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Lifelong training improves anti-inflammatory environment and maintains the number of regulatory T cells in Masters athletes

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Abstract

Purpose: The purpose of this study was to quantify and characterize peripheral blood regulatory T cells (Tregs), as well as the IL-10 plasma concentration, in Masters athletes at rest and after an acute exhaustive exercise test. **Methods:** Eighteen Masters athletes (self-reported training: 24.6±1.83 years; 10.27±0.24 months and 5.45±0.42 hours/week per each month trained) and an age-matched control group of 10 subjects (that never took part in regular physical training) volunteered for this study. All subjects performed an incremental test to exhaustion on a cycle ergometer. Blood samples were obtained before (Pre), 10min into recovery (Post) and 1h after the test (1h). **Results:** Absolute numbers of Tregs were similar in both groups at rest. Acute exercise induced a significant increase in absolute numbers of Tregs at Post (0.049±0.021 to 0.056±0.024 x10⁹/L, P=0.029 for Masters; 0.048±0.017 to 0.058±0.020 x10⁹/L, P=0.037 for control) in both groups. Treg mRNA expression for Foxp3, IL-10 and TGF-β in sorted Tregs was similar throughout the trials in both groups. Masters athletes showed a higher percentage of subjects expressing the FoxP3 (100% for Masters vs. 78% for Controls, P=0.038) and TGF-β (89% for Masters vs. 56% for Controls, P=0.002) after exercise and a higher plasma IL-10 concentration (15.390±7.032 for Masters vs. 2.411±1.117 for control P=0.001, ES =2.57) at all time-points. KLRG1 expression in Tregs was unchanged. **Conclusion:** Our findings showed that Masters athletes have elevated anti-inflammatory markers and maintain the number of Tregs, may be an adaptive response to lifelong training.

Key words: Treg Cells; master athletes; aging; Foxp3; TGF-beta; Interleukin-10.

Abbreviations:	
1h	1h post-exercise
APCs	Antigen-presenting cells
KLRG1	Killer cell lectin-like receptor G1
IL	Interleukin
Pre	Before exercise
Post	10 min into recovery
VO _{2max}	Maximal Oxygen Consumption
URTI	Upper respiratory tract infection
Tregs	Regulatory T cells

1 INTRODUCTION

Strenuous exercise is commonly associated with increased symptoms of upper respiratory tract infection (URTI) in athletes (Nieman et al. 1989; Gleeson et al. 2012), suggesting that high-volume exercise may have an immunosuppressive effect (Gleeson 2007). The immunological mechanisms that underlie this apparent increase in infection susceptibility in athletes are probably multi-factorial and include perturbations of the T-cell compartment (Cosgrove et al. 2012; Tossige-Gomes et al. 2014; Simpson et al. 2015).

Regulatory T cells (Tregs) are crucial in maintaining immunological tolerance and negative control of pathological immune responses (Fehérvari and Sakaguchi 2004; de Moura Braz et al. 2014). Tregs secrete IL-10 and TGF-β as additional mechanisms to suppress target cell activity (Corthay 2009). Besides expressing the IL-2 receptor α-chain (CD25), Tregs also express the FoxP3 (Forkhead Box P3) transcription factor, a control gene that is key to their development and function (Campbell and Ziegler 2007). TGF-β triggers FoxP3 expression in precursors (CD4⁺CD25⁻) and together with IL-2, is a key regulator of signalling pathways that maintain FoxP3 expression and suppressive function in Tregs (Fu et al. 2004; Perry et al. 2013). Tregs can also express the KLRG1, a marker of senescence and differentiation of IL-10 production capacity by Tregs (Tauro et al. 2013). The Killer cell lectin-like receptor G1 (KLRG1), ITIM-bearing receptor for cadherin-family proteins. It is expressed on NK cells and on a minority of CD4⁺ and CD8⁺ T cells (Henson and Akbar 2009). Given the central role of Tregs in immune homeostasis, any age-related loss of Treg function would be predicted to contribute to over-active immune function encountered in elderly humans as a syndrome of chronic inflammation and the age-related increase in the risk for autoimmune disorders (Fessler et al. 2013). Conversely, the age-related increase in Treg numbers could result in compromised immune responses, increasing the risk of malignancies and infections amongst the elderly. This expansion of the Treg population can only be considered physiologically relevant if the suppressive function of the accumulated aged Tregs remains intact. Indeed, IL-10 production by Tregs and FoxP3 expression has been shown to be preserved with aging (Jagger et al. 2014). However, IL-10 expression was elevated among KLRG1⁺ Treg cells (Tauro et al. 2013). Though KLRG1 serves as a marker for senescence, signalling through KLRG1 could result in altered T-cell function (Tauro et al.

2013). Actually, KLRG1+ Treg cells are somewhat more potent than KLRG1- cells in suppressing naive T-cell proliferation *in vitro* (Beyersdorf et al. 2007).

Aging is a natural process, but with increasing life expectancy worldwide, activities that can mitigate the effects of aging on the immune system are gaining attention. This includes the practice of exercise/physical activity. The impact of exercise on immune system has been extensively studied. However, most studies have investigated the acute effects of exercise and there are few research related to chronic effects or longitudinal baseline studies. Evidence suggests that circulating numbers of Tregs increase following an acute bout of strenuous exercise in humans (Handzlik et al. 2013). Moreover, evidence of an association between high training load, increased *in vitro* antigen-stimulated IL-10 production and greater numbers of circulating Tregs, support the hypothesis that Tregs mediate inhibitory effects through increases IL-10 production in highly physically active individuals, and this may play an important role in modifying respiratory infection risk (Gleeson and Walsh 2012; Handzlik et al. 2013).

The immune response to acute exercise is transitory and variable, being influenced by a range of factors such as the intensity, duration and mode of the exercise. The effects of exercise training on immunosenescence are inconsistent, probably due to differences in experimental design among these studies (Simpson et al. 2012; de Araújo et al. 2013; Silva et al. 2016).

Masters athletes, represent an interesting sub-demographic group of adults because many of them express a highly unique physiological phenotype that could be termed 'exceptionally successful ageing' (Tanaka and Seals 2008). Thus, the study of Masters athletes provides an opportunity to investigate the effects long-term training on the ageing immune system *per se*, and the influence of long-term training adaptations on acute exercise responses. Therefore, the purpose of this study was to quantify and functionally characterize peripheral blood Tregs, as well as the expression of a senescence marker, in Masters athletes after an exercise incremental test to exhaustion.

2 MATERIALS AND METHODS

2.1 Subjects

Nineteen Masters athletes (3 female, 16 male) over 40 years old who had participated in training and competitions for more than 20 years volunteered for this study. The study also included a healthy, non-smoking and body weight- and age-matched control group of 10 subjects (4 female, 6 male) (Table 1). All participants completed a comprehensive health-screening questionnaire before testing commenced used for inclusion/exclusion criteria. The inclusion criteria for masters was a minimum of twenty years of regular training and competition participation. Moreover, the master athletes should be currently participating in regular training and competition (judo, swimmers, athletics). The inclusion criteria for the control group, was to have performed no regular physical training in the last 20 years. Participants were only included if they were non-smokers and had no known cardiovascular, musculoskeletal or neurological diseases or where taking any medication like statins.

On average, master athletes had an experience of training of 24.6 ± 1.8 years. Each year, the athletes trained 10.3 ± 0.2 months and approximately 5 hours per week (5.5 ± 0.4 hours) per month trained. All subjects provided their written, informed consent to participate in the study, which was approved by the Ethics and Human Subjects Review Board at the Faculty of Sports Science and Physical Education, University of Coimbra.

* Table 1 about here *

2.2 Experimental design

Participants arrived at the laboratory at 09:00 having refrained from caffeine and alcohol in the previous 24 h and avoided strenuous exercise 72 h prior to the laboratory procedures. Participants sat quietly for 15 min while they completed a comprehensive health-screening questionnaire. Resting venous blood samples (15 mL) were taken from an antecubital vein by venipuncture in a seated position into 3 vacutainer K₃EDTA tubes (Becton–Dickinson, Oxford, UK). A maximal protocol test was performed for determination of VO_{2max}. Ten min and 1h into completion of exercise protocol 15-mL blood samples were collected in the same manner as described before. Haematological analysis, plasma IL-10 concentration, flow cytometry procedures, cell purification and mRNA expression in purified Treg cells are described below.

2.3 VO_{2max} determination

VO_{2max} was determined using an incremental cycling protocol performed on an electro-magnetically braked cycle ergometer (Lode Excalibur Sport V4.67, Groningen, The Netherlands). After the measurements of body weight, stature and a pre-test blood sample, participants began cycling at 75W; power output was increased in 25W every 3 min until volitional fatigue. Expired gas was collected via breath-by-breath automated gas-analysis system (Quark CPET COSMED, COSMED, Rome, Italy). Heart-rate, using short range telemetry (COSMED, Rome, Italy), and ratings of perceived exertion Borg Cr-10 scale (RPE) were recorded each 3-min stage and during the final of the test.

2.4 Haematological analysis

Immediately after collection, blood cell counts: total leukocytes, lymphocytes, monocytes, granulocytes were determined using an automated cell counter (Coulter ACT Diff, Beckman Coulter, USA).

2.5 Cytokines

Plasma was extracted from one of the tubes by centrifugation at 4°C, at 2500rpm for 15min, and stored at -80°C until further use.

Plasma IL-10 concentration was evaluated using a commercially available ELISA kit, according to the manufacturer's instructions (Invitrogen, CA). The minimum detection limit for IL-10 was < 1pg/ml. Plasma samples were assayed in duplicate. The mean coefficient variation for the duplicate analysis was 3.55%.

2.6 Flow cytometry

Immunofluorescent staining of whole blood was performed using an eight-colour immunophenotyping strategy on a FACSCanto II (BD Bioscience, San Jose, CA, USA) identified specific lymphocyte populations and Tregs. The peripheral blood cells were stained with the following monoclonal antibodies (mAb): anti-human CD3-PB (pacific blue, clone UCHT1, Pharmingen, San Diego, C.A. USA), anti-human CD4-APC-H7 (allophycocyanin-hilite 7, Clone 13B8.2, Beckman Coulter, Miami, FL, USA), anti-human CD8-KO (Krome Orange, Clone 5M3.332, Beckman Coulter, Miami, FL, USA), anti-human CD25-PE (phycoerythrin, Clone 2A3, BD Bioscience, San Jose, CA, USA), anti-human CD127-FITC (fluorescein isothiocyanate, Clone R.34.34, Beckman Coulter), anti-human CD27-PECy5 (phycoerythrin-cyanine 5, clone R.8.01, Beckman Coulter), anti-human CD45RA-PECy7 (phycoerythrin-cyanine 7, Clone L48, BD Bioscience) and anti-human/mouse KLRG1-APC (allophycocyanin, Clone 2F1/KLRG1, Biolegend, San Diego, CA). Briefly, peripheral blood cells were labelled and incubated for 10 min at room temperature in the dark. After this, 2 ml of FACS lysing Solution (BD Biosciences) was added; cells were incubated at room temperature for a further 10 min and then washed with 2ml of PBS. The tubes were then centrifuged at 540 g for 5 min and the supernatant was discarded. The cells were resuspended in 0.5 ml PBS, and immediately acquired in a FACSCanto II (BD) flow cytometer.

For acquisition initially, 1,000,000 events, corresponding to all nucleated cells present in the sample, were collected and information stored. A lymphocyte population was first gated from the PB cells according to their low forward and side scatter. T lymphocytes were identified according to their positivity for CD3 and typical light scatter. Tregs cells were defined based in the following phenotype: CD4⁺CD25⁺⁺CD127^{-/low} and subdivided into naive (CD45RA⁺) and memory (CD45RA⁻) cells. An inversion relationship between CD127 and FoxP3 expressed in CD4⁺CD25⁺⁺ cells has been described (Liu et al. 2006), allowing accurate estimation of this Treg cell population via flow cytometry (Handzlik et al. 2013) (Figure 1). Senescent T cells were detected based on the expression of KLRG1⁺. The FACSCanto II was routinely calibrated using Calibrite beads (BD Biosciences) and single labelled antibody tubes were used for further compensation adjustments. Data analysis was performed in the Infinicyt version 1.7 software (Cytognos, Salamanca, Spain). Absolute counts were calculated using a dual platform methodology (flow cytometry and haematological cell analyser – Beckman Coulter LH 750, Miami, USA).

* Figure 1 about here*

2.7 Cell purification by fluorescence-activated cell sorting

CD4⁺CD25⁺CD127^{-/low} Tregs-cell populations were purified by FACS (using a FACSAria II flow cytometer; BD) according to their typical phenotype. The purified cell populations were subsequently used for mRNA expression studies.

2.8 Analysis of mRNA expression in purified Treg cells

The content of purified cell sorted CD4⁺CD25⁺CD127^{-/low} Tregs-cell population, were transferred to a 1.5-mL Eppendorf tube and centrifuged for 5 min at 300 g, and the pellet was resuspended in 350 μ L of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen) in accordance with the instructions of the manufacturer. Total RNA was eluted in a 20- μ L volume of RNase-free water and was reverse-transcribed with Tetra cDNA Synthesis (Bioline, London, UK) in accordance with the instructions of the manufacturer. Relative quantification of gene expression by real-time polymerase chain reaction (PCR) was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCRs were carried out by using 1x QuantiTect SYBR Green PCR Master Mix (Qiagen) and 1x QuantiTect Primer Assay (TGF- β 1: QT00025718; IL-10: QT00041685; FoxP3: QT00048286) (Qiagen) in a final volume of 10 μ L. The reactions were performed by using the following thermal profile: one cycle of 10 min at 95°C, 50 cycles of 10 s at 95°C, 20 s at 55°C and 30 s at 72°C, one cycle of 5 s at 95°C, 1 min at 65°C and continuous at 97°C, and one cycle of 10 s at 21°C. Real-time PCR results were analysed with the LightCycler software (Roche Diagnostics). GeNorm software (Primer Design Ltd., Southampton, UK) was used to select the reference genes to normalize data. The reference genes used for gene expression analysis were cytochrome c1 cytochrome c1 (CYC1; QT00209454) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; QT012504271). The normalized expression levels of the genes of interest were calculated by using the delta Ct (change in threshold cycle) method.

2.9 Statistical analysis

All data were expressed as mean \pm standard deviation. Group comparisons were assessed using the Mann-Whitney U test. Sequential changes by acute exercise were assessed by repeated-measures analysis of variance for data with normal distribution. When the distributions deviated from normal distribution, a Friedman test with Dunn's post hoc test was undertaken to evaluate the statistical differences in time. Because no differences between genders were found when analysing the Treg subpopulations, final statistical analysis where done without separating the samples by gender. Significance was accepted at P<0.05. Correlation between subsets populations and age were assessed according to Spearman's rank correlation coefficient. Percentage of differences was tested using cross-tabulation and Chi-Square Test. All statistical analyses were performed using SPSS 23.0 (SPSS, Chicago, IL). To report the strength of the exercise intervention or magnitude of effect of condition (Masters vs. control) the Cohen's effect size (ES) was calculated and was categorized by convention as (d=0.2 small effect; d=0.5 medium effect; d=0.8 or more, large effect) using G*Power Version 3.1.9.2. The graphs are building using the GraphPad Prism version 5.0.

3 RESULTS

There were no significant differences for height, body weight and BMI between groups (Table 1). VO_{2max} was higher for master athletes when compared to the older group (P=0.001; Table 1). The exercise induced a significant increase of the leukocyte numbers (P=0.003, ES =1.21). *Post hoc* analysis revealed that the total leukocyte count returned to pre-exercise values at 1h (control, P=0.001, ES =1.57; Masters, P=0.001, ES =0.73). Lymphocytes exhibited a significant biphasic response to exercise, whereby exercise induced an increase in lymphocyte count in both groups at Post (control, P=0.001, ES =1.32; Masters P=0.005, ES =0.82) compared to baseline values; however, 1h post-exercise values were significantly lower than Pre for Masters athletes only (P=0.002, ES =0.53), but returned to baseline for control subjects (P=0.426). Monocytes and granulocytes showed an increase at Post (control, P=0.045, ES =1.11, Masters, P=0.054, ES =0.70 for monocytes; control, P=0.045, ES =0.50, Masters, P=0.024, ES =0.40 for granulocytes; Table 2).

* Table 2 about here *

To assess any effects of long-term exercise on Tregs, we first looked at differences at baseline. There were no significant differences in the proportion (%) of CD4⁺CD25⁺CD127^{-low} Tregs either within the total lymphocytes population or within the CD4⁺ T cell population, between the two studied groups at baseline (Table 3, P=0.890). The frequency of naïve (CD45RA⁺) and memory (CD45RA⁻) Tregs subpopulations was also similar for both groups (Table 3).

* Table 3 about here *

There was no significant effect of acute exercise on the proportion of CD4⁺CD25⁺CD127^{-low} Tregs within total lymphocytes and CD4⁺ T cells (Table 3). However, acute exercise induced a significant increase in the numbers of Tregs at Post (Table 3, P=0.034, ES =0.31 for Masters; P=0.033, ES =0.53 for control) and returned to pre-exercise values at 1 h post (Table 3).

The ability of exercise to induce FoxP3, TGF-β and IL-10 mRNA expression was evaluated in purified CD4⁺CD25⁺CD127^{-low} Treg cells. FoxP3 and TGF-β mRNA expression was similar in all measurements from both groups. Similarly, IL-10 mRNA expression was not altered in response to exercise (Figure 2, Table 4). Interestingly, in the Masters group the percentage of cases where IL-10 mRNA expression was detected remained unchanged (Pre =61.1%; Post =66.7% and 1h Post =50% of all subjects), while in the control group there was a tendency for a fewer cases expressing mRNA for IL-10 (Pre =22.2%; Post =55.6% and 1h =22.2%) (Table 4). For FoxP3 and TGF-β after exercise the number of detected cases was also different between groups, with higher results for Masters athletes (Table 4). Since the expression of reference genes CYC1 and GAPDH was achieved in all the samples, the negative cases for FoxP3, TGF-β and IL-10 mRNA are truly undetectable.

* Figure 2 about here *

* Table 4 about here *

Plasma IL-10 concentration was significantly higher in Masters athletes compared with the control group before and after the exercise test (P=0.001, ES =2.57). Acute exercise had no influence on IL-10 concentrations (figure 3).

* Figure 3 about here *

KLRG1 was not highly expressed on Tregs with expression around 2% of Tregs in Masters athletes at baseline and 3.1% of Tregs in the control group. This remained unchanged Post and 1h post (Figure 4). Of interest however, was the number of subjects expressing this marker of senescence: 6 out of 18 (33%) in the Masters group versus 5 out of 10 (50%) in the control group (Table 4).

* Figure 4 about here *

4 DISCUSSION

The purpose of this study was to quantify and characterize peripheral blood Tregs after acute, maximal exercise in two groups with distinct differences in lifelong exercise habits; Masters athletes and an age-matched healthy control group. The main findings of this study demonstrate that both groups have similar levels of resting circulating Tregs as well as similar levels of lymphocytes and CD4⁺ T cells. The Masters athlete group have increased activation levels of Tregs at baseline when compared to the control group (increased percentage of subjects expressing the FoxP3, TGF-β and a higher plasma IL-10 concentration).

Masters and Controls had similar numbers of circulating CD4⁺CD25⁺CD127^{-low} Tregs at baseline, suggesting that lifelong training does not affect these cells numbers. Tregs maintain self-tolerance and control excessive immune responses to foreign antigens. Age-dependent gain of Treg activity may result in weakened immunity, such as increased risk of developing malignancies and infections amongst the elderly. Conversely, age-related loss of Treg function would be predicted to cause an over active immune state, which has been reported in elderly as higher inflammation status and increased risk for autoimmunity (Jagger et al. 2014).

The number of studies examining the effect of exercise on Treg function, phenotype and suppressive effects is scarce. Some researchers showed that exercise increases the number and function of Treg cells in murine models (Wang et al. 2012; Uchiyama et al. 2015), murine asthma models (Lowder et al. 2010) and humans (Lages et al. 2008; Handzlik et al. 2013; Teixeira et al. 2014).

We found that absolute counts of CD4⁺CD25⁺CD127^{-low} cells increased immediately post-exercise in both groups. It is well known that maximal efforts have profound effects on total leukocytes, marked by an acute inflammatory response that is characterized by leukocytosis and leukocyte activation, release of

inflammatory mediators and tissue damage (Tossige-Gomes et al. 2014). In our study an increase in the absolute number of granulocytes and monocytes, and lymphocytes was observed, arguing that some of the leukocytosis observed in the Masters and Control groups may be due to a local inflammatory response caused by tissue injury. However, this is a temporary response and 1 hour after exercise the values returned to pre-exercise values. Because of this, the increase in Tregs observed at Post might simply reflect the increase in total numbers of lymphocytes (Handzlik et al. 2013).

Additionally, the phenotype of circulating Tregs remained unchanged, as assessed by the functional markers FoxP3 and TGF- β . In fact, we did not find any differences in Treg FoxP3, TGF- β and IL-10 mRNA expression in both studied groups following exercise, but the number of detected cases (number of subjects where the expression of these markers was detected) was different between groups (Table 4). The higher number of detected cases in Masters athletes for FoxP3 after exercise implies that lifelong exercise influences acute exercise responses for transcription factor FoxP3 in Masters athletes. Yeh et al (2009) demonstrated that exercise training increased the T cell expression of FoxP3, in the periphery, of type-2 diabetic patients. Yeh et al. (2006) also showed that the production of regulatory T cell mediators TGF- β and IL-10 under varicella zoster virus stimulation was increased after a 12 week-programme of tai chi chuan exercise in middle-aged volunteers. Our results showed that acute exercise maintained TGF- β detection rates in Masters athletes, but not in the control group, where TGF- β detection rates decreased 11% in Post and 34% 1h post-exercise (Table 4). TGF- β has potent immunoregulatory properties and has been shown to induce IgA secretion. This suggests improved mucosal immunity defences leading to a lower risk of autoimmune and inflammatory disorders (Yeh et al. 2006). Also, TGF- β can play an essential role in the suppression of inflammation. TGF- β inhibits activity of T and B-lymphocytes and natural killer cells. Additionally, TGF- β also influences the production of other cytokines via Foxp3-dependent and -independent mechanisms (Yoshimura et al. 2010).

One of main molecules identified as responsible for immunomodulation by Tregs is IL-10. It is therefore possible, that elevation in IL-10 secretion may be associated with increased numbers of circulating Treg cells, the primary source of IL-10 within the body (Gleeson et al. 2011). IL-10 appears to be as an immunosuppressive cytokine with broad anti-inflammatory properties, in particular by the inhibition of macrophage and DCs functions. IL-10 down regulates the expression of MHC class II and costimulatory molecules such as CD80 and CD86, affecting the T cell-stimulating capacity of APCs.

In our study, IL-10 gene expression was similar between both groups at baseline (Pre), but IL-10 plasma concentrations were higher in the Masters athletes group. Handzlik et al (2013) showed that high training loads are associated with higher resting Treg cell counts and greater IL-10 production following antigen stimulation. By showing a strong relationship between the increase in the number of Treg and high production of IL-10 *in vitro*, the authors suggested that athletes engaged in high training loads had a greater ability to secrete IL-10 because of the increase in Treg numbers. On the other hand, Hwang et al. showed that Tregs from elderly individuals (age > 65) inhibited the proliferation of responder cells and that the production of IL-10 was reduced. This agrees with our results where the lower concentrations of IL-10 were found in the control group (Figure 3). Together, these results support the anti-inflammatory effects of exercise theory proposed by Gleeson and colleagues (Gleeson et al. 2011).

Recently, the expression of KLRG1 in Tregs has been linked with altered function and senescence (Tauro et al. 2013). In a model of autoimmune disease, KLRG1⁺Tregs expressed higher levels of activation markers (FoxP3 and CD25) and were at a terminal differentiation stage. Furthermore, KLRG1⁺ Tregs produced more IL-10. Our results showed that a very small percentage of regulatory T cells were KLRG1⁺ and differences (albeit non-significant) were observed in the number of cases expressing KLRG1 between the groups, the Masters group having approximately 50% less Tregs KLRG1⁺ when compared with the control group.

The discrimination between naïve and memory Tregs is biologically relevant. Although both naïve and memory Tregs are suppressive, they possess distinct homing receptors that allow migration into either lymphoid organs or inflamed tissues, respectively (Miyara et al. 2009; van der Geest et al. 2014). Naïve Tregs might play an important role in the maintenance of the Treg pool in adults, whereas the increase of memory Treg cells was associated with a decreased humoral response to vaccine (van der Geest et al. 2014). Based on the expression of CD45RA, we divided CD4⁺ T cells into naïve (CD45RA⁺) and memory (CD45RA⁻) cells, but no differences in naïve and memory populations were found between controls and Masters. Consistent with previous studies (Hwang et al. 2009), in the present study most of the CD4⁺CD25⁺⁺CD127^{-/low} cells had the memory phenotype (CD45RA⁻). Unlike other studies (Miyara et al. 2009; Booth et al. 2010; van der Geest et al. 2014) that showed that the proportions of circulating naïve Tregs decreased with age, whereas memory Treg cells were increased (a tendency observed for our control group), we observed a tendency to maintain / increase the proportions of the naïve cells in the Masters athlete group, while a maintenance / reduction was observed in the control group (Figure 4). This however was not statistically significant and a higher number of subjects will need to be analysed to substantiate, or not, these findings.

The primary limitation of this study was the small sample size due to the difficulty in finding masters athletes with the training characteristics required for this study. The same occurred for the control group, since

these individuals had to have the social, healthy and physical characteristics matched with the athletes. Furthermore, the questionnaire to determine the characteristics of training is based on participant recall. It is difficult to establish the training history after a mean training period of 24.6 ± 1.83 years. Our intention was to analyse an overview of lifelong training providing data of the past and current physical activities of participants. Because the training-specific adaptations are related to the nature (aerobic-based, resistance-based, balance-based training), intensity, and duration of the exercise training (Chodzko-Zajko et al. 2009), cautious interpretation of the results obtained is warranted.

5 Conclusions

Overall, despite the limitations described above, the results of this study have important implications. We show that lifelong training appears to be an important non-pharmacological intervention that clearly induces a health profile. Masters athletes showed high levels of the immunoregulatory cytokine IL-10 at rest and in response to a maximal exercise test when compared to the age-matched control group. The numbers of Tregs were the same in Masters and control group suggesting that lifelong training does not interfere in age-related loss or increase in Tregs numbers. Moreover, Masters athletes had increased percentage of subjects expressing FoxP3 and TGF- β mRNA. This is the first study to show that Masters athletes have elevated anti-inflammatory markers and maintain the number and markers of activation of regulatory T cells as adaptive responses to lifelong training. Future research should include larger samples and functional testing.

6 Acknowledgments

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7 Conflict of interest

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Table 1. Participant characteristics.

	Control	Masters	P value
Age (years)	54.2 ± 5.94	53.2 ± 9.08	0.759
Height (cm)	168.6 ± 8.53	170.4 ± 5.29	0.480
Body mass (kg)	70.4 ± 12.93	74.9 ± 15.55	0.444
BMI (kg.m ⁻²)	24.6 ± 3.18	25.7 ± 4.65	0.526
VO _{2max} (ml.kg.min ⁻¹)	29.3 ± 4.14	40.2 ± 11.45*	0.001

Values are Mean ± Standard Deviation (SD). N= Masters (19), Control (10). BMI= Body Mass Index; VO_{2max} = Maximal Oxygen Consumption * P<0.001 compared to Control group.

Table 2. Haematological variables and leukocyte counts

	Pre	Post	1h
<i>WBC (x 10⁹/L)</i>			
Control	6.70±1.41	8.80± 1.93*	5.88±1.76
Masters	6.94±1.89	8.94± 3.13*	6.88±2.19
<i>Lymphocytes (x 10⁹/L)</i>			
Control	2.00± 0.66	3.20±1.03*	1.77± 0.66
Masters	2.00± 0.74	3.26± 1.75*	1.55± 0.92§
<i>Monocytes (x 10⁹/L)</i>			
Control	0.41± 0.17	0.60± 0.17*	0.35± 0.10
Masters	0.45± 0.19	0.58 ± 0.18*	0.38± 0.18
<i>Granulocytes (x 10⁹/L)</i>			
Control	3.80±1.22	4.40±1.17*	3.77±1.64
Masters	4.05±1.17	4.73± 1.91*	4.38±1.68

Values are Mean±SD n = Masters (19), control (10). Pre (before), Post (10 min into recovery), 1h (1 h after the test). * P < 0.05 from Pre to Post, § p < 0.05 from Pre. WBC: white blood cells

Table 3. Frequency (among total lymphocytes or CD4+ T cells) and absolute counts (x10³/μl) of CD25⁺CD127^{-/low} Treg cells, in response to acute exercise.

Regulatory T cells	Pre	Post	1h
<i>%Total</i>			
Control	0.69 ± 0.25	0.63 ± 0.20	0.65 ± 0.27
Masters	0.68 ± 0.23	0.61 ± 0.23	0.58 ± 0.25
<i>%CD4</i>			
Control	6.13 ± 1.36	5.72 ± 1.22	6.40 ± 1.80
Masters	6.80 ± 1.43	6.44 ± 1.42	7.43 ± 0.91
<i>Absolute Counts</i>			
Control	0.048 ± 0.017	0.058 ± 0.020*	0.042 ± 0.025
Masters	0.049 ± 0.021	0.056 ± 0.024*	0.044 ± 0.023
<i>Naïve (% of Tregs)</i>			
Control	11.50 ± 4.91	15.20 ± 8.72	10.17 ± 4.55
Masters	14.37 ± 7.05	14.15 ± 6.74	11.96 ± 6.11
<i>Memory (% of Tregs)</i>			
Control	88.49 ± 4.91	84.79 ± 8.72	89.82 ± 4.55
Masters	85.62 ± 7.05	85.84 ± 6.74	87.83 ± 6.02

Values are Mean±SD n = Masters (19), control (10). Pre (before), Post (10 minutes into recovery), 1h (1 h after the test). * P < 0.05 from Pre to Post.

Table 4. Number of detected cases for Foxp3, TGF- β and IL-10 mRNA expression.

Genes	Pre			Post			1h		
	Control N (%)	Masters N (%)	<i>P</i>	Control N (%)	Masters N (%)	<i>P</i>	Control N (%)	Masters N (%)	<i>P</i>
<i>Foxp3</i>									
Yes	9 (100)	17 (94)	<i>0.471</i>	7 (78)	18 (100)	<i>0.038</i>	5 (56)	16 (89)	<i>0.050</i>
No	0 (00)	1 (06)		2 (22)	0 (00)		4 (44)	2 (11)	
<i>TGFβ</i>									
Yes	6 (67)	18 (100)	<i>0.009</i>	5 (56)	18 (100)	<i>0.002</i>	3 (33)	18 (100)	<i>0.003</i>
No	3 (33)	0 (00)		4 (44)	0 (00)		6 (67)	0 (00)	
<i>IL-10</i>									
Yes	2 (22)	11 (61)	<i>0.057</i>	5 (56)	12 (67)	<i>0.573</i>	2 (22)	9 (50)	<i>0.166</i>
No	7 (78)	7 (39)		4 (44)	6 (33)		7 (78)	9 (50)	
<i>KLRG1</i>									
Yes	5 (50)	6 (33)	<i>0.387</i>	5 (50)	6 (33)	<i>0.387</i>	5 (50)	6 (33)	<i>0.387</i>
No	5 (50)	12 (67)		5 (50)	12 (67)		5 (50)	12 (67)	

P= Pearson Chi-Square

Pre (before exercise), Post (post-exercise); 1h (1 h post-exercise)

Figure 1. Identification of Treg cells via flow cytometry.

a CD127 versus CD25 identifying CD3⁺CD4⁺CD25⁺⁺CD127^{low/-} (Treg) cells. b FSC-H versus CD45RA gating naïve (CD4⁺ CD45RA⁻) and memory (CD4⁺ CD45RA⁺) Treg cells.

Figure 2. Effect of acute exercise on FoxP3, TGF-β and IL-10 gene expression in purified CD4⁺CD25⁺⁺CD127^{low/-} Treg cells.

Data are shown as Mean ±SD (Grey box = Control group; Black box = Masters group). Pre (before), Post (10 minutes into recovery), 1h (1 h after the test).

Figure 3. Acute exercise effects on IL-10 plasma levels.

Data are shown as Mean ±SD (Grey box = control group; Black box = Masters group). Pre (before, Masters n= 16; control n= 8), Post (10 minutes into recovery, Masters n= 16; control n= 10); 1h (1 h after the test, Masters n= 16; control n= 4). * Different from Control, p<0.001.

Figure 4. Percentage of KLRG1+ Treg cells among CD4+ T cells.

Data are shown as Mean ± SD. Pre (before); Post (10 minutes into recovery); 1h (1 h after the test). Black squares represent group control (n=6 from 18 of total; 01 outlier); Black circles represent Masters group (n=5 positive cases from a total of 10)







