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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Exercise: a Powerful Tool to Counteract Immune Ageing via IL-15-induced Autophagy

Mariken Lemmens

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Helena SLAETS

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Exercise: a Powerful Tool to Counteract Immune Ageing via IL-15-induced Autophagy*

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*Running title: *Defeat Immune Ageing via IL-15-induced Autophagy*

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ABSTRACT

Immunosenescence, the gradual dysfunction of the immune system with age, makes immune cells less effective in clearing naturally occurring senescent somatic cells, which exacerbates inflammageing. This makes the elderly more susceptible to developing age-related diseases. Currently, no cure exists to rejuvenate the immune system, although regular exercise has been reported to stimulate autophagy – a cellular process crucial for maintaining cellular homeostasis – in human peripheral blood mononuclear cells (PBMCs). However, underlying mechanisms remain unclear. This study proposed interleukin (IL)-15, a protein produced by skeletal muscle tissue, as a potential mechanism to stimulate immune function via increased T cell autophagy. First, young (2 months old) and aged (15-24 months old) male and female mice were assigned to a sedentary or acute exercise group (n = 6-10/group). Results indicated that muscle IL-15 levels were not significantly influenced four hours post-exercise; however, baseline mRNA levels differed between biological sexes. Moreover, an increasing trend in IL-15 protein levels was detected in young male mice and results showed that IL-15 is predominately produced by type IIx muscle fibres. Finally, it was examined whether treatment with IL-15 (25 ng/ml) for three days induced autophagy differently in PBMCs from young and aged male donors (n = 2). Data suggested that aged PMBCs increased autophagy more than young PMBCs upon IL-15 stimulation. In conclusion, these results indicate that biological sex, age, and exercise duration may influence IL-15 expression and suggest that autophagy response to IL-15 differs between young and old individuals.

INTRODUCTION

In recent decades, human life expectancy has increased markedly, especially in developed countries. However, the healthy life expectancy has not followed this trend. For instance, global life expectancy at birth was approximately 73.4 years in 2019, whereas healthy life expectancy was only 63.7 years (1, 2). This marked difference between lifespan and health span can be attributed to physiological dysfunctions in different tissues and organs, such as the immune system, with age. Especially the T cell compartment is dramatically affected by ageing (3). As we age, the immune system remodels; for example, the thymus shrinks, leading to reduced production of naive T cells and the adaptive immune system loses diversity, resulting in reduced recognition of diverse antigens (4). This phenomenon is known as immunosenescence. In addition, the T lymphocytes are ineffective in reducing senescent somatic cells (SSCs), which naturally arise by ageing, exacerbating inflammageing (5, 6). Hence, immunosenescence is characterised by reduced protective immune responses, persistent low-grade inflammation, and autoantibody production. As a

result, older people are more prone to develop infections, malignancies, and autoimmune diseases and are more likely to respond poorly to vaccinations (4). Consequently, global ageing poses major challenges to healthcare systems and governments as age-related diseases continue to rise (7).

Research shows that frequent exercise positively influences the immune system's function. For example, regular exercise is indicated to improve the immune system's antiviral responses and reduce the risk of infection (2, 8). Particularly, T cells are important in this antiviral response and exercise is associated with an increase in the number of T cells (2, 4). Notably, the frequency, intensity, duration, and type of physical activity all influence these beneficial health outcomes on the immune system (9). More specifically, a single bout of exercise also referred to as acute exercise, leads to a transient increase in selective lymphocyte subsets in the blood and, therefore, improves immunosurveillance (9-11). On the other hand, when single bouts of exercise are repeated regularly, the physical activity is defined as chronic exercise (9, 11). Chronic exercise is associated with decreased circulating inflammatory markers such as prostaglandin and C-reactive protein and thus reduced inflammageing (9, 12). On top of that, regular exercise has been linked to lower numbers of senescent T cells and the delayed onset of immunosenescence (10, 11).

In recent years, skeletal muscle has gained attention for its secretory function. In particular, skeletal muscle cells have been shown to produce elevated cytokines and other small proteins, often referred to as myokines, upon contraction (13, 14). These myokines are indicated to have autocrine, paracrine, and endocrine functions. For example, myokines can auto-regulate muscle metabolism but they are also released into the circulation, which enables communication with other cells/organs such as the brain and circulating immune cells (2, 15, 16). Often-described myokines are interleukin (IL)-6 and IL-15 (13, 14). IL-15 is a pro-inflammatory cytokine which binds cells that express the IL-2/IL-15 receptor (IL-2/15R) β and γC subunits after transpresentation via the IL-15Rα on activated monocytes and dendritic cells (17, 18). Due to the shared receptor subunits, IL-15 exerts similar functions to IL-2, such as the capability to stimulate multiple lymphocyte lineages. For instance, IL-15 is shown to regulate the activation and proliferation of both natural killer (NK) and T cells (18-20). Notably, LeBris and colleagues reported a progressive decline in murine muscle and serum IL-15 levels with age (21). Furthermore, circulating IL-15 levels are demonstrated to be reduced in older people (> 65 years) compared to younger individuals. However, the interaction between physical activity and age on the regulation of IL-15 remains unclear (22). In addition, not only age but also biological sex can influence myokine production since males and females differ in muscle fibre type composition and physiological responses to exercise training (23, 24).

More recently, *in vitro* studies have shown that IL-15 stimulates autophagy in NK and T cells (25, 26). Autophagy, a self-eating process, is a cellular pathway responsible for degrading dysfunctional organelles and misfolded proteins (27). More specifically, the dysfunctional cytoplasmatic components are engulfed in a double membrane vesicle, which is named the autophagosome. Ultimately, autophagosomes fuse with lysosomes to form autolysosomes, which degrade the encapsulated material (28). Hence, autophagy is indicated to be highly important for maintaining cellular homeostasis (29). Notably, ageing is associated with reduced autophagy activity. As a result, ageing is accompanied by the accumulation of harmful proteins and subsequent cellular senescence (27). Moreover, impaired autophagy has been linked to the development of age-related diseases such as cancer and neurodegenerative diseases (29-31). It is speculated that enhancing autophagy in age-affected immune cells, specifically T cells, could improve their function and thus the overall immune response (32). Interestingly, treadmill exercise is indicated to increase autophagy in mice's skeletal muscles, and the research group of Mejías-Peña *et al*. has demonstrated that regular resistance training stimulates autophagy in peripheral blood mononuclear cells (PBMCs) of elderly subjects (33, 34).

Little is known about the underlying mechanisms of exercise-mediated prevention of immunosenescence. We hypothesise that exercise stimulates IL-15 production by skeletal muscles of aged mice and that IL-15, in turn, stimulates autophagy in T cells. Hence, this study aims to investigate the effect of acute exercise on IL-15

expression in young and aged mice. Furthermore, the association of exercise-induced IL-15 with improvement in autophagy is investigated. The capacity of IL-15 to stimulate autophagy in aged T cells is expected to be lower than in healthy young T cells. In the future, endurance training should elucidate the potential beneficial effects of exercise-induced IL-15 on immune ageing.

EXPERIMENTAL PROCEDURES

Animals and housing – Male and female C57BL/6 mice were obtained from Janvier-Labs (Le Genest-Saint-Isle, France) at 2, 15, and 24 months. The animal care and experimental procedures performed were approved by the Ethical Committee for Animal Experimentation and were in accordance with the ethical regulations and guidelines for animal research. Animals were housed at a maximum of 10 mice per cage in a standard pathogen-free environment under controlled temperature (20-24°C), humidity (40-60%), and light (12:12-h light/dark cycles), and were fed a standard chow diet and water *ad libitum*. Aged (15 months old or older) male mice were housed individually. Mice were acclimated to the housing conditions for one week, after which mice were randomly assigned to the following experimental groups: sedentary control (SED) or treadmill exercise (EX) group (n = 6-7/group).

Animal exercise protocol – Mice from the EX group were allowed to familiarise with the treadmill for three non-consecutive days. During this familiarisation period, mice ran at 10 m/min. After the adaptation training, an incremental speed test was performed to determine the maximal workload. Next, mice were subjected to an acute exercise training at 80% of the maximum workload. SED mice were placed in the same room to ensure exposure to similar stress levels associated with the laboratory setting.

Mouse skeletal muscle isolation – Four hours after the exercise training, mice were given a lethal dose of dolethal and sacrificed by cardiac perfusion with 1x PBS and heparin. Next, gastrocnemius (GAS), soleus (SOL), extensor digitorum longus (EDL), and tibialis anterior (TA) muscles were dissected of both hindlimbs. Left muscle tissues were immediately frozen in liquid nitrogen and later stored at -80°C. Right muscle tissues were embedded in Tissue-Tek O.C.T Compound (Leica) and stored at -80°C.

Human PBMC culture and stimulation – For *in vitro* experiments, PBMCs from young (< 65 y/o) and old (> 65 y/o) healthy male individuals who did not perform a specific exercise protocol were obtained from the UBiLim Biobank. Cells were stimulated for three days with recombinant human (rh) IL-15 (25 ng/ml) (Bio-Techne Ltd., 247-ILB) or rapamycin (100 nM) (Sigma-Aldrich, 553210) and two hours with bafilomycin A_1 (100 nM) (Invivogen). Unstimulated cells were used as a control. PBMCs were cultured in RPMI 1640 medium enriched with 10% fetal bovine serum, 0.5-1% penicillin-streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate. Cells were seeded at 1.10^6 cells/ml for immunocytochemical analysis and 5.10^5 cells/ml for western blot.

Immunocytochemical/Immunohistochemical analysis (ICC/IHC) – Prior to immunostaining, PBMCs were fixed with ice-cold methanol for 15 min at -20°C and frozen muscle tissues were cut into sections (10 µm) using a Leica CM3050 S cryostat (Leica Biosystems). Next, cells/tissues were washed and permeabilised in PBS 0.5% Triton X-100 (PBST) for 15 min at room temperature (RT). After washing with PBST (0.05%), cells/tissues were blocked with 10% blocking serum for 30 min and incubated with relevant primary antibodies overnight at 4°C. Next, cells/tissues were incubated with secondary antibodies for 1h at RT. Primary and secondary antibodies used are listed in supplementary information (SI), Table S1 and Table S2. After washing, cells/tissues were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at RT and mounted with fluorescence mounting medium. Images of muscle sections were captured using a Leica fluorescence microscope (Leica Microsystems) at 5x and 10x magnification. Images of PBMCs were captured using the Zeis LSM 900 Airyscan 2 laser scanning confocal microscope at 63x magnification.

Western blot – PBMCs were lysed in RIPA buffer with protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics) and centrifugated at 16,000 x g for 20 min at 4 °C. Protein concentration was determined using a

Pierce BCA Protein assay Kit (Thermo Scientific, 23227). Cell protein lysate was diluted with sample buffer (1:5), boiled at 95°C for 5 min, separated on 15% SDS-page gels and blotted on polyvinylidene difluoride (PVDF) membranes. Next, membranes were blocked in blocking buffer (PBS 0.05% Tween 20 with 5% milk) for 1h. The membranes were then incubated overnight at 4°C in blocking buffer containing primary antibodies. Then, membranes were washed with PBS 0.05% Tween 20 and incubated with HRP-conjugated secondary antibodies for 1 h at RT. Primary and secondary antibodies used are listed in SI, Table S1 and Table S2. Pierce ECL plus western blotting substrate (1 ml reagent A and 25 µl reagent B) (Thermo Scientific) was used for immunodetection. β actin was used as the loading control. Quantification was done using an Amersham Imager and ImageJ/Fiji software.

RNA isolation and complementary DNA (cDNA) synthesis – Total RNA was extracted from mouse muscle tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germany). The purity and concentration of the RNA were determined by Nanodrop. RNA was reverse transcribed into cDNA using the qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's protocol.

Quantitative PCR (qPCR) – To measure muscle IL-15 and IL-15Rα mRNA expressions, qPCR was carried out using a PowerUp SYBR green master mix (Applied Biosystems, Fisher Scientific) following the manufacturer's instructions in a QuantStudio 3 (Thermo Fisher Scientific). PCR reactions were performed under the following cycling conditions: denaturation for 2 min time at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing for 1 min at 60 °C. The mRNA expression levels were normalised to the GADPH and 18s mRNA levels. Relative gene expression was analysed with the $2^{-\Delta\Delta CT}$ method. Specific primer sequences used for PCR are listed in SI, Table S3.

Statistical analyses – Statistical analysis was performed using GraphPad Prism 10 (GraphPad Software, version 10.1.0). Data are presented as mean ± standard error of the mean (SEM). Outliers were identified using Grubb's test, and normality was checked using the Shapiro-Wilk test. If normally distributed, data were analysed by an independent t-test and two/three-way Analysis of Variance (ANOVA). Otherwise, non-parametric data alternatives were used. Šídák's multiple comparisons test was used to correct for multiple comparisons. P values < 0.05 were considered statistically significant (*, $P < 0.05$; **, $P < 0.01$).

RESULTS

An acute bout of exercise does not significantly increase mouse IL-15/IL-15Rα mRNA or IL-15 protein levels in gastrocnemius muscle – To determine the effect of acute exercise, age, and biological sex on IL-15 myokine production, IL-15 mRNA and protein levels were measured in mouse gastrocnemius (GAS) muscles.

First, IL-15 and IL-15Rα mRNA expression levels were measured using qPCR. Our results showed no significant effect of acute exercise on IL-15 expression. Nevertheless, a decreasing trend was observed after exercise ($p = 0.0906$) (Fig. 1A). To comprehensively assess the impact of acute exercise, age, and biological sex on IL-15 production, a three-way analysis of variance (ANOVA) was conducted. This approach allowed us to evaluate the individual and interactive contributions of the variables. Note that no significant differences were observed between the IL-15/IL-15Rα mRNA levels of 15- and 24-month-old male or female mice. Therefore, both age groups were pooled and further referred to as the 'old' group (data not shown). Results showed no significant influence of exercise, age, or sex on IL-15 mRNA expression. However, a decreasing trend of IL-15 mRNA levels was observed after acute exercise in all groups (Fig. 1B). In addition, sedentary female mice, especially older females, seemed to express higher IL-15 mRNA levels (Fig. 1B). After reviewing the sources of variation, a significant effect of biological sex was detected ($p = 0.0263$) (Table S4). Consequently, a two-way ANOVA, excluding the age variable, was performed. Data showed that sedentary female mice have significantly higher IL-15 mRNA levels in m. gastrocnemius compared to sedentary male mice ($p = 0.0107$). Moreover, female mice tended to show lower mRNA levels of IL-15 after acute exercise compared to sedentary controls of the same biological sex ($p = 0.0743$) (Fig. 1C). Furthermore, our results showed no significant

effect of acute exercise on IL-15Rα mRNA expression (Fig. 1D). Three-way ANOVA also showed no significant impact of exercise, age, or biological sex on IL-15Rα mRNA levels (Fig. 1E). Nevertheless, similar to IL-15 mRNA levels, the source of variation showed a significant effect of biological sex on IL-15Rα mRNA levels (p = 0.0279) (Table S5). After excluding the age factor, a significantly higher mRNA expression of IL-15Rα was detected in sedentary female mice compared to sedentary male mice (Fig. 1F). Thus, gene expression of both IL-15 as well as its transpresenting receptor was higher in GAS muscle of sedentary female mice than sedentary male mice.

Subsequently, protein levels of IL-15 were determined by IHC on mouse GAS muscle sections. Representative images are shown in Fig. 2A-H. Intensity analysis of IL-15 showed no significant differences between study groups. However, an increasing trend of corrected total cell fluorescence (CTCF) was detected in the muscles of young male mice after acute exercise, which was not visible in the muscles of female mice. Notably, old male mice appeared to have higher baseline levels of IL-15 CTCF compared to young male mice. Furthermore, unlike young male mice, no increasing trend in IL-15 CTCF was detected in the muscles of old mice after acute exercise (Fig. 2I). To further investigate the effect of acute exercise on IL-15 protein levels, the amount of IL-15 positive fibres corrected for muscle area was calculated. No exercise effect was measured (Fig. 2J). Consequently, a three-way ANOVA was conducted to get more detailed information on the effect of all variables separately. The results showed no significant differences in the percentage of IL-15 positive fibres between the muscle of EX and SED mice of the same age and sex category (Fig. 2K). Nevertheless, the source of variation data showed a significant effect of age and an interactive effect of age and exercise on the amount of IL-15 positive fibres (p = 0.0242 and 0.0390, respectively) (Table S6). Two-way ANOVA, excluding the sex variable, showed significantly higher numbers of IL-15 positive

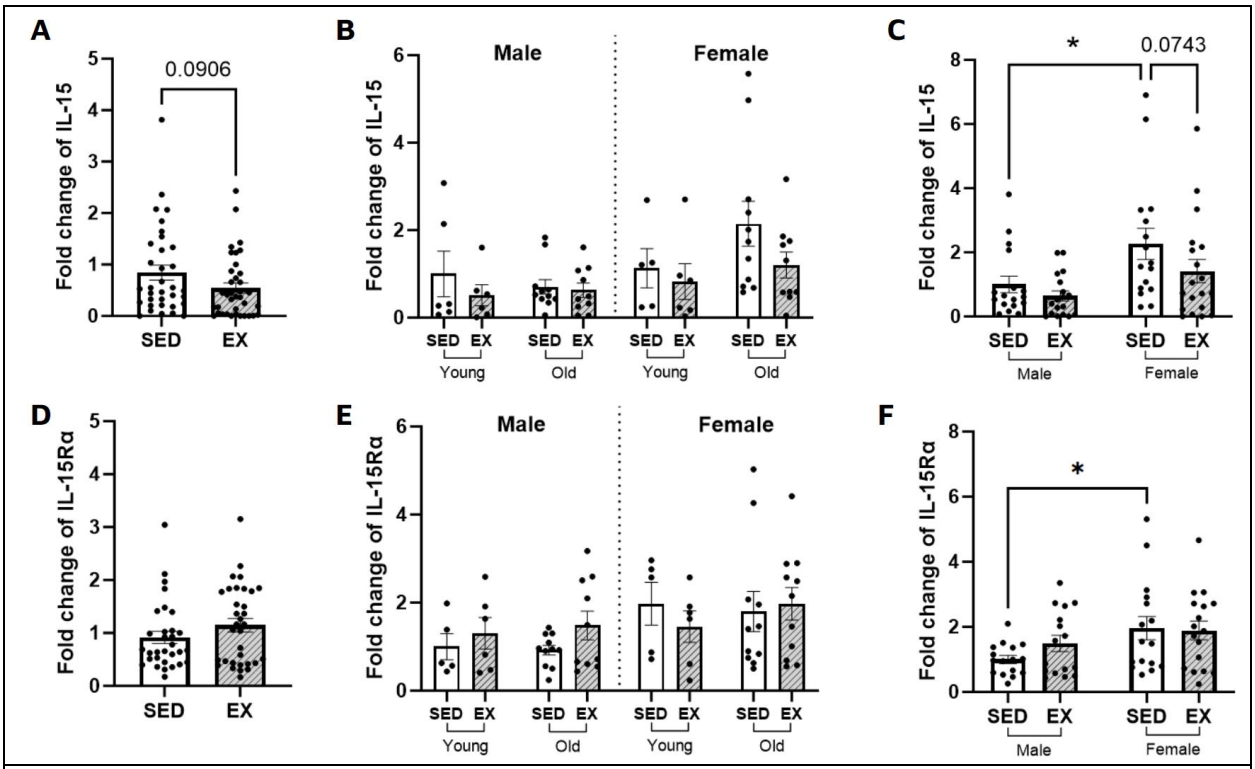

Fig. 1 – Sedentary female mice have higher IL-15 and IL-15Rα mRNA levels in m. gastrocnemius compared to their male counterparts. IL-15 (A-C) and IL-15Rα (D-F) mRNA levels in gastrocnemius muscles of C57BL/6 mice, divided into SED and EX groups, were measured using a qPCR assay. Young (2 months old) and old (15 to 24 months old) male and female mice are visualised separately (n = 6-17/group). Data are represented as mean ± SEM and analysed using a three-way ANOVA followed by Šídák's multiple comparisons test or a two-way ANOVA (*, P < 0.05). EX, Exercise group; IL-15, Interleukin 15; IL-15Rα, Interleukin 15 receptor alpha; SED, Sedentary control group.

fibres in old SED mice compared to young SED mice (p = 0.0036). Additionally, a decreasing trend in the amount of IL-15 positive fibres was noted in old mice after acute exercise (p = 0.0693) (Fig. 2L).

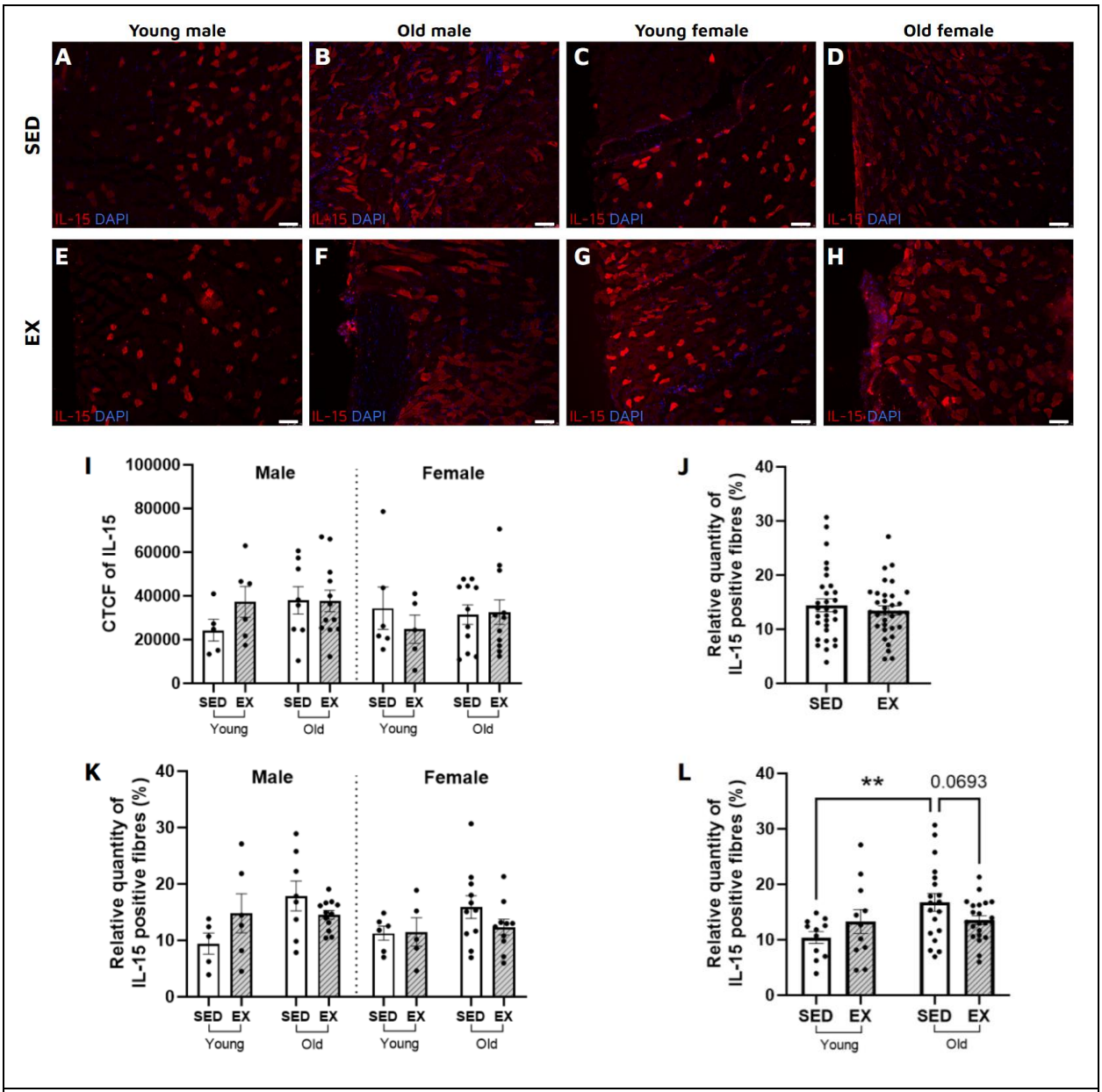

Fig. 2 – Increasing trend of IL-15 levels in m. gastrocnemius of young male mice four hours after acute exercise. (A-H) Representative images of immunohistochemical analysis of IL-15 in gastrocnemius muscles of C57BL/6 mice divided into SED (A-D) and EX (E-H) groups. Tissues of young male (A, E), old male (B, F), young female (C, G), and old female (D, H) mice are shown. Tissues were stained for IL-15 (red), and nuclei were counterstained with DAPI (blue). Scale bar = 100 µm (I) Fluorescence intensity analysis of IL-15 in m. gastrocnemius. (J-L) Percentage of IL-15 positive fibres per muscle area. Young (2 months old) and old (15 to 24 months old) male and female mice are visualised separately (n = 6-19/group). Data are represented as mean ± SEM and analysed using a three-way ANOVA followed by Šídák's multiple comparisons test or a two-way ANOVA (**, P < 0.01). CTCF; corrected total cell fluorescence; DAPI, 4′,6-diamidino-2-phenylindole; EX, Exercise group; IHC, Immunohistochemistry; IL-15, Interleukin 15; SED, Sedentary control group.

IL-15 is mainly produced by type IIx fibres – To investigate which fibre types produced IL-15 and possible differences among exercise status, age, and biological sex, an immunohistochemical comparison was performed by matching IL-15 immunostaining and muscle fibre type staining on neighbouring muscle sections. Representative images are shown in Fig. 3A-B.

After the descriptive analysis, results indicated an increase in IL-15 expressing type IIx fibres after acute exercise for all groups, with the highest effect among young male and old female mice (Fig. 3C-G, Fig. 3F-J). Old female mice seemed to express more type IIa fibres than young female mice (Fig. 3E-F and I-J). Raw data can be consulted in supplementary Table S7.

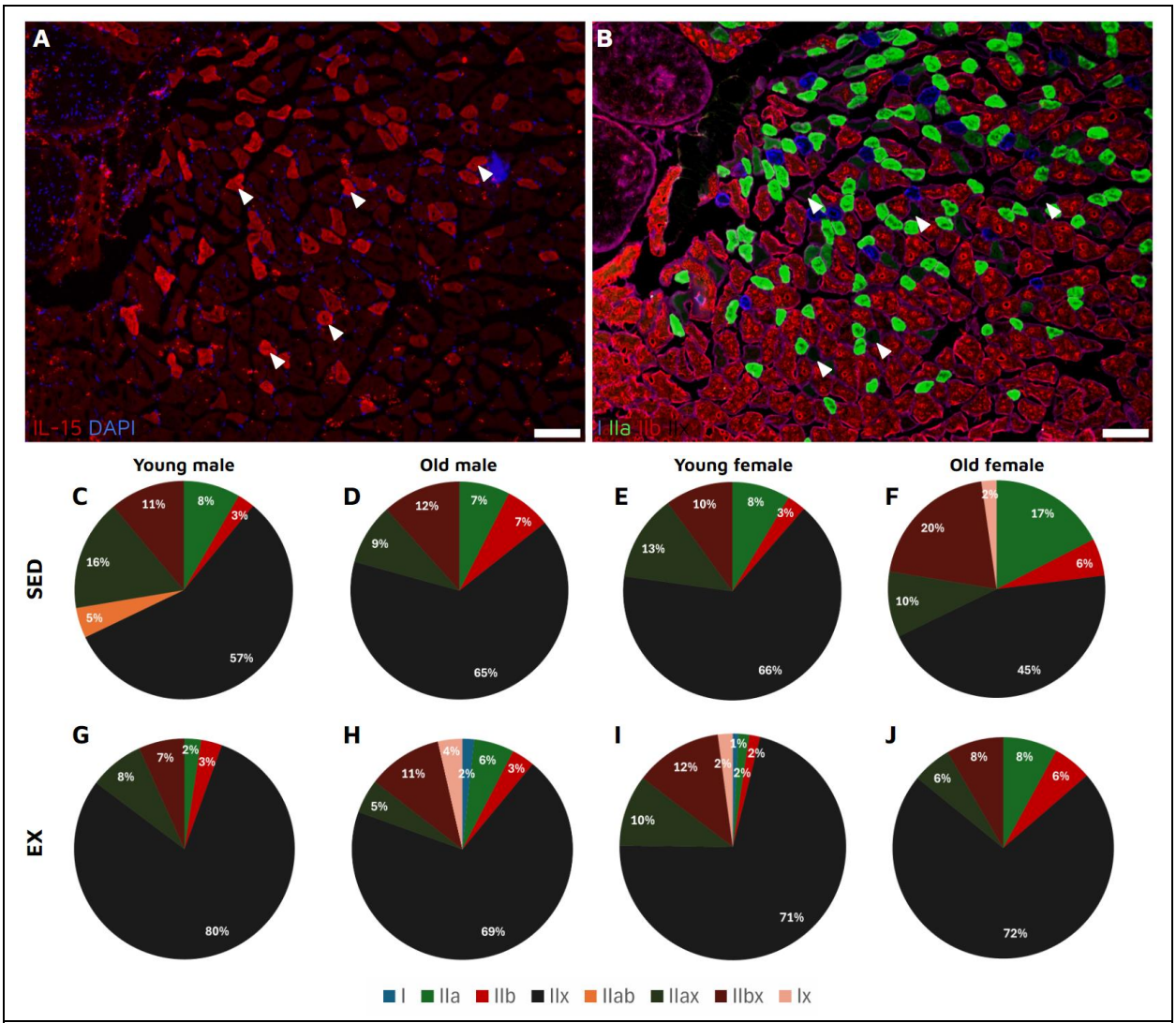

Fig. 3 – **Myokine IL-15 is predominantly produced by type IIx muscle fibres.** (A-B) Representative images of IL-15 staining (A) and fibre-type staining (B) in neighbouring tissue sections from a sedentary young male mouse are shown. Nuclei were counterstained with DAPI (blue). White arrows depict matching IL-15 expressing type IIx muscle fibres. Scale bar = 100 µm. (C-J) Fibre type analysis in gastrocnemius muscles of C57BL/6 mice, divided into SED (C-F) and EX (G-J) groups. Pie charts depict the fibre types expressing IL-15 in young male (C, G), old male (D, H), young female (E, I), and old female (F, J) mice. Data are presented as mean (n = 3-8/group). DAPI, 4′,6-diamidino-2-phenylindole; EX, Exercise group; IL-15, Interleukin 15; SED, Sedentary control group.

IL-15 boosts autophagy in PBMCs of old donors – Next, the capacity of IL-15 to induce autophagy in young and aged PBMCs was investigated.

First, autophagy was monitored by immunoblotting of the markers 'microtubule-associated protein light chain 3' (LC3) and 'ubiquitin-binding protein 62' (p62) (Fig. 4A). Data suggested that rhIL-15 increased LC3-II expression in aged PBMCs but not in PBMCs of young donors (Fig. 4B). P62 displayed similar results across all conditions, with no distinct effect of age observed (Fig. 4C). To confirm this data, autophagic activity was also measured by counting the number of $LC3^+$ puncta in $CD3^+$ T cells by fluorescence microscopy (Fig. 4D-E). T cells from old donors tended to exhibit higher amounts of $LC3^+$ puncta upon IL-15 treatment with bafilomycin A_1, which was not the case in young donors (Fig. 4D).

DISCUSSION

In the present study, we investigated IL-15 production in response to exercise and proposed this myokine as a potential tool to stimulate immune function via increased T cell autophagy. It was hypothesised that acute exercise promotes IL-15 production in the skeletal muscles of both young and aged mice. Additionally, it was anticipated that IL-15-induced autophagy would be lower in T cells from aged donors compared to young donors. The key findings of this study were that (1) baseline levels of skeletal muscle IL-15 and IL-15Rα mRNA were higher in female mice than male mice, (2) young male mice exhibited a higher trend in IL-15 protein levels after acute exercise, (3) IL-15 was predominantly produced by type IIx fibres, and (4) autophagy was increased in PBMCs from old donors compared to PBMCs from young donors after treatment with rhIL-15.

Skeletal muscle tissue is often reported as a secretory organ which produces myokines after contraction (13, 35). Our results, however, showed no significant influence of acute exercise on IL-15 or IL-15Rα mRNA levels in mouse GAS muscles four hours after an acute bout of treadmill running. This contrasts with current literature, stating that IL-15 mRNA levels of rat GAS muscles significantly increased after treadmill running. It should be noted, however, that the rats were subjected to chronic exercise (five days/week for eight weeks) (36). Nevertheless, several clinical trials have reported a two-fold upregulation in IL-15 and/or IL-15Rα mRNA levels in vastus lateralis muscle biopsies 24 hours after an acute bout of resistance exercise (35, 37). Pérez-López and colleagues even reported a significant increase in IL-15 mRNA levels of the human vastus lateralis muscle four hours after acute resistance exercise (37). However, it is possible that a 24-hour post-exercise time point may be more appropriate for detecting the effects of acute treadmill running on IL-15 and IL-15Rα mRNA levels. Apart from this, we showed that female mice have significantly higher IL-15 and IL-15Rα mRNA levels at baseline than male mice. To our knowledge, we are the first to demonstrate this sex-dependent difference in IL-15 and IL-15Rα mRNA expression in mouse GAS muscle. The higher levels of IL-15 and IL-15Rα mRNA in female mice could be ascribed to a difference in sex hormones such as oestrogen or testosterone. The oestrogen receptor beta is shown to be present in skeletal muscle tissue in both males and females (38-40). Hence, many studies have reported that sex hormones can influence skeletal muscle mass and strength in both mice and humans (41-43). More recently, a study by Norton *et al*. also suggested that oestrogen signalling in mouse tibialis anterior muscle regulated the myokine expression, specifically of myokines that could alter bone cells (44). Other possibilities are muscle type composition or metabolic differences between males and females (24, 45).

Furthermore, the current study investigated protein levels of IL-15 after acute exercise. No significant differences were found between study groups. However, young male mice showed an increasing trend of IL-15 levels in GAS muscle four hours post-exercise, while their aged counterparts failed to show this exercise effect. In line with our results, Nielsen *et al*. have reported no change in IL-15 protein levels after an acute bout of resistance exercise in humans (35). Similarly, Rinnov and colleagues have shown that an acute cycle exercise did not significantly alter human muscle IL-15 protein expression. Despite this, the study demonstrated that 12 weeks of endurance training significantly increased IL-15 protein levels, indicating that repeated bouts of exercise might be necessary to observe significant changes in IL-15 protein expression in skeletal muscle (14). In addition, we found that ageing mice exhibited higher levels of IL-15. In contrast, other studies

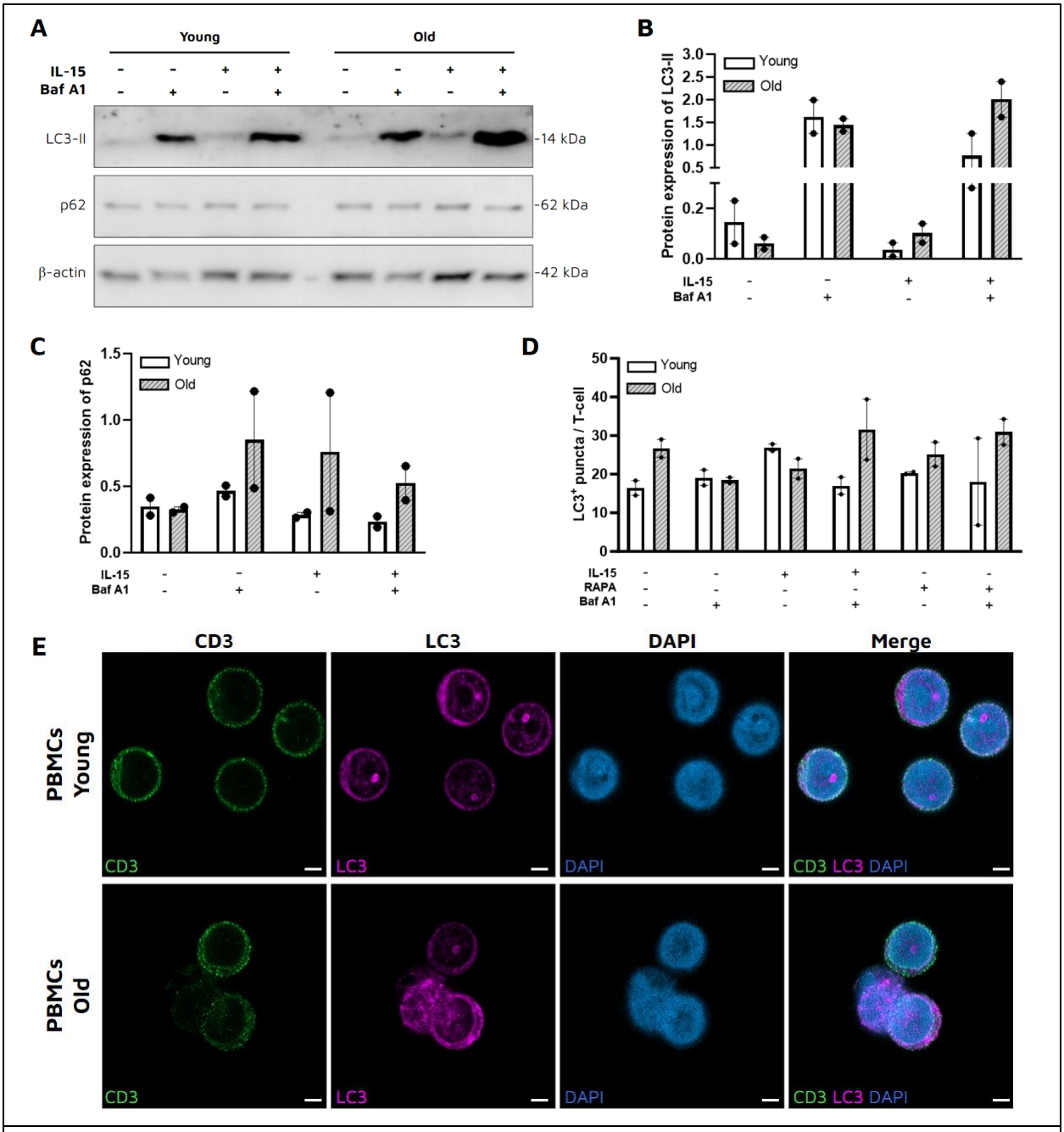

Fig. 4 – IL-15 promoted autophagy in PBMCs of old, but not young, donors. (A) Western blotting was used to detect the protein expression of p62 and LC3-II in PBMCs of young (25-26 y/o) and old (67 y/o) healthy male donors. β-actin was used as the loading control. (B) Graph showing the relative protein expression of LC3-II in PBMCs of young and old healthy male donors as assessed by western blot. (C) Graph showing the relative protein expression of p62 in PBMCs of young and old healthy male donors as measured by western blot. (D) Quantification of $LC3^+$ puncta in T cells of young and old healthy male donors as calculated by immunocytochemical analysis. (E) Representative images of CD3 (green) and LC3 (fuchsia) co-staining in PBMCs of young and aged healthy male donors. Nuclei were counterstained with DAPI (blue). Cells were stimulated with rhIL-15 (25 ng/ml) for three days and treated with bafilomycin A_1 (100 nM) for two hours. Scale bar = 2 µm. Data are represented as mean ± SEM, n=2/age category. Baf A1, Bafilomycin A_1; CD3, Cluster of differentiation 3; DAPI, 4′,6-diamidino-2-phenylindole; IL-15, Interleukin 15; LC3, microtubule-associated protein light chain 3; PBMCs, Peripheral blood mononuclear cells; Rapa, Rapamycin.

have reported a decrease in muscle IL-15 protein levels in ageing mice. For example, Quinn *et al*. indicated that quadriceps muscle IL-15 protein levels were significantly lower in 28-month-old compared to 18-month-old mice. Notably, no animals younger than 12-month-old were included in the study (21). As a result, they may have missed the potential increase in IL-15 levels between mice of 2 and 15 months of age. Additionally, a study by Marzetti *et al*. demonstrated a significant decrease in IL-15 expression in rat GAS muscle with age. Specifically, they have shown that 37-month-old rats had lower IL-15 levels than 18 and 29-month-old rats. However, it should be noted that, although not significant, an increasing trend in IL-15 levels was detected between 8 and 18-month-old rats (46). Therefore, we postulate that it might be possible that mice around 15 to 24 months of age experience a transient increase in muscle IL-15 levels, which slowly decline with ageing. Since C57BL/6J mice aged 18-24 months old are considered old, the transiently increased levels of IL-15 may be linked to inflammageing (47). Moreover, the decreasing muscle IL-15 expression with ageing might be ascribed to the emergence of sarcopenia with ageing. Several studies suggest that low levels of IL-15 are associated with sarcopenia and that increasing IL-15 levels in ageing muscles might inhibit this age-related loss of muscle mass and strength (21, 48, 49). Aside from this, unlike IL-15 mRNA expression, no influence of biological sex on IL-15 protein levels was detected. This may be due to various posttranscriptional regulations of IL-15, which might impact IL-15 protein expression. Current literature has often reported that IL-15 mRNA and protein levels in immune cells, as well as skeletal muscle, showed no correlation (21, 35, 50, 51).

Skeletal muscles are composed of many different fibre types. These fibre types are categorised depending on their myosin heavy chain (MHC) isoform expression. In mammalian skeletal muscle, four main categories of fibre types are defined: type I (MHC-I), type IIa (MHC-IIa), type IIb (MHC-IIb), and type IIx (MHC-IIx) (24, 52). However, hybrid fibres expressing two MHC isoforms have also been identified (53). Although literature describing IL-15 production by specific fibre types is limited, Nielsen *et al*. reported that IL-15 mRNA was higher in human skeletal muscles dominated by type II fibres compared to type I fibres (35). This is consistent with our results, which showed that IL-15 is mainly expressed in type IIx fibres. Notably, IL-15 is thus mostly produced by fast-twitch type II fibres, which are characterised by an anaerobic metabolism and stimulated by resistance training. However, in the present study, mice were subjected to treadmill running, an aerobic-exercise intervention which stimulates slow-twitch type I muscle fibres (54). Hence, this could also explain why no significant exercise effect was observed on IL-15 mRNA or protein level. Besides, different skeletal muscles are also characterised by different muscle fibre type compositions (35, 55). Augusto and colleagues reported that GAS muscles, as well as tibialis anterior (TA) and extensor digitorum longus (EDL) muscles, of C57BL6J mice contained predominantly type II fibres, specifically IIb and IIbx fibres. On the contrary, in mice soleus (SOL) muscles, there was an equal dominance of type IIa and type I fibres. Percentages of pure IIx fibres were similar between all muscle types (55). Haizlip *et al*. described similar fibre type distributions (24). Consequently, studying the GAS muscles in this research was a well-founded choice, particularly due to its diverse type II fibre composition compared to the EDL and TA muscles (55). In addition, although we did not report marked differences in IL-15 expressing fibre type composition between males and females or by ageing, Miljkovic *et al*. stated that ageing is linked to a shift from fast-twitch to slow-twitch muscle fibres, predominantly affecting type IIx fibres (56). Similarly, Lexell *et al*. reported that increasing age is linked with reduced number and size of type II human muscle fibres (57). Possibly, this could also explain the absence of increased IL-15 expression in the GAS muscles of our old male mice in comparison to their young counterparts after exercise. Furthermore, despite literature analysing sex-based differences in GAS fibre type composition is rather limited, Sciote *et al*. reported no significant differences in GAS fibre type composition between male and female mice (58). However, other studies have described sex-based differences in fibre type composition in other skeletal muscles. For example, Haizlip *et al*. stated that MHC-IIa expression is higher in SOL and TA muscles of male mice compared to female mice (24). Additionally, Staron and colleagues concluded that the percentages of fast and slow

fibre types were similar in vastus lateralis muscle between men and women. However, they noted that the area of slow fibres was larger in muscles from women compared to muscles from men.

After identifying muscle IL-15 expression upon acute exercise, the capacity of IL-15 to induce autophagy and potential differences in PBMCs from donors of different ages were explored since IL-15 is anticipated to enter the bloodstream and bind to circulating immune cells. The most widely used markers to monitor autophagy are LC3 and p62. During autophagy, LC3-I is converted into LC3-II, which binds to the autophagosomal membrane, thereby reflecting the formation of autophagosomes. LC3-II is typically increased with increased autophagy. P62, on the other hand, binds to ubiquitinated proteins and serves as a linker between these proteins and the autophagosomal membrane. Consequently, p62 is selectively degraded by autophagy (59). The current study found that LC3-II, and thus autophagy activity, was lower in untreated PBMCs from aged healthy male individuals compared to cells from younger individuals. In line with our results, other studies report an age-related decline of autophagy in different tissues and cells, including PBMCs (34, 60-63). Note that p62 levels were not considered decisive since the data remained stable between the different conditions and study groups. Moreover, literature states that p62 should be interpreted with caution as it is also influenced by several other processes independent of autophagy (64). On top of that, Klionsky and colleagues reported that in some cell types, no change in p62 levels is measured despite strong autophagy induction, which is consisted with our results (65). Remarkably, the present study indicated that autophagy increased in PBMCs from old donors but not in PBMCs from young donors upon treatment with 25 ng/ml rhIL-15 for three days. Similarly, a study by Swadling *et al*. demonstrated an increase in LC3 levels in PBMCs of healthy controls after treatment with 50 ng/ml of rhIL-15 for three days. However, the age of the donors was not specified in their materials and methods, making it impossible to make comparisons regarding age differences (25). Zhu *et al*. also demonstrated a boost in autophagy after 24h of stimulation with IL-15 in mouse NK T cells. Nevertheless, again, age was not taken into account (26). A potential explanation for the observed increase in autophagy in PBMCs from older donors is that aged cells may exhibit an enhanced response to IL-15. For example, Lee and colleagues stated that senescent human $CD8^+$ T cells have a greater responsiveness to IL-15 compared to non-senescent cells (66, 67). Similarly, Quinn *et al*. also reported an age-dependent increase in IL-15 sensitivity in human virtual memory T cells (T_{VM}), a subset of antigen-naive, semi-differentiated $CD8^+$ T cells. To understand age-mediated changes in IL-15 cell signalling, alterations in receptor subunits were investigated. The study demonstrated a significant increase in IL-15Rβ expression on both mice and human T cell subsets with age (68). Therefore, possibly, the lower autophagy noted in PBMCs from young compared to old donors may be linked to lower IL-15R subunit expressions.

Despite the insights provided in the present study, three main limitations should be acknowledged. The first limitation is the considerable variation observed in the data concerning mouse IL-15 mRNA and protein levels. Although all mice were genetically the same and housed under the same conditions, muscle IL-15 may be naturally more variable expressed as its gene expression is also suggested to be regulated by cytokines and hormones (44, 69). Therefore, it is possible that the effect of exercise on IL-15 is rather subtle, and a larger sample size might be required to detect these small differences adequately. Secondly, it should be emphasised that the autophagy results are rather exploratory since the sample size was only two per age group and a maximum of 11 cells per donor were examined due to time limitations. Moreover, a high variability in $LC3^+$ puncta was observed between cells from the same donor. For this reason, the findings cannot be generalised. Lastly, it should be noted that one autophagy analysis was performed by measuring $LC3^+$ puncta in the cytoplasm of $CD3^+$ expressing PBMCs, which represented T cells (70). Generally, T cells have high nucleus-to-cytoplasm ratios, which might make it difficult to distinguish between different $LC3^+$ puncta due to the accumulation of these puncta in the relatively small cytoplasm (71).

For future studies, it is important to consider the time point of muscle sampling after acute treadmill exercise. Additionally, it might be relevant to investigate the effects of chronic exercise on IL-15 levels since the literature suggests that acute exercise does not significantly

influence protein levels of IL-15. Moreover, sample sizes of IL-15 and autophagy experiments should be greatly increased to provide more statistical power and reliable results, respectively. For future studies, it might also be useful to conduct a western blot and flow cytometric analysis of autophagy on isolated T cells to re-examine the effects of rhIL-15 on T cell autophagy as immunocytochemical analysis in T cells may be unreliable. Eventually, comparisons could be made between different T cell subsets. Lastly, additional research is needed to clarify the link between exercise-induced IL-15 and IL-15-induced autophagy. Therefore, it is recommended to perform experiments that consider the release of exercise-induced IL-15 into the circulation and its binding to immune cells, using techniques such as ELISA and flow cytometry, respectively.

CONCLUSION

In summary, the present study aimed to investigate the potential of exercise-induced IL-15 to counteract immune ageing by boosting T cell autophagy. Specifically, it was investigated whether acute exercise stimulated muscle IL-15 production and whether there were age-related differences in IL-15-induced autophagy in T cells. Collectively, the results suggested that baseline levels of IL-15 and IL-15Rα mRNA were influenced by biological sex and that acute exercise seemed to increase IL-15 protein levels in young male mice while this effect was no longer detectable in older mice due to increased baseline levels. Furthermore, it was demonstrated that protein levels of IL-15 were mainly expressed by type IIx muscle fibres. Additionally, our data indicated that IL-15 increased autophagy in PBMCs from old donors compared to cells from young donors. Altogether, the results offered new insights into the impact of acute exercise on IL-15 production and the effect of IL-15 on immune cell autophagy. Ultimately, further research is needed to explore the effects of exercise-induced IL-15 on immune ageing in both mice and humans in order to translate these findings into effective training programmes and potentially reveal novel therapeutic targets.

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SUPPLEMENTARY INFORMATION

Table S1 – List of primary antibodies used in the study.

Primary antibody target	Type	Dilution		Supplier, product code
		ICC/IHC	WB	
IL-15	Goat anti-mouse IgG	1:250	/	Thermo Fiser, PA5-47014
MHC Type I	Mouse anti-mouse MIgG2b	1:50	/	Developmental Studies Hybridoma Bank, BA-D5
MHC Type IIa	Mouse anti-mouse MIgG1	1:100	/	Developmental Studies Hybridoma Bank, SC-71
MHC Type IIb	Mouse anti-mouse MIgM	1:100	/	Developmental Studies Hybridoma Bank, BF-F3
Laminin	Rabbit anti-mouse IgG	1:100	/	Thermo Fisher, PA1-16730
LC3B-II	Rabbit anti-human	1:500	1:1000	Sigma-Aldrich, L7543
SQSTM1/p62	Mouse anti-human IgG2a	/	1:1000	Abcam, ab56416
β-actin	Mouse anti-human IgG1	/	1:2000	Santa Cruz Biotechnology, sc-47778
CD3	Rat anti-human IgG1	1:100	/	Bio-Rad, MCA1477

For 10% blocking solution of antibodies, IL-15 antibody was diluted in rabbit serum, MHC antibodies and laminin were diluted in goat serum. Ig, Immunoglobulin; Myosin heavy chain, MHC.

Table S2 – List of secondary antibodies used in the study.

Secondary antibody	Type	Dilution		Supplier, product code
		ICC/IHC	WB	
AF 555	Rabbit anti-goat IgG	1:250	/	Life technology, A21431
AF 350	Goat anti-mouse IgG2b	1:250	/	Thermo Fisher, A-21140
AF 488	Goat anti-mouse IgG1	1:250	/	Thermo Fisher, A-21121
AF 555	Goat anti-mouse IgM	1:250	/	Thermo fisher, A-21426
AF 647	Goat anti-rabbit IgG	1:250	/	Thermo Fisher, A-21245
HRP-conjugated	Goat anti-rabbit	/	1:5000	Agilent, P0488
HRP-conjugated	Rabbit anti-mouse	/	1:5000* 1:2000**	Agilent, P0260
AF 555	Goat anti-rabbit IgG	1:250	/	Thermo Fisher, A-21430
AF 488	Goat anti-rat IgG	1:250	/	Thermo Fisher, A-11006

AF, Alexa fluor; HRP, Horseradish peroxidase; Ig, Immunoglobulin; *, SQSTM1/p62; **, β-actin.

Table S3 – List of primer sequences used for qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
IL-15	5'-TCT CCC TAA AAC AGA GGC CAA -3'	5'-TGC AAC TGG GAT GAA AGT CAC-3'
IL-15Rα	5'-TGA ACT CCA GGG AGA GGT ATG-3'	5'-CTA GGG AGG GGT CTC TGA TGC-3'

IL-15, Interleukin 15; IL-15Rα, Interleukin 15 receptor alpha.

Table S4 – Analysis of variance (ANOVA) results of IL-15.

Source of variation	% of total variation	P value	P value summary
Exercise	3.802	0.0983	ns
Age	1.691	0.2670	ns
Sex	7.005	0.0263	*
Exercise x Age	0.05886	0.8351	ns
Exercise x Sex	0.5407	0.5287	ns
Age x Sex	2.866	0.1500	ns
Exercise x Age x Sex	1.269	0.3357	ns

*, $P < 0.05$; ns, non significant.

Table S5 – Analysis of variance (ANOVA) results of IL-15Rα.

Source of variation	% of total variation	P value	P value summary
Exercise	0.3306	0.6427	ns
Age	0.2419	0.6913	ns
Sex	7.736	0.0279	*
Exercise x Age	1.123	0.3935	ns
Exercise x Sex	1.836	0.2763	ns
Age x Sex	0.07153	0.8290	ns
Exercise x Age x Sex	0.2395	0.6928	ns

*, $P < 0.05$; ns, non significant.

Table S6 – Analysis of variance (ANOVA) results of IL-15 positive muscle fibres.

Source of variation	% of total variation	P value	P value summary
Exercise	0.06722	0.8325	ns
Age	7.997	0.0242	*
Sex	1.355	0.3443	ns
Exercise x Age	6.659	0.0390	*
Exercise x Sex	1.209	0.3714	ns
Age x Sex	0.3117	0.6491	ns
Exercise x Age x Sex	1.036	0.4077	ns

*, $P < 0.05$; ns, non significant.

Table S7 – Percentage of IL-15 positive fibres per muscle fibre type in mouse gastrocnemius muscle.

Group		%Type I	%Type IIa	%Type IIb	%Type IIx	%Type IIab	%Type IIax	%Type IIbx	%Type Ix
SED	Young male (n=3)	0,00 ± 0	8,42 ± 8	2,54 ± 3	56,93 ± 20	4,48 ± 5	16,46 ± 4	11,16 ± 9	0,00 ± 0
	Old male (n=7)	0,21 ± 1	7,51 ± 9	6,79 ± 6	64,80 ± 24	0,00 ± 0	9,05 ± 7	11,64 ± 11	0,00 ± 0
	Young female (n=5)	0,22 ± 0	8,58 ± 3	2,74 ± 2	65,29 ± 13	0,53 ± 1	12,68 ± 7	9,97 ± 11	0,00 ± 0
	Old female (n=6)	0,00 ± 0	17,46 ± 12	5,54 ± 3	44,82 ± 21	0,00 ± 0	9,77 ± 5	20,15 ± 15	2,25 ± 6
EX	Young male (n=3)	0,00 ± 0	2,44 ± 2	3,07 ± 5	79,72 ± 17	0,00 ± 0	8,03 ± 9	6,73 ± 3	0,00 ± 0
	Old male (n=7)	1,72 ± 4	5,90 ± 6	3,36 ± 4	69,51 ± 21	0,00 ± 0	4,99 ± 5	10,95 ± 12	3,57 ± 9
	Young female (n=4)	0,80 ± 2	1,54 ± 1	1,55 ± 2	71,28 ± 16	0,00 ± 0	10,20 ± 9	12,50 ± 5	2,13 ± 4
	Old female (n=8)	0,00 ± 0	7,97 ± 7	5,68 ± 7	72,24 ± 17	0,00 ± 0	5,72 ± 4	8,39 ± 6	0,00 ± 0

Data are presented as mean ± standard deviation (SD). EX, Exercise group; IL-15, Interleukin 15; SED, Sedentary control group.