutrophil antibody (n = 10), exhaustive exercise with control antibody (n = 10), and exhaustive exercise with anti-neutrophil antibody (n = 10). Anti-neutrophil (1A8) or control antibodies were administered intraperitoneally to mice 48 h before treadmill running experiments. To determine the effects of macrophage depletion, C57BL/6J mice were divided into four groups: sedentary with control liposomes (n = 8), sedentary with clodronate liposomes (n = 8), exhaustive exercise with control liposomes (n = 8), and exhaustive exercise with clodronate liposomes (n = 8). The mice were intraperitoneally administered clodronate or control liposomes 48 h before exhaustive exercise. The blood, heart, and lungs were collected 24 h after running until exhaustion on a treadmill with a 7% gradient and a speed of 24 m/min. Although a previous study has reported cardiac and pulmonary injuries during 7 days of exhaustive exercise (37), it was not clear whether these injuries occur during acute exhaustive exercise. As the present study was conducted as an exploratory study using cardiac and pulmonary injury markers and inflammatory cytokines to determine whether these injuries also occur during acute exhaustive exercise, no histological samples were prepared. Moreover, the levels of MPO in the heart and lung and CK-MB and cTnI in the plasma were measured using enzyme-linked immunosorbent assay (ELISA) (details are reported in the Supplementary Methods). The mRNA expression levels of Ly-6G, a neutrophil marker; F4/80, a macrophage marker; receptors for advanced glycation end-products (RAGE), a marker of lung injury; and inflammatory cytokines in the lungs and heart were measured using quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). The NADPH oxidase activity and hydrogen peroxide levels in the heart were also measured. Details are shown in Supplementary Methods.

RESULTS

Effect of neutrophil depletion on exhaustive exercise-induced heart and lung injuries

To determine the effect of 1A8 antibody treatment on exhaustive exercise-induced neutrophil infiltration in the heart and lungs, we analyzed the mRNA expression of Ly-6G, a specific marker of neutrophils. Exhaustive exercise significantly increased Ly-6G levels in the heart and lungs, whereas treatment with the 1A8 antibody decreased these levels (Figures 1 and 2). Consistently, while exhaustive exercise increased MPO levels in the heart and lung, injection of the 1A8 antibody reduced them (Figures 1 and 2). IL-8 mRNA expression in the heart and lung was not significantly altered by exercise or 1A8 treatment. Plasma CK-MB and cTnI levels, which are specific indicators of myocardial injury, increased with exhaustive exercise; however, these increases were significantly suppressed by treatment with 1A8 (Figure 1). In addition, the wet/dry weight ratio of the lung, a marker of pulmonary edema, and level of RAGE, a marker of lung injury, were significantly increased by exhaustive exercise and suppressed by anti-neutrophil antibody administration. The changes in the mRNA expression of several cytokines are shown in Figures 1 and 2. The inflammatory response induced by exhaustive exercise was suppressed by treatment with the 1A8 antibody (Figures 1 and 2). Moreover, NADPH oxidase and hydrogen peroxide levels in the heart were measured. Exhaustive exercise significantly increased cardiac NADPH oxidase and hydrogen peroxide levels, whereas treatment with the 1A8 antibody significantly suppressed these effects (Figure 1). The effect of exhaustive exercise and neutrophil depletion on macrophage infiltration in the heart and lung was evaluated using F4/80 mRNA. Although exhaustive exercise increased the F4/80 mRNA level in the heart and lung, injection of the 1A8 antibody reduced them (Figures 1 and 2). The mRNA expression of MCP-1 was also increased by exercise but ameliorated by the 1A8 antibody.

Effect of macrophage depletion on exhaustive exercise-induced heart and lung injuries

The effects of exhaustive exercise and clodronate liposome treatment were assessed based on F4/80 expression, which was significantly higher in the heart and lungs of the exhaustive exercise group than in those of the sedentary group. However, F4/80 expression was markedly lower in the exhaustive exercise with the clodronate liposome administration group (Figures 3 and 4). Exhaustive exercise significantly increased MCP-1 mRNA levels in both cardiac and pulmonary tissue, and these increases were suppressed by clodronate liposome treatment (Figures 3 and 4). Exhaustive exercise increased the CK-MB, cTnI, and RAGE levels and wet/dry weight ratio of the lung, confirming the results described above, and this increase was suppressed by clodronate liposome treatment (Figure 3). To elucidate the tissue inflammatory response after exercise, we measured the expression of TNF-α IL-6, and IL-1β in these tissues. In both tissues, the TNF-α and IL-1β levels were significantly increased after exhaustive exercise, and these increases were suppressed by macrophage depletion (Figures 3 and 4).

Discussion of original data

Exhaustive exercise induces cardiac and lung injuries [44]; however, it is not clear whether secondary injury by neutrophils and macrophages contributes to these injuries. In this study, we aimed to further elucidate the mechanisms underlying exhaustive exercise-induced tissue injury using neutrophil and macrophage depletion models. We observed increased Ly-6G mRNA expression in the heart and lung tissues of model mice after exhaustive exercise. In addition, treatment with an anti-neutrophil antibody suppressed these effects. MPO, one of the most abundant proteins in neutrophils reported to be associated with the number of infiltrating neutrophils in the heart and lungs [2, 101], was assessed, and the results were consistent with the Ly-6G levels. F4/80 mRNA level has been reported to be associated with the number of macrophages infiltrating the heart and lungs [24, 51]. In this study, its level was increased by exhaustive exercise and suppressed by clodronate liposome administration. Thus, these results suggest the efficacy of our protocol in blocking neutrophil and macrophage infiltration into tissues. In this study, no increase in IL-8 was observed after exhaustive exercise, neither in the heart nor in the lungs. We have previously reported increased CD11b and CD62L expression on blood neutrophils after exhaustive exercise [59], suggesting that adhesion factors might play a vital role in neutrophil infiltration into these tissues after exercise. In the present study, immunostaining and flow cytometric analysis were not performed to confirm neutrophil and macrophage

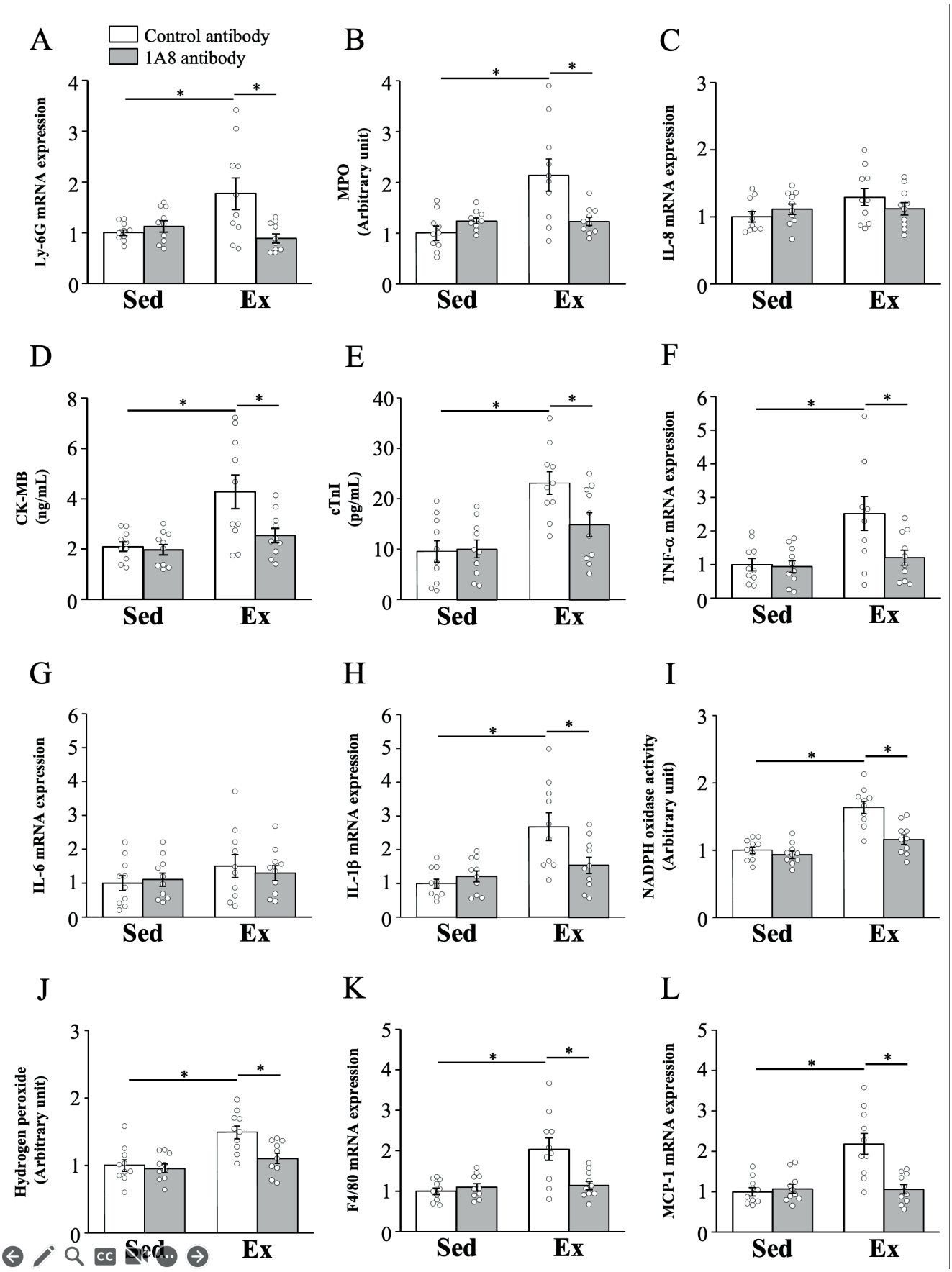

Figure 1. Effects of exhaustive exercise and treatment with the 1A8 antibody on heart injury. (A) Ly-6G expression in the heart. (B) MPO level. (C) IL-8 mRNA expression in the heart. (D and E) Plasma CK-MB and cTnI concentrations. (F) TNF-α, (G) IL-6, and (H) IL-1β expression in the heart. (I) NADPH oxidase activity, and (J) hydrogen peroxide concentration in the heart. (K) F4/80 and (L) MCP-1 mRNA expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *$P < 0.05$. Sed, sedentary; Ex, exercise; MPO, myeloperoxidase; CK-MB, creatine kinase MB isozyme; cTnI, cardiac troponin I; TNF, tumor necrosis factor; IL, interleukin; NADPH, nicotinamide adenine dinucleotide phosphate; MCP, monocyte chemoattractant protein.

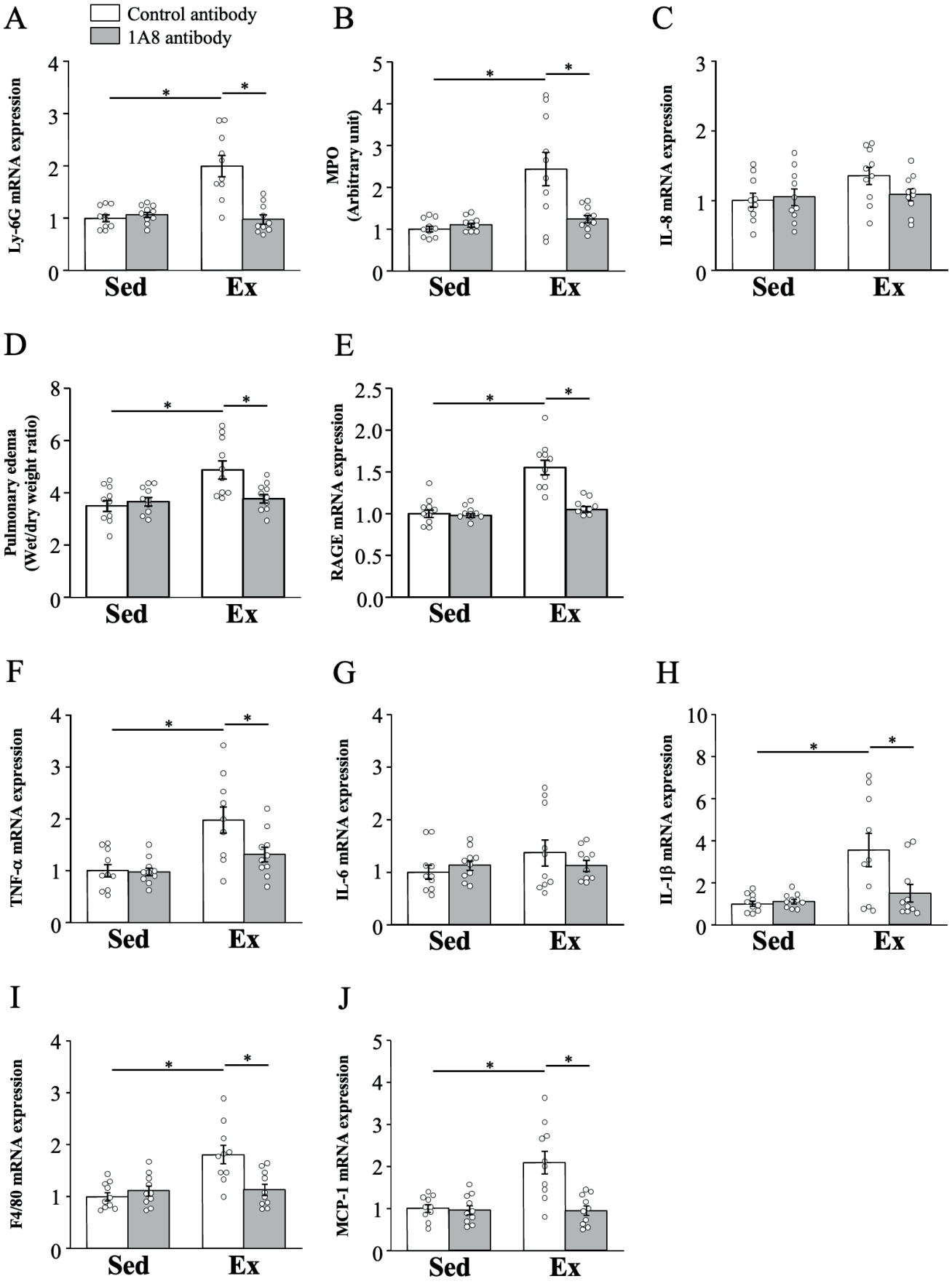

Figure 2. Effects of exhaustive exercise and treatment with the 1A8 antibody on lung injury. (A) Ly-6G expression in the lungs. (B) MPO level. (C) IL-8 mRNA expression in the lung. (D) Pulmonary edema. (E) RAGE, (F) TNF-α, (G) IL-6, (H) IL-1β, (I) F4/80, and (L) MCP-1 expression in the lung. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MPO, myeloperoxidase; RAGE, receptors for advanced glycation end-products; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemoattractant protein.

tissue infiltration because this study was conducted as an exploratory study examining the involvement of neutrophils and macrophages in acute exhaustive exercise-induced cardiac and pulmonary injuries, rather than continuous exhaustive exercise. As mentioned above, the number of neutrophils and monocytes in the blood increases after exercise [7, 61]; therefore, the Ly-6G, MPO and F4/80 results obtained in this study may reflect the dynamics of these cells in the blood. Thus, further studies on the infiltration of immune cells in various tissues after exhaustive exercise are required.

A previous study has reported cardiac injury with increased blood levels of CK-MB and cTnI in rats subjected to exhaustive exercise for 7 days [44]. In the present study, increased CK-MB and cTnI levels were observed after acute exhaustive exercise, suggesting that cardiac injury may have occurred. During ischemia/reperfusion-induced cardiac injury, neutrophils are recruited to the injured myocardium and activated, further exacerbating the cardiac injury by producing inflammatory cytokines and ROS [8, 21, 94]. Hiroi et al. have shown that H2O2-mediated activation of the neutrophil transient receptor potential melastatin-2 exacerbates ischemia/reperfusion-induced cardiac injury and promotes neutrophil infiltration and inflammation [21]. In addition, neutrophils secrete harmful degradative enzymes, such as MPO [8], and produce ROS via NADPH oxidase [94]. The superoxide generated by NADPH oxidase directly inactivates NO, thereby causing secondary endothelial dysfunction. Furthermore, the lack of NADPH oxidase suppresses ischemia/reperfusion-induced endothelial injury in humans [49]. Consistent with these reports, we demonstrated that anti-neutrophil antibody administration reduced exhaustive exercise-induced ROS production in terms of NADPH oxidase and H2O2 levels, as well as inflammatory cytokine expression, resulting in reduced cardiac injury in mice. Moreover, macrophage recruitment during the initial inflammatory phase of ischemia/reperfusion-induced cardiac injury may further damage the cardiomyocytes by releasing proteolytic enzymes, ROS, and inflammatory cytokines [64]. In the present study, macrophage depletion suppressed the mRNA expression of inflammatory cytokines and levels of cardiac injury markers in the blood, similar to the results observed in the anti-neutrophil antibody administration model. Notably, the administration of anti-neutrophil antibodies in this study prevented increases in F4/80 and MCP-1 mRNA after exhaustive exercise. Neutrophils have been reported to play a role in monocyte recruitment in ischemia/reperfusion-induced cardiac injury [1] and macrophage infiltration in exercise-induced cardiac injury via the production of MCP-1. However, further comprehensive studies are required to elucidate the interactions between neutrophils and macrophages. Few reports have examined changes over time in the mobilization and tissue invasion of these cells after exhaustive exercise; therefore, data collection over extended periods is necessary to fill this knowledge gap. In addition, future studies should aim to accumulate data over time in scenarios where either neutrophils or macrophages are depleted, as was done in this study.

Seven days of exhaustive exercise results in not only cardiac injury but also lung injury, with pulmonary alveolar swelling, inflammatory cell infiltration, and extensive necrosis of lung tissue [44]. In the present study, we assessed the levels of the wet/dry weight ratio of the lung, a marker of pulmonary edema, and RAGE, a membrane receptor expressed on alveolar type-1 epithelial cells in the lungs and a marker of epithelial damage [90], and found them to be increased by exhaustive exercise. These results suggest that acute exhaustive exercise induces lung injury. Neutrophil involvement has also been reported in lung injury [8], and Eppinger et al. have found that neutrophil depletion had no protective effect 30 min after reperfusion, but it attenuated the injury after 4 h [13]. Moreover, neutrophil depletion in the blood using a leukocyte filter alleviates post-reperfusion-induced lung injury [13]. Similarly, macrophages reportedly contribute to lung injury, and Eppinger et al. have reported the contribution of TNF-α and MCP-1 in lung injury [13], indicating an important role of alveolar macrophages immediately post-reperfusion. In the present study, treatment with anti-neutrophil antibody, as well as macrophage depletion, suppressed TNF-α and IL-1βmRNA expression, suggesting that neutrophils and macrophages may contribute to the inflammatory response in the lungs after exhaustive exercise. Furthermore, as in the heart, F4/80 and MCP-1 mRNA expression were suppressed in the anti-neutrophil antibody-treated group. Therefore, even in exercise-induced lung injury, neutrophils may contribute to macrophage infiltration through the production of MCP-1.

In this study, exhaustive exercise-induced cardiac and pulmonary injuries were reduced by neutrophil and macrophage depletion. In particular, injury marker levels in the macrophage-depleted group were decreased to the same level as in the control group. These results suggest that a secondary injury, involving inflammatory cytokine and ROS production by immune cells, might significantly contribute to cardiac and pulmonary injuries induced by exhaustive exercise. However, as the running exercise used in this study elicits a systemic stimulus, it is difficult to determine whether a primary or secondary injury is the major contributor. In a previous study, the administration of anti-inflammatory and antioxidant substances before exhaustive exercise prevented exercise-induced pulmonary and cardiac injuries [32, 101], supporting the hypothesis that inflammatory mediators released by neutrophils and macrophages may play an important role in exercise-induced tissue injury. Liao et al. reported cardiac and pulmonary injuries following seven consecutive days of exhaustive exercise, making comparisons between uphill and downhill exercise types [44]. The degree of lung lesion due to exhaustive exercise was higher in downhill exercise types than in uphill exercise types, and the CK-MB level was significantly higher during downhill exercise than during uphill exercise in cardiac injury [44]. Moreover, previous studies have reported an increase in blood neutrophil and monocyte counts after downhill exercise compared to those after uphill running [44, 76], suggesting that these variations in the infiltrating cells may influence differences in the extent of injuries associated with the type of exercise.

Histological changes in the heart and lungs have been reported seven days after exhaustive exercise [44]. In the present study, acute exhaustive exercise may have caused heart and lung tissue injuries, which may have been suppressed by the depletion of neutrophils and macrophages. However, the lack of histological evaluation showing tissue damage is a limitation of the present study, and a more detailed study is required.

Another important issue that should be addressed in future studies is the extent to which the exhaustive exercise

model used in this study applies to humans. In this study, exhaustive exercise increased plasma CK-MB and pulmonary edema by approximately 1.7–to 2.0-fold and 1.5-fold, respectively. Exhaustive exercise-induced tissue injuries are expected to be comparable or less severe than physical injury, such as ischemia/reperfusion, as CK-MB was increased by approximately 2.5-fold in ischemia/reperfusion-induced cardiac injury and edema was increased by approximately 1.5-fold in pulmonary injury [95, 97]. In human studies, approximately 3.75-fold and 14-fold increases in CK-MB levels have been reported immediately after a marathon race and a triathlon, respectively [65, 75]. In addition, CK-MB increased by 1.8-fold immediately after a half-ironman triathlon, but this increase was maintained up to 48 h after exercise [98]. Therefore, the CK-MB values suggest that the exhaustive exercise used in this study may not be of an intensity beyond that of exhaustive exercises in humans. While pulmonary function has been reported to be reduced after prolonged endurance exercise in humans [88], it is not clear whether lung injury occurs, partly due to the lack of useful biomarkers. In this study, pulmonary RAGE mRNA expression, a marker of lung injury, increased after exhaustive exercise; to the best of our knowledge, this is the first report of such an increase following exhaustive exercise. Plasma RAGE concentrations are increased in patients with acute liver injury and higher concentrations have been detected in patients with more severe lung dysfunction [56], highlighting the potential of RAGE as a blood biomarker for acute lung injury [8]. Unfortunately, in the present study, plasma RAGE levels could not be measured owing to insufficient sample volume. In future studies, we will aim to investigate whether plasma RAGE level can act as a marker of post-exercise lung injury and whether it increases after prolonged intense exercise.

This review focused on the involvement of neutrophils and macrophages in the initial response in exhaustive exercise-induced acute tissue injury. Although excessive inflammation causes tissue injury, the inflammatory response to moderate exercise contributes to tissue adaptation and recovery [9]. Notably, non-steroidal anti-inflammatory drugs have been shown to adversely affect skeletal muscle regeneration and adaptation [50, 89, 91]. Therefore, completely blocking inflammation may prevent certain tissues from adapting to exercise. The threshold of the inflammatory response is not clear and requires further investigation. Moreover, macrophages are believed to be associated with tissue repair and protection [59, 66], and the effects of these cell functions may not be entirely detrimental, as they may aid in the resolution of inflammatory reactions. The contribution of these cells to the recovery phase of exhaustive exercise-induced tissue injury is not clear and should be investigated in future studies.

CONCLUSION

This review summarizes the involvement of neutrophils and macrophages in exhaustive exercise-induced tissue injuries and provides original findings supporting the involvement of neutrophils and macrophages in exhaustive exercise-induced cardiac and pulmonary injuries.

SUPPLEMENTARY METHODS

Animals

Male C57BL/6J mice, aged 10 weeks, were purchased from Kiwa Laboratory Animals (Wakayama, Japan) and housed, 4 mice per cage, in a controlled environment under a 12-h light/dark cycle (lights on at 9:00 h and off at 21:00 h). All mice had free access to standard chow and water. The experimental procedures complied with the Guiding Principles for the Care and Use of Animals at Waseda University and were approved by the Institutional Animal Care and Use Committee of Waseda University (2013-A110).

Neutrophil depletion

The neutrophil-specific antibody anti-Ly-6G (clone 1A8) and isotype control antibody (clone 2A3) were purchased from Bio X Cell (Sunnyvale, CA, USA). Furthermore, 1A8 (0.5 μg) and 2A3 (0.5 μg) antibodies were individually diluted in phosphate-buffered saline, and the mice were intraperitoneally administered 150 μL of either antibody solution, according to their respective experimental groups. In our previous study under the same conditions, the efficiency of neutrophil depletion in blood was 49% immediately after exercise. After 24 h of exercise, it was 25% in the control group [61].

Macrophage depletion

To deplete macrophages, 150 μL of Clophosome-A (TM)-Clodronate Liposomes (Anionic) (Funakoshi, Tokyo, Japan) was administered intraperitoneally to the mice under anesthesia with 2% isoflurane inhalation at 0.8 L/min (Abbott Japan, Tokyo, Japan) using a gas anesthesia system for small laboratory animals (DS Pharma Biomedical, Osaka, Japan). Control mice were administered 150 μL of plain control Clophosome-A liposomes (Funakoshi) in a similar manner. In a preliminary study, we investigated the effects of the administration of clodronate liposomes or control liposomes on blood monocyte counts. The results are shown in Supplemental Table 1.

Exercise protocol

Mice in the sedentary groups remained under resting conditions in the cage, whereas mice in the exercise groups were subjected to exhaustive exercise 48 h after injection. One week before the exhaustive exercise regimen, the mice in all groups were familiarized with running on a motorized treadmill (Natsume, Tokyo, Japan). On the day of the experiment, the mice were forced, using a shock grid,

Table 1: Effects of clodronate liposomes or control liposomes on blood monocytes

	Blood monocytes (10^2/μL)			
	After administration of clodronate liposomes or control liposomes			
	Pre	24h	48h	72h
Control liposome administration group	3.4 ± 0.3	3.2 ± 0.4	3.5 ± 0.3	3.4 ± 0.3
Clodronate liposome administration group	3.3 ± 0.2	1.7 ± 0.2*, §	1.9 ± 0.3*, §	2.0 ± 0.4*, §

Values represent means ± SEM (n=5). Analyses were performed using mixed effect models for repeated measures.
*P<0.05 vs Pre, §P<0.05 vs Control liposome administration group at the same time point

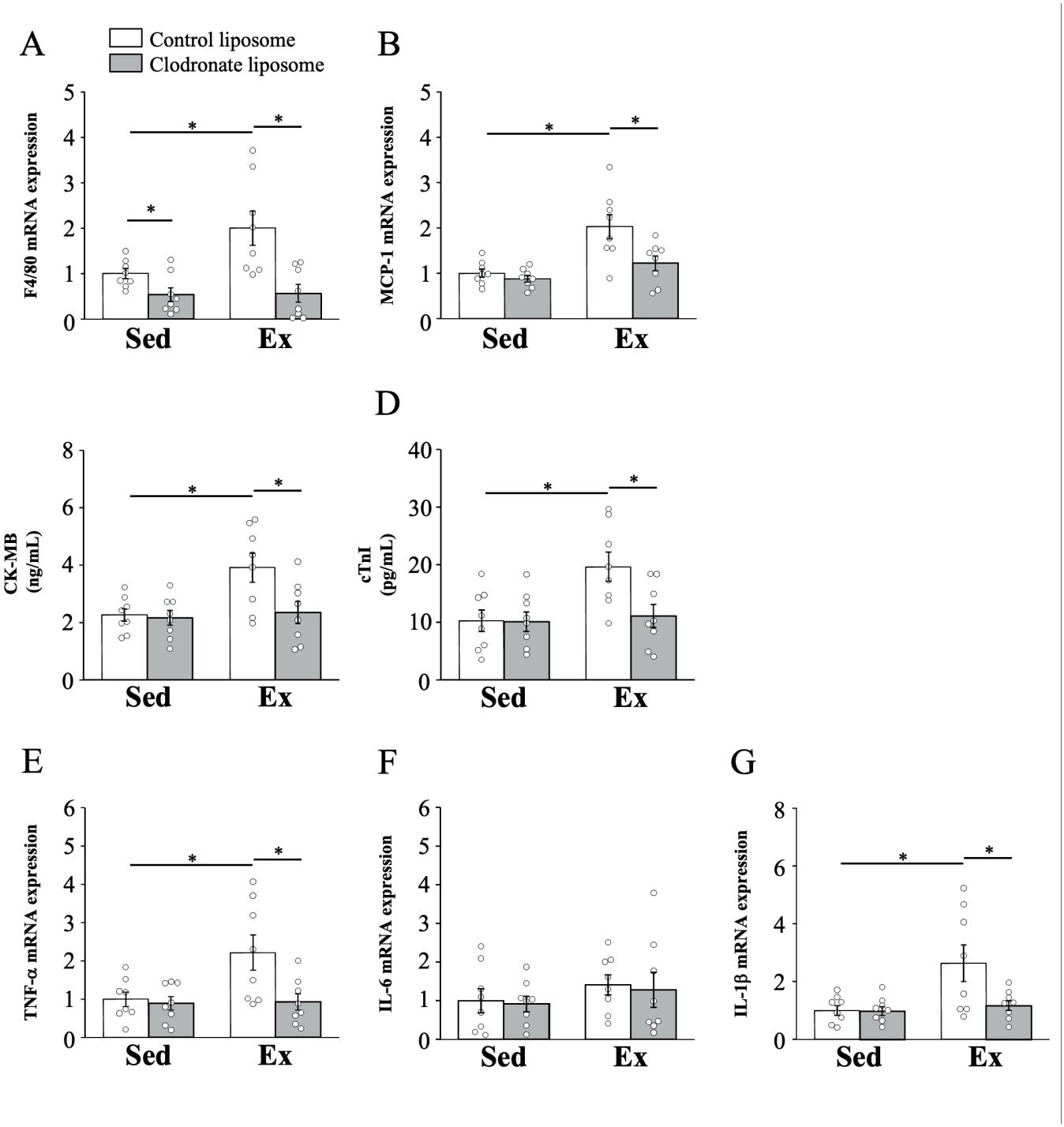

Figure 3. Effects of exhaustive exercise and clodronate liposome administration on heart injury. (A) F4/80 and (B) MCP-1 mRNA expression in the heart. (C and D) Plasma CK-MB and cTnI concentrations. (E) TNF-α, (F) IL-6, and (G) IL-1β expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MCP, monocyte chemoattractant protein; CK-MB, creatine kinase MB isozyme; cTnI, cardiac troponin I; TNF, tumor necrosis factor; IL, interleukin.

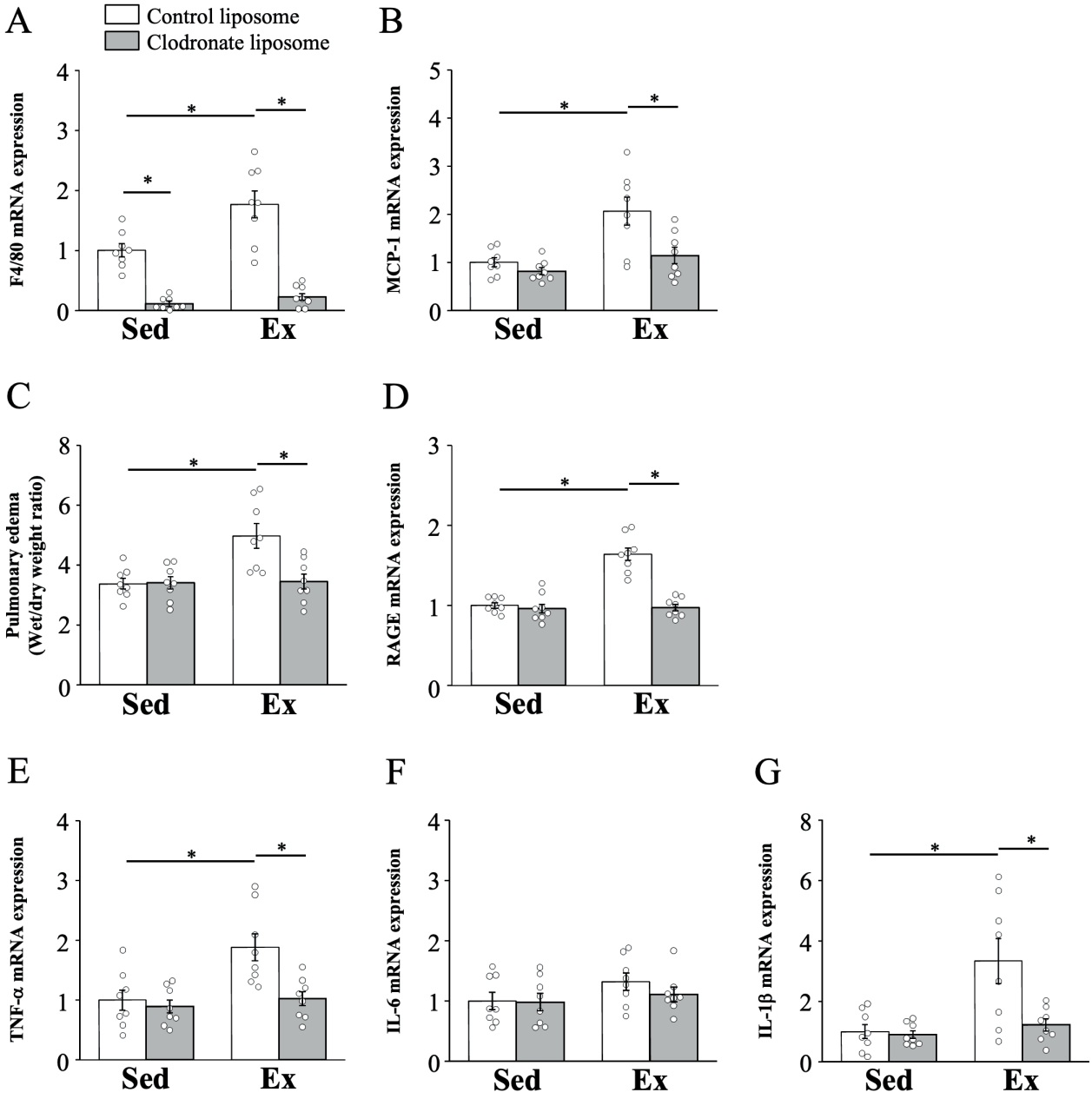

Figure 4. Effects of exhaustive exercise and clodronate liposome administration on lung injury. (A) F4/80 and (B) MCP-1 mRNA expression in the heart. (C) Pulmonary edema. (D) RAGE, (E) TNF-α, (F) IL-6, and (G) IL-1β expression in the heart. Values represent mean ± standard error of the mean. Analyses were performed using a two-way analysis of variance for multiple comparisons. *P < 0.05. Sed, sedentary; Ex, exercise; MCP, monocyte chemoattractant protein; RAGE, receptors for advanced glycation end-products; TNF, tumor necrosis factor; IL, interleukin.

to run on a treadmill with a 7% gradient at a speed of 10 m/min for 15 min, followed by 15 m/min for 15 min, 20 m/min for 15 min, and finally, 24 m/min until exhaustion. Exhaustion was defined as the point at which the mouse refused to run despite touching the shock grid five times. In an experiment examining the effects of neutrophil depletion, the mean running time until mice were exhausted was 338.5 ± 50.2 min in the control antibody group and 342.43± 11.2 min in the 1A8 antibody group. However, in an experiment examining the effects of macrophage depletion, the mean running time until the mice became exhausted was 161.0 ± 14.2 min in the control liposomes groups and 187.7 ± 13.8 min in the clodronate groups, which were not significantly different.

Blood, heart, and lung tissue sampling

The mice in all groups were euthanized 24 h after exhaustive exercise. Anesthesia was maintained by inhalation of 2% isoflurane at 0.8 L/min until exsanguination. The depth of anesthesia in this study was determined to be adequate based on the absence of any flexion response to a noxious stimulus, such as pinching the digit for approximately 2 s. Blood samples were collected from the abdominal aorta using a 1-mL syringe mounted with a 20-gauge needle and coated with heparin (5000 UI/mL; Nipro, Osaka, Japan). Blood samples were transferred to a heparin-coated tube and centrifuged at 2,600 × g for 10 min, and the plasma was stored at -80°C until analysis. The heart and lung tissues were snap-frozen by immersing in liquid nitrogen and stored at -80°C until analysis.

Assessment of blood biomarkers
Plasma CK-MB level was assayed using the Mouse Creatine Kinase MB ELISA Kit (Abcam, Cambridge, UK). Plasma cTnI level was measured using an ELISA kit (CSB-E08421m; CUSABIO, China). All procedures were performed according to the manufacturer's instructions. Optical density was analyzed using a SpectraMax 190 microplate reader (Molecular Devices LLC., San Jose, CA, USA). No standardization by protein content was performed. The inter- and intra-assay coefficient of variation for the same sample was less than 5%.

Measurement of pulmonary edema
After removal, the lungs were blotted briefly on a paper towel; then, they were weighted (wet lung weight). After 72 h of drying at 80°C in an incubator (dry lung weight), the lungs were weighed again, and the wet and dry lung weight ratio was determined.

NADPH oxidase activity
NADPH oxidase activity in the heart tissue was determined based on NADPH oxidation, measured at 340 nm, in a reaction mixture containing 50 mM Tris, 50 mM 2-(N-morpholino) ethanesulfonic acid (pH 7.0), 1 mM KCN (to inhibit low levels of mitochondrial oxidase activity), and 150 mM NADPH (Sigma) for 1 min at 37°C.

Hydrogen peroxide assay
To examine hydrogen peroxide levels in the heart, heart tissues were homogenized using a tissue protein extraction reagent with a protease inhibitor (Thermo, Rockford, IL, USA). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo). Hydrogen peroxide levels were measured using the SensoLyte ADHP Hydrogen Peroxide Assay Kit (Fremont, CA, USA) according to the manufacturer's instructions.

Myeloperoxidase level
Myeloperoxidase (MPO) level in the heart and lung was measured in the homogenate using the Myeloperoxidase Mouse ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA). MPO levels were normalized to protein concentrations using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)
Total RNA was extracted from the heart and lung tissues of mice using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE, USA), and samples with A260/A280 ratios between 1.9 and 2.1 were used for analysis. Total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). RT-qPCR was performed with the Fast 7500 real-time PCR system (Applied Biosystems) using 10 ng of cDNA and Fast SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile consisted of denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 3 s and annealing at 60°C for 15 s. The expression of target genes was normalized to that of 18S ribosomal RNA, a housekeeping control, using the 2-ΔΔCT method. The data are presented as fold change relative to the expression level in the sedentary group treated with the control antibody. The specific PCR primer pairs used for each gene are listed in Supplemental Table 2.

Statistical analyses
All data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using SAS software version 9.4. To evaluate the statistical significance of the relationship between exhaustive exercise and neutrophil or macrophage depletion, data were analyzed using a two-way analysis of variance. If significant interactions were observed, further comparisons were performed using Tukey's HSD post-hoc test.

Conflicts of interest statement
The authors declare no conflict of interest.

Gene	Forward	Reverse
18s ribosomal RNA	CGGCTACCA-CATCCAAGGA	AGCTGGAATTAC-CGCGGC
Ly-6G	TGGACTCTCA-CAGAAGCAAAG	GCAGAG-GTCTTCCTTC-CAACA
F4/80	CTTTGGCTATGG-GCTTCCAGTC	GCAAGGAGGA-CAGAG-TTTATC-GTG
RAGE	ACTACCGAGTC-CGAGTCTACC	GTAGCTTCCCT-CAGACACACA
TNF-α	TCTTCTCAT-TCCTGCTTGTGG	GAGGCCATTTG-GGAACTTCT
IL-6	AACGATGATG-CACTTGCAGA	TGGTACTC-CAGAAGACCA-GAGG
IL-1β	GGGCCTCAAAG-GAAAGAATC	TTGCTTGGGATC-CACACTCT
IL-8	AGAAGTTTT-GAAGAGGGCT-GAGA	AGTTTCACTG-GCATCTTCACT-GATT
MCP-1	CTTCTGGGCCT-GCTGTTCA	CCAGCCTACT-CATTGGGATCA

Tumor necrosis factor (TNF); interleukin (IL); receptors for advanced glycation end-products (RAGE); monocyte chemoattractant protein (MCP)

Table 2: Primer sequences for RT-PCR analysis.

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Immune Response to COVID-19 Vaccination in Elite Athletes

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ABSTRACT

Purpose: This study analyses the immune response of elite athletes after COVID-19 vaccination with double-dose mRNA and a single-dose vector vaccine.

Methods: Immunoglobulin G (IgG) antibody titers, neutralizing activity, CD4 and CD8 T-cells were examined in blood samples from 72 athletes before and after vaccination against COVID-19 (56 mRNA (BNT162b2 / mRNA-1273), 16 vector (Ad26.COV.2) vaccines). Side effects and training time loss was also recorded.

Results: Induction of IgG antibodies (mRNA: 5702 BAU/ml; 4343 BAU/ml (hereafter: median), vector: 61 BAU/ml; 52 BAU/ml, $p<0.01$), their neutralizing activity (99.7%; 10.6%, $p<0.01$), and SARS-CoV-2 spike-specific CD4 T-cells (0.13%; 0.05%; $p<0.01$) after mRNA double-dose vaccines was significantly more pronounced than after a single-dose vector vaccine. SARS-CoV-2 spike-specific CD8 T-cell levels after a vector vaccine (0.15%) were significantly higher than after mRNA vaccines (0.02%; $p<0.01$). When athletes who had initially received the vector vaccine were boostered with an mRNA vaccine, IgG antibodies (to 3456 BAU/ml; $p<0.01$), neutralizing activity (to 100%; $p<0.01$), CD4 (to 0.13%; $p<0.01$) and CD8 T-cells (to 0.43%; $p<0.01$) significantly increased. When compared with dual-dose mRNA regimen, IgG antibody response was lower ($p<0.01$), the neutralizing activity ($p<0.01$) and CD8 T-cell ($p<0.01$) response higher and no significant difference in CD4 T-cell response ($p=0.54$) between the two regimens. Cumulative training loss (3 days) did not significantly differ between vaccination regimens ($p=0.46$).

Conclusion: mRNA and vector vaccines against SARS-CoV-2 appear to induce different patterns of immune response in athletes. Lower immune induction after a single-shot vector vaccine was clearly optimized by a heterologous booster. Vaccine reactions were mild and short-lived.

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INTRODUCTION

The world-wide coronavirus pandemic led to many medical, social, and health care system challenges. An infection with SARS-CoV-2 can cause severe COVID-19 with pathology including pulmonary inflammation, pulmonary fibrosis, or vascular thrombosis (1). Moreover, neurologic complications (2), olfactory and gustatory dysfunctions (3), and cardiac manifestations like myocarditis(4) may result. Important preventative/hygiene measures like frequent disinfection, wearing face masks and social distancing were recommended and used in the beginning of the pandemic(5) while different types of vaccines (vector-vaccine, mRNA vaccine, protein-based) were developed with some delay knowing that vaccinations are one of the most effective means to prevent the spread and severe courses of many infection diseases(6).

Due to vaccine shortage, it was initially necessary to prioritize older people, medical staff and other high-risk populations for vaccinations, mostly without individual choice of vaccine type. In May 2021, during the last preparation stages for the Olympic and Paralympic Games in Tokyo, aspirants for the German Olympic and Paralympic team were prioritized for vaccination based on a political decision of the German government, considering that vaccinating athletes against COVID-19 had been strongly advised (7). With different vaccine types available (and very little experience with mRNA vaccines in general), their immunogenicity and reactogenicity could be expected to differ and potentially differ in their impact on the training (e.g. time loss due to vaccine reactions) and the safety of athletes (e.g. protection from acquiring an infection) prior to and during the Olympic Games. The double-dose mRNA vaccines BNT162b2 (Comirnaty® by BioNTech) and mRNA-1273 (Spikevax® by Moderna) are based on non-replicating mRNA delivered via lipid-based nanoparticles. SARS-CoV-2 spike-encoding mRNA are translated by muscle cells or tissue resident antigen-presenting cells followed by its secretion and/or presentation on the cell surface. These viral spike proteins are recognised as foreign antigens and trigger cellular and humoral immune response(8). The mRNA vaccines were approved based on pivotal trials showing vaccination efficacy of 95%(9) and 94%(10), respectively. Overall, vaccine reactions were reported to be mild and short-lived (mean of 2-3 days) in these investigations (9, 10). The single-dose vector vaccine Ad26.COV.2 (Janssen® by Johnson&Johnson (renamed in 2022 as Jcovden®) is a recombinant, replication-incompetent human adenovirus type 26-based vector that encodes the SARS-CoV-2 spike protein, inducing expression and an immune response. It was officially approved with an effectiveness of 67% in the pivotal trial (11). At the time of the first athlete prioritization, only BNT162b2, mRNA-1273, and Ad26.COV.2 were available. It must be noted that at this time the double-dose ChAdOx1 nCoV-19 vector vaccine (by AstraZeneca) was no longer recommended for people under 60 years of age in Germany (12). Despite the considerably lower effectiveness of Ad26.COV.2 as demonstrated in the registration studies, Ad26.COV.2 was considered a practical choice for members of the German Olympic team in summer 2021 in Germany (7). A single-shot vaccination was considered promising by many athletes (and medical advisors) due to a potential induction of less vaccine side effects and possibly a faster build-up of SARS-CoV-2-specific immunity. The aspect of formally receiving a vaccinated state (meaning a certificate needed for traveling) more quickly added to the positive image of the vector vaccine particularly in the athletes.

Understanding that vaccinating athletes against SARS-CoV-2 is important, it also needs to be mentioned that sport may lead to changes in the immune system of athletes. Intensive training programs in the preparation phase for major competitions may result in an increased susceptibility to infections due to a reduction in the number of immune cells and an associated reduction in functionality(13). Therefore, it is important to understand the influence of COVID-19 vaccines on the immune system of athletes. In general data about vaccinating athletes is limited due to concerns in athletes about safety and efficacy of vaccinations - but it is important to understand more about the immune system of athletes (4, 14).

The aim of this study was to determine the immune response of elite athletes after COVID-19 vaccination as well as comparing the humoral und cellular immune response between double-dose mRNA vaccines and a single-dose vector vaccine in this population. We hypothesized a significant induction of the immune response after both vaccine types with a stronger induction of the immune response after double dose regimen compared to a single dose vector vaccine. We further hypothesized that vaccine related adverse events will overall be mild and short-lived but that training restrictions will be lower after a single dose compared to a double dose vaccine. Later changes in official vaccination policies putting more emphasis on booster vaccinations enabled us to carry out some comparison between homologous and heterologous booster vaccination in our elite athlete population.

METHODS

Participants

72 healthy elite athletes older than 16 years participated in this prospective study. Among individuals who were vaccinated with an mRNA vaccine (mean of 21 years ± 6 years (standard deviation)), 29 were females (28: BNT162b2, 1: mRNA-1273) and 27 were males (25: BNT162b2, 2: mRNA-1273). The mean age of the 5 female and 11 male athletes of the Ad26.COV.2 group was 28±5 years (standard deviation). In their respective sports discipline, the athletes performed on international or national level. Recruitment was supported by the Olympic Training Centre Saarbrücken, the University Hospital Charité Berlin and the Institute of Applied Training Science (IAT) in Leipzig mainly via personal communication with the athletes from May 2021 to September 2021. Exclusion criteria were hypersensitivity or allergy to one of the ingredients of the vaccines, a clinically relevant immunodeficiency, or an acute illness. Medication intake was not verified by means of blood profiling, but participants were explicitly asked about serious illnesses and possible treatments.

Ethics approval.

The study was carried out in accordance with the Helsinki

declaration and approved by the local ethics committee (133/21, Ärztekammer des Saarlandes, Saarbrücken, Germany). All participants were informed about the study procedures, prior to giving written informed consent. Parents signed informed consent for participants under the age of 18 years.

Study design

All participants received one out of three approved and vaccine regimens recommended at the time of the study. The regimen was chosen depending upon availability or personal preference, as a randomized controlled assignment of the vaccine was not intended and not possible under the circumstances in mid 2021. The available vaccines were mRNA-1273 (Spikevax® by Moderna, 3 athletes), BNT162b2 (Comirnaty® by BioNTech/Pfizer, 53 athletes) and Ad26.COV.2 (Jcovden® by Janssen, 16 athletes). mRNA-1273 and BNT162b2 are double-dose mRNA vaccines whereas Ad26.COV.2 was approved as a single-dose vector vaccine. Blood samples were taken before vaccination to determine baseline reactivity and exclude previous contact with SARS-CoV-2 antigens during asymptomatic infection. Moreover, short-term immunogenicity was analysed two weeks after the second dose in case of mRNA vaccines, and three weeks after the single dose vector vaccine (due to known differences in vaccine-induced peak immune responses after the first and the second vaccination(15)). Follow-up analyses were performed 6 months after the last vaccination. Further evidence for prior infection with SARS-CoV-2 was tested using an NCAP-ELISA that was performed at least once (primarily after second mRNA vaccination, or after the first Ad26.COV.2 vaccination to test for the presence of antibodies to the SARS-CoV-2 nucleocapsid protein). The study design is illustrated in figure 1. The athletes recorded all local and systemic adverse events such as pain, redness and swelling at the injection site as well as headache, fatigue, muscle pain, chills, and nausea during the first week after each vaccination by completing a standardized questionnaire. Each adverse event was rated by means of four different levels of severity. Experiencing no side effects was rated 0, whereas mild, moderate, or severe side effects were graded with 1, 2, and 3, respectively. Mild side effects were defined as adverse reactions that did not interfere with training and daily routine, moderate side effects impaired but still allowed training and daily routine, whereas severe side effects prevented training and daily routine for at least one day. Therefore, training restrictions in the context of this study were solely based on occurrence of moderate or severe side effects, whereas restrictions based on precaution were not considered. For regimens with two vaccination time points, all days with training restrictions were added to determine the total number of days lost.

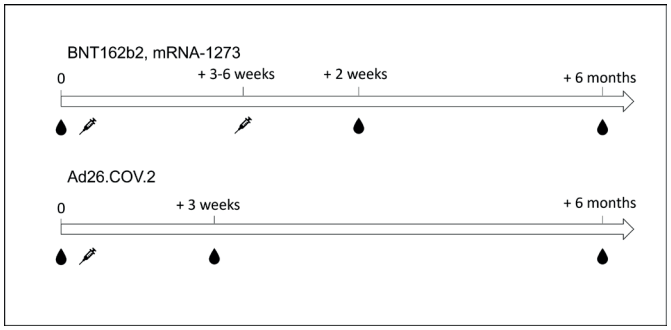

Figure 1. Overview of the study design with the vaccine regimens and their matching blood samples.

Necessary adjustments during the course of the study

After collecting the samples 2/3 weeks after vaccination and analysing the humoral and cellular immune response we found that the single-dose vector vaccine led to an insufficient humoral immune response in our athletes (e. g. median IgG antibodies after double-dose mRNA vaccination: 5702 BAU/ml, median IgG antibodies after single-dose vector vaccination: 61 BAU/ml). To provide adequate protection from COVID-19, recommendations for athletes were modified (and the study design had to be adjusted accordingly) by offering a heterologous boost vaccination to optimize the immune response in these athletes. This was carried out in 11 out of 16 athletes with the BNT162b2 vaccine after a median time of 119 days. An additional blood sample was taken two weeks after the heterologous boost to analyse the immune response. The adjusted study design can be seen in figure 2. The study adjustment was approved by the local ethics committee on September 6, 2021.

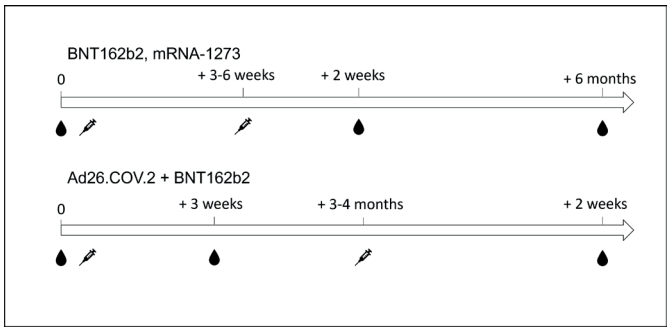

Figure 2. Overview of the adjusted study design with timelines for vaccination and blood sampling.

Procedures for immunological analyses

Lymphocyte subpopulations as well as vaccine-induced IgG antibody titers, neutralizing activity, and CD4 and CD8 T-cells were analysed from heparinized blood as previously described(16). Blood samples (9ml) were taken from an antecubital vein. The time of day was variable and deemed acceptable for our targeted parameters.

Vaccine-induced humoral immune responses were tested using ELISA assays as described by the manufacturer´s instruction (Euroimmun, Lübeck, Germany). An enzyme-linked immunosorbent assay (ELISA, SARS-CoV-2-QuantiVac) was used to quantify SARS-CoV-2 specific IgG antibodies against the receptor binding domain. Thresholds were set at <25.2 BAU/ml for being negative, ≥25.2 to <35.2 BAU/ml for being intermediate and ≥35.2 BAU/ml for being positive. An anti-SARS-CoV-2-NCP-ELISA was used to quantify SARS-CoV-2 specific IgG towards the nucleocapsid (N) protein. A surrogate neutralization assay that is based on antibody-mediated inhibition of soluble ACE2 binding to the plate bound S1 receptor binding domain (SARS-CoV-2-NeutraLISA) was used at a single serum dilution. Surrogate neutralizing capacity was calculated as percentage of inhibition (IH) by 1 minus the ratio of the extinction of the respective sample and the extinction of the blank value (16). The stimulus threshold was set according to manufacturer instructions with IH being negative under 20%, intermediate between 20 and 35% and positive over 35 %.

The protocol for quantification of SARS-CoV-2 spike-specific CD4 and CD8 T cells has been described before

(16). In brief, spike-19 specific CD4 and CD8 T-cells were quantified after a 6h stimulation with SARS-CoV-2 spike-derived overlapping peptides (each peptide 2 µg/ml, JPT, Berlin, Germany). Stimulation with 0.64% dimethyl sulfoxide (DMSO) and with 2.5 µg/ml of Staphylococcus aureus Enterotoxin B was used as a negative and positive control, respectively, to secure the specificity of the stimulation. Immunostaining was performed using anti-CD4 (clone SK3, 1:33.3), anti-CD8 (clone SK1, 1:12.5), anti-CD69 (clone L78, 1:33.3) and anti-IFNγ clone 4S.B3, 1:100, all antibodies from BD), and analyzed using flow-cytometry (BD FACS Canto II including BD FACSDiva software 6.1.3) (16). SARS-CoV-2-reactive CD4 or CD8 T-cells were identified as activated CD69-positive T-cells producing IFNγ. The percentage of specific T-cells was quantified by subtracting the percentage of T-cells after negative control stimulation from that after spike-specific stimulation. Detection limit was set at 0.03% as described before (16, 17).

Statistics

Statistical analysis was performed using R studio (version 4.0.5). Normal distribution of data was assessed using the Shapiro-Wilk test. No target parameter was distributed normally. Consequently, the nonparametric Wilcoxon test was used to analyse the quantitative parameters IgG antibody titre, neutralizing activity, CD4 and CD8 T-cells before and after vaccination. The Mann-Whitney-U-test was used to compare the immune response of the different vaccines and to analyse the vaccine side effects. The significance level was set at $p < 0.05$ for the α error. The effect size r for the Mann-Whitney-U and the Wilcoxon test was calculated with $|Z|/\sqrt{n}$ with Z being the standardised value and n the number of cases. Z was calculated with $x - \mu / \delta$. The effect size r is defined with r being small >0.10, medium >0.30 and large >0.50. No sample size analysis was performed because targeting a specific effect was not possible and intended; no comparable studies were available at that time.

RESULTS

Comparison of the immune response before and after vaccination

None of the athletes were tested NCAP-positive, which excluded a history of SARS-CoV-2 infection. The mRNA vaccines induced a significant immune response as indicated by an increase in IgG antibodies ($z=-6.5$, $p<0.01$, $r=0.87$), neutralizing antibodies ($z=-6.5$, $p<0.01$, $r=0.87$), as well as spike protein-specific CD4 ($z=-6.5$, $p<0.01$, $r=0.87$) and specific CD8 T-cells ($z=-4.9$, $p<0.01$, $r=0.70$). The aforementioned mRNA group comprises two vaccines, with mRNA-1273 being obtained from only three athletes. The IgG antibodies and neutralizing antibodies of the three athletes fall within the interquartile range of the mRNA group – which they belong to. Nevertheless, the values of the CD4 and CD8 T cells exhibit slight discrepancies, and thus, they are presented separately here (CD4 T cells: 0.05%, 0.48%, 0.67%; CD8 T cells: 0.01%, 0.05%, 0.11%). The Ad26.COV.2 vaccine also induced a significant increase in IgG antibodies ($z=-4.2$, $p<0.01$, $r=0.88$), neutralizing activity ($z=-4.2$, $p<0.01$, $r=0.88$), CD4 spike T-cells ($z=-3.4$, $p<0.01$, $r=0.87$) and CD8 spike T-cells ($z=-4.2$, $p<0.01$, $r=0.88$). Data are shown in table 1.

Comparison of the short-term immune response between the different vaccine regimens

When comparing immune-responses after vaccination, median IgG-levels were significantly higher after the mRNA vaccination ($z=-6.1$, $p<0.01$, $r=0.71$) than after the Ad26.COV.2 vaccination. This also held true for median neutralizing activity ($z=-6.1$, $p<0.01$, $r=0.71$), and CD4 T-cells ($z=-4.4$, $p<0.01$, $r=0.52$). In contrast, the Ad26.COV.2 vaccine induced a significantly higher CD8 T-cell response as compared to the mRNA vaccine ($z=-4.1$, $p<0.01$, $r=0.48$). Spike-specific IgG antibody levels and neutralizing activity as well as spike-specific CD4 and CD8 T-cell levels after vaccination are illustrated in figure 3.

Immune response after heterologous vaccination

A second heterologous mRNA vaccination with BNT162b2 was recommended for all individuals who had received a single dose of Ad26.COV.2. This led to a significant increase in both humoral and cellular immune responses (figure 3). IgG-levels increased from a

	mRNA			Ad26.COV.2		
Parameter	before	after	p-value	before	after	p-value
Spike specific IgG antibodies	4 BAU/ml (IQR 4 BAU/ml)	5702 BAU/ml (IQR 4343 BAU/ml)	<0.01	4 BAU/ml (IQR 2 BAU/ml)	61 BAU/ml (IQR 52 BAU/ml)	<0.01
Spike specific Neutralizing antibodies	0 % (IQR 0%)	99% (IQR 0.48%)	<0.01	0 % (IQR 0%)	11% (IQR 24%)´	<0.01
Spike specific CD4 T-cells	0 % (IQR 0.01%)	0.13 % (IQR 0.12%)	<0.01	0 % (IQR 0.01%)	0.05% (IQR 0.05%)	<0.01
Spike specific CD8 T-cells	0 % (IQR 0.005%)	0.02% (IQR 0.06%)	<0.01	0 % (IQR 0.003%)	0.15% (IQR 0.19%)	<0.01

Table 1 Blood parameters before and after vaccination with mRNA and Ad26.COV.2. Spike-specific IgG antibody levels [BAU/ml], neutralizing activity [%IC50], and the percentage of spike-specific CD4 and CD8 T-cells were quantified after two doses of a mRNA vaccine (n=56; BNT n=53, mRNA-1273 n=3) and after a single dose of Ad26.COV.2 (n=16), as well as before those vaccinations. Median values and interquartile range (IQR) are given.

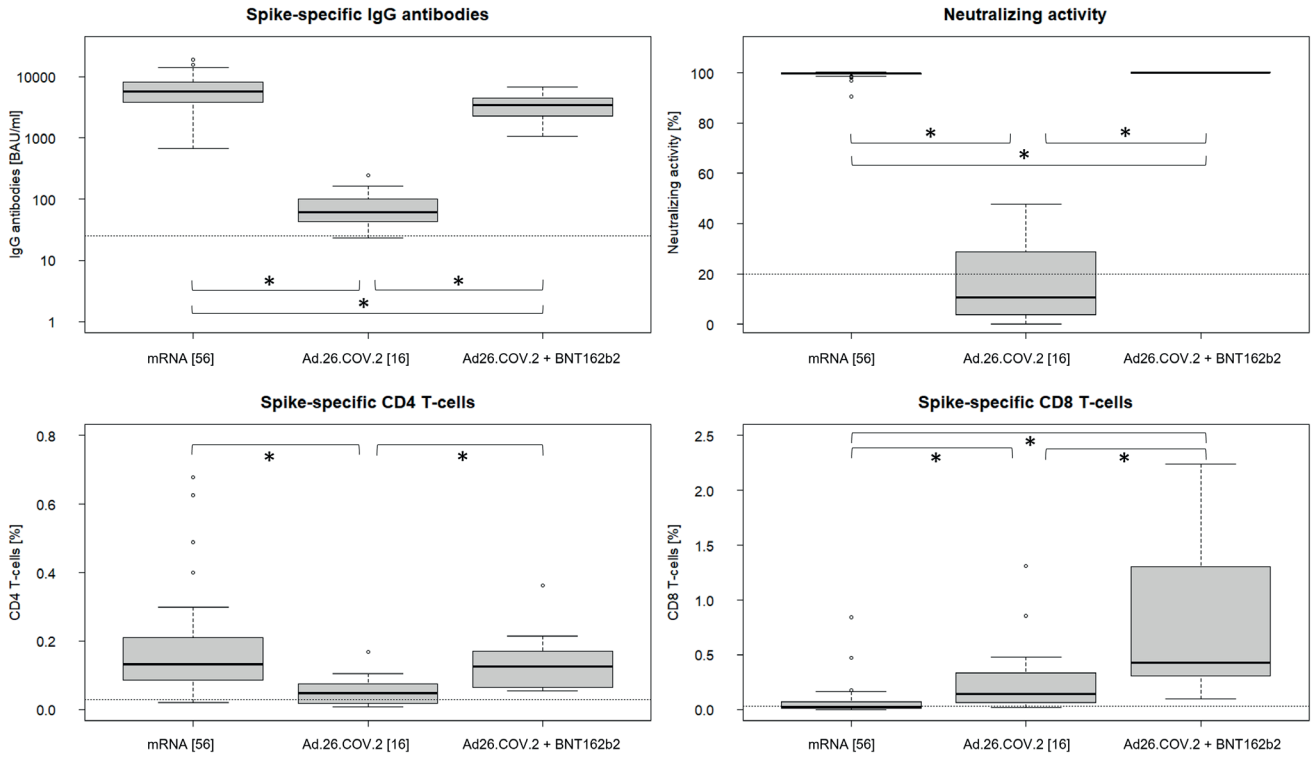

Figure 3. Vaccine-induced antibodies and T cells. Median spike-specific IgG antibody levels [BAU/ml], neutralizing activity [%IC50], and the percentage of spike-specific CD4 and CD8 T-cells were quantified after two doses of a mRNA vaccine (n=56; BNT n=53, mRNA-1273 n=3), a single dose of Ad26.COV.2 (n=16) or after heterologous combination of Ad26.COV.2 followed by BNT (n=11). Thresholds defining a negative response are indicated by a stippled line. Asterisks mark significance <0.05.

median of 61 BAU/ml (IQR 52 BAU/ml) to a median of 3456 BAU/ml (IQR 2209, z=-3.3, p<0.01, r=0.88) and the neutralizing activity from a median of 11% (IQR 24) to 100% (IQR 0.24, z=-3.3, p<0.01, r=0.88). Likewise, spike-specific CD4 T-cells increased from a median of 0.05% (IQR:0.05) to 0.13% (IQR 0.1, z=-2.6, p<0.01, r=0.75) and the CD8 T-cells from a median of 0.15% (IQR:0.19) to 0.43% (IQR 1, z=-2.6, p<0.01, r=0.75).

Comparison of the immune response after mRNA vaccine regimen and adjusted regimen

When compared to the homologous mRNA double dose vaccination regimen, IgG antibody levels after heterologous vaccination were moderately lower (z=-2.6, p<0.01, r=0.32), while the neutralizing activity (z=-3.6, p<0.01, r=0.45) and the CD8 T-cell response (z=-4.8, p<0.01, r=0.58) were significantly more pronounced. No difference was observed in CD4 T-cell levels (z=-0.6, p=0.54).

Long-term immune response after mRNA vaccine regimen

For the mRNA vaccines, all four chosen indicators significantly decreased after 6 months: IgG from a median of 5702 BAU/ml (IQR 4343 BAU/ml) to 1043 BAU/ml ((IQR 1112 BAU/ml), z=-7.7, p<0.01, r=0.87), neutralizing activity from a median of 99% (IQR 0.48) to 98% ((IQR 6), z=-4.8, p<0.01, r=0.70), CD4 T-cells from a median of 0.13 % (IQR 0.12) to 0.03% ((IQR 0.03),z=-5.9, p<0.01, r=0.86) and CD8 T-cells from a median of 0.02% (IQR:0.06) to 0.01% ((IQR 0.02),z=-3, p<0.01, r=0.45).

Due to necessary adaptions of the study design and limited numbers, a long-term follow-up after a single dose-vector vaccine (marginal reaction after 3 weeks) or heterologous regimen after Ad.26.COV.2 prime (too much delay) was not performed.

Adverse vaccine reactions

After the first dose of the mRNA vaccine, all athletes reported pain at the injection site lasting for a median time of 3 days (IQR 1). The most frequently reported systemic side effect was fatigue with 70% (median time: 2 days, IQR 3 days) and headache with 45% (median time: 0 days, IQR 1 day). The second mRNA dose caused pain at the injection site in 76% of cases for a median time of 2 days (IQR 2 days). Fatigue was reported by 71% (median time: 2 days, IQR 3 days) and headache by 59% (median time: 1 days, IQR 3 days) of the athletes. After the Ad26.COV.2 dose, all athletes reported pain at the injection site for a median time of 4 days (IQR 2 days). Fatigue was reported by 93% (median time: 3 days, IQR 2 days) and headache by 87% (median time: 3 days, IQR 1 day) of the athletes. The second heterologous mRNA dose led to local pain in 92% (median time: 2 days, IQR 1 day). Fatigue was reported by 84% (median time: 3 days, IQR 3 days) and headache by 75% (median time: 2 days, IQR 2.5 days) of the athletes. Occurrence of all collected local and systemic side effects is shown in figure 4.

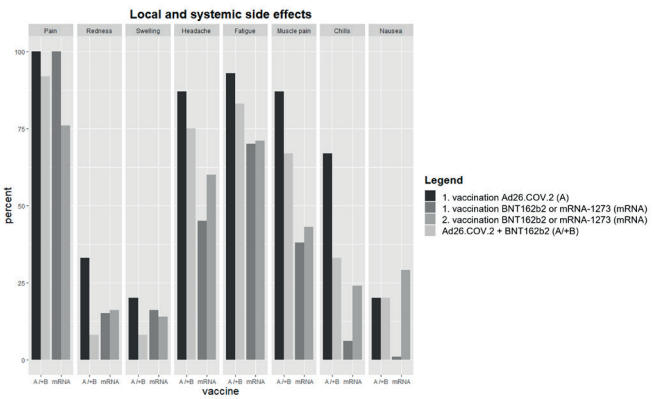

Figure 4. Local and systemic side effects. The different vaccine regimens are shown with their occurrence of local and systemic side effects.

Training Restrictions

Training restriction due to adverse events after the first and second mRNA vaccination lasted for a median of 2 days (IQR 1). The single dose Ad26.COV.2 vaccine led to a comparable training restriction of 2 (IQR1) days (z=-0.09, p=0.9, r=0.01). The heterologous regimen after Ad26.COV.2 priming was followed by a cumulative restriction of training of 3 days (IQR 1), which was not significantly different from the two dose mRNA vaccines (z=-0.73, p=0.46, r=0.1).

DISCUSSION

The aim of our study was to evaluate the humoral and cellular response in elite athletes after vaccination with different regimes against COVID-19. The main findings were (i) the humoral and cellular immune response in athletes was induced after double-dose mRNA and single-dose vector vaccines, (ii) the mRNA and vector vaccines differed in their immunogenicity, with Ad26.COV.2 as single-dose being less potent for increasing IgG antibodies, neutralizing activity and CD4 T-cells, but more potent in inducing the CD8 T-cell response, (iii) a heterologous mRNA vaccination after Ad26.COV.2 priming was able to bring the humoral and cellular immune response close to double-dose mRNA vaccinations in all parameters, and (iv) there were no differences in training restrictions between the vaccine regimens. All side effects were minor and did not lead to substantial training loss.

Lo Sasso et al. (2021)(18) state that an effective immune response can be inferred from the increase in IgG antibodies and their related neutralizing activity, as well as from induction of CD4 and CD8 T-cells. Particularly, the neutralizing antibody titers are considered important for the protection against acquisition of SARS-CoV-2 infection due to their ability to inhibit spike protein attachment to the ACE-2 receptor, and consequently inhibit entry of the coronavirus (18). Initial studies on the immunogenicity of a single dose of the Ad26.COV.2 vaccine among non-athlete healthy individuals reported adequate induction of neutralizing antibody titers against the wild type and the Alpha variant, and some studies even showed durable and sufficient responses against new variants of the coronavirus (19–21). In contrast, the current study showed that the single dose of the Ad26.COV.2 vaccine only induced poor neutralizing antibody activity in elite athletes, which may indicate insufficient protection against infection and transmission. Similar findings have been reported for immunocompetent individuals in general by Self et al. (2021) (21) who claim that the single-dose vector vaccine is the least immunogenic one of the available vaccines. On the other hand, it induced a comparably strong CD8 T-cell response, which in concert with a low neutralizing antibody function may still protect from severe courses of COVID-19 disease once infected. Thus, the Ad26.COV.2 vaccine may protect athletes from serious outcomes of the infection, but it is potentially less effective in protecting against an acquisition of the infection and transmitting it to other athletes; it should therefore not be considered an effective choice for elite athletes participating in major sport events who want to avoid SARS-CoV-2 infections.

The double-dose mRNA vaccines showed a clearly stronger induction of neutralizing antibody titers and CD4 T-helper cells compared to the single-dose vector vaccine. This aligns with findings from Tada et al. (2021)(23) who showed significantly lower neutralizing antibody titers against all variants after Ad26.COV.2 compared to BNT162b2 and mRNA-1273. Collectively, findings support the notion of an inadequate humoral immune response after a single-dose vector vaccine, thereby explaining the increased rate of breakthrough infections (24), thus necessitating a second immunization following Ad26.COV.2 vaccine to increase protection from virus acquisition. Moreover, it is likely that transmission between athletes cannot be effectively prevented by the Ad26.COV.2 vaccine to control the virus spread in settings typical for sport and major sports events.

However, CD4 and CD8 T cells also contribute to the effectiveness of vaccinations. Grifoni et al. (2020)(25) showed that individuals who had contact with the virus develop CD4 T-cells in 100% and CD8 T-cells in 70% of cases and inferred that this mobilisation of the adaptive immune system may assist in the prevention of severe courses of COVID-19. In our study, the double-dose mRNA vaccinations led to a larger induction of CD4 T-cells than the single dose vector vaccine, whereas the latter induced a moderately higher CD8 T-cell response. Therefore, prevention of severe courses can be assumed for both vaccine regimen.

Under consideration of these findings, athletes vaccinated with Ad26.COV.2 were offered an additional vaccination to improve their immune response. A study by Atmar et al. (26) showed that the humoral immune response can be significantly improved with a heterologous boost after Ad26.COV.2 priming, leading to similar immune responses as homologous mRNA booster vaccination. Our data confirm these findings by showing a large improvement in all investigated immunological parameters. Moreover, a comparison of vaccine-induced immune responses after homologous mRNA vaccination with heterologous vector/mRNA vaccination in immunocompetent non-athlete individuals using exactly the same analysis methods also revealed significantly higher CD8 T-cell levels after heterologous vaccination, which is in line with our findings in elite athletes (16, 27). Accordingly, in October 2021, the Standing Committee on Vaccination (STIKO) at the Robert Koch Institute, the relevant council for vaccination policies in Germany, recommended a heterologous mRNA boost vaccination to all persons who have received the Ad26.COV.2 vaccine to optimize immunity against SARS-CoV-2 (24).

Typical vaccine related adverse events may lead to training restrictions and are therefore important aspects to consider when vaccinating athletes, particularly during their preparation for major sport events like Olympic Games. In the current study, there were no significant differences in (cumulative) training restrictions between the double-dose homologous mRNA, the single dose-vector vaccine, and the heterologous vector-mRNA regimens. Median training restriction was 2-3 days. In our study only training restrictions were considered that were caused by side effects with a score larger than 1, although it has to be taken into account that there may be additional reasons for athletes not to train than only side effects, e.g. general caution after vaccination. Comparable results have been found in British Olympic athletes where side effects after mRNA vaccination lasted for 1-2 days (28). Thus, adverse events in elite athletes appear to be generally

mild and short-lived with limited impact on training. However, individual athletes may be affected considerably longer (up to 9 days; (28)) so that - if possible - vaccinations should be planned well in advance of the next competition. Of note, training restrictions after vaccination are considerably lower and more predictable compared to an infection with SARS-CoV-2 (29).

Lastly, there was an expected large decline in the immune response 6 months after the double-dose mRNA vaccines. Accordingly, a third vaccine dose with BNT16b2 or mRNA-1273 can be considered to boost the immune response and increase the protective effect(30), which was generally recommended at a later stage of the pandemic.

Limitations

Due to the vaccine shortage and local differences in vaccine availability at the time of prioritizing Olympic Games aspirants for vaccination, it was not possible to control and randomize assignment of the vaccine regimens, which precluded a more rigorous study design. This is similar to many COVID-19 related studies, which arose from the circumstances at that time. Moreover, the time interval of the heterologous boost after the first dose of the Ad26.COV.2 vaccine was longer than between the first and second mRNA vaccinations, which may contribute to altered immune responses as compared to the dual dose mRNA regimen (31). However, at the time of planning the study, the less pronounced immune response after single-dose vector vaccine was unforeseeable. Altogether, some unpredictable changes in the national COVID-19 policy had a relevant influence on our study protocol without invalidating the measurements per se (but weakening the conclusions).

Perspective

This study helps to understand the induced immune response after COVID-19 vaccinations in athletes, and vaccine related training restrictions and side effects. In addition, it would be interesting to investigate the association of the analysed immune response with the number of athletes that experience SARS-CoV-2 infection, as well as the severity and duration of their symptoms. This could provide better insights in the actual risk of infections after vaccination and the protection that is assumed by the immune response. Another new question that can be explored in the future is more detailed analysis of the side effects. Detection of side effects and training limitations was performed in our study using paper-based questionnaires. It would also be interesting to investigate limitations using objective measurement devices including fitness watches or other biometric devices. These can detect parameters such as heart rate, heart rate variability, sleep phases and skin temperature that may be associated with the vaccination and documented side effects. This has been previously investigated using a wrist-worn biometric device, but not specifically in elite athletes(32).

Conclusion

In contrast to double-dose mRNA vaccination, a single-dose vector vaccination does not seem to protect athletes sufficiently against acquisition of COVID-19. Receiving a booster dose seems to induce a sufficient immune response in all cases. There were no indications for a compromised immune response to vaccination in elite athletes. Based on both the strong immunogenicity and limited side effets, this study does not provide any evidence against vaccinating elite athletes against COVID-19.

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Conflict of Interest and Source of Funding

M.S. has received honoraria for lectures or participation in advisory boards for Takeda, MSD, Moderna, Biotest, Novartis or Qiagen. BCG hast received honoraria for lectures or participation in advisory boards from Sanofi, Seqirus, GSK and BionTech. All other authors declare no conflicts of interest. This study was financially supported by the German Federal Institute of Sport Sciences (Bundesinstitut für Sportwissenschaften; reference: 2521BI0106) and part of a larger study being registered in the German Clinical Trials register (DRKS00023717).

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