Effects of lifelong training on senescence and mobilization of T lymphocytes in response to acute exercise.

Luciele G. Minuzzi[1], Luís Rama[1], Matheus Uba Chupel[1], Fátima Rosado[1], João Valente dos Santos[1,2], Richard Simpson[3], António Martinho[4], Artur Paiva[5], Ana M. Teixeira[1]

[1] Research Center for Sport and Physical Activity (IUD/DTP/04213/2016) Faculty of Sports Science and Physical Education, University of Coimbra, Portugal
[2] Faculty of Physical Education and Sport, Lusófona University of Humanities and Technologies, Lisbon, Portugal
[3] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Texas, USA.
[4] Portuguese Institute for Blood and Transplantation, São Martinho do Bispo, Coimbra, Portugal
[5] Flow Cytometry Unit-Clinical Pathology Service, University Hospital Centre of Coimbra, Portugal

ABSTRACT

Background/Purpose: *Ageing has profound impact on the immune system, mainly on T-cells. However, it has been suggested that chronic exercise may delay immunosenescence. Master athletes represent an interesting sub-demographic group to test this theory since they maintain a high training frequency and load throughout life. The purpose of this study was to evaluate the effects of lifelong training on the senescence and mobilization of T lymphocytes in response to acute exercise.*

Material and Methods: *Nineteen athletes who regularly participated in training and competitions for more than 20 years throughout their lives and a control group of 10 healthy individuals participated in this study. All subjects performed a progressive test to exhaustion on a cycle ergometer. Blood samples were obtained before (Pre), 10 min after the test (Post) and 1 h after the test (1h). Phenotypic study of peripheral blood T-cells was performed by flow cytometry. Genes of interest expression was done on T-cells purified by cell sorting.*

Results: *Master athletes had a lower percentage of senescent naïve, central memory and effector memory $CD8^+$ T-cells and senescent naïve and effector memory $CD4^+$ T-cells. Age had a positive effect on SLEC $CD8^+$ T-cells and a negative effect on naïve $CD8^+$ T-cells. VO_{2max} positively correlated with the proportion of naïve $CD4^+$ T-cells and negatively correlated with the percentage of total lymphocytes. No differences were founded for $CD4^+$ and $CD8^+$ T-cells and their subsets between master athletes and the control group at all times of measurement. No differences were observed in the CD45RA expressing effector memory cells (EMRA) for the various study conditions. The mRNA expression of the CCR7 gene for naïve $CD8^+$ T-cells and the Fas-L gene for effector-terminal $CD8^+$ T-cells was not different between masters and controls and did not change in response to the maximal protocol test.*

Conclusion: *In conclusion, maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells.*

Keywords: Immunosenescence; KLRG1; Cytomegalovirus; CCR7; Fas-L; Immune space.

INTRODUCTION

The age-associated decline in immune function, referred to as immunosenescence, is well characterized in the adaptive immune system and, in particular, within T-cells. The characteristics of immunosenescence in the T cell pool, include low numbers and proportions of naïve T-cells (especially $CD8^+$ T-cells) and a large number of memory T-cells ($CD8^+$ T-cells especially in late stage of differentiation) (1, 9, 21, 22); poor vaccination responses (18, 41); and a CD4:CD8 ratio <1.0 (17). These changes are largely driven by infection with cytomegalovirus (455). Depletion of the naïve T cell pool may result in fewer naïve cells capable of responding to new antigens; in fact, the extended pool of memory cells may actually prevent populations of naïve cells from being able to proliferate and expand in sufficient numbers (26).

The onset of senescent T cells occurs due to excess clonal expansions occurring as part of a normal immune response to pathogens that invade or reactivate infections in the body throughout life (33). Repeated exposure to antigenic stimuli throughout life (like reactivation of latent viral infections) leads to further rounds of cell division and premature senescence. As these senescent T cells still preserve effector cell functions (like death of virus-infected cells) and are highly pro-inflammatory, their accumulation in blood and tissues

Corresponding author:
Ana Maria Teixeira, Pavilhão III, Estádio Universitário de Coimbra, Santa-Clara, 3040-156 Coimbra, Portugal
E-mail: ateixeira@fcdef.uc.pt
Tel.: +351 239 802 770, Fax: +351 239 802 779

may also contribute to a number of pathologies associated with inflammation (33).

Spielmann et al. (2011) demonstrated that the proportions of senescent $CD4^+$ and $CD8^+$ T-cells increased with advanced age, at a corresponding rate of 10% and 10.2% per decade, respectively. This was accompanied by a reduction in the proportions of naive $CD4^+$ and $CD8^+$ T-cells (10% and 9.9% per decade, respectively). Interestingly, the authors found that subjects who had above-average VO_{2max} values had fewer senescent $CD4^+$ and $CD8^+$ T-cells and increased numbers of naive $CD8^+$ T-cells than those with lower VO_{2max} values, even after adjustment for age, body mass index, and percentage of body fat. Surprisingly, the authors found that the well-accepted association between age and senescent T-cells no longer existed when age was adjusted for VO_{2max}, indicating that aerobic fitness may be a strong determinant of T-cell phenotypic changes with greater impact than chronological age. This effect was limited to senescent cells, and VO_{2max} was not associated with increased memory cells after adjustment for age. The study was the first to show that aerobic fitness is associated with a moderation of natural age-related accumulation of senescent T cells in peripheral blood, highlighting the beneficial effects of maintaining a physically active lifestyle on immunosenescence (38).

Many aspects of immune function change with age, and some of these changes can be restored temporarily by exercise by delaying the onset of immune ageing or by rejuvenating aged immune profiles (20, 28, 34, 36, 40). This theory has been developed based on the evidence that exercise "stimulates" the immune function (43, 44). For example, moderate or intense training throughout life leads to strong response to vaccines against influenza, resulting in higher percentages of protected subjects (2). Furthermore, the immunosurveillance of lymphocytes, the cells which seek tissue antigens derived from viruses, bacteria or malignant transformation is thought to be facilitated by lymphocytosis and subsequent transient exercise-induced lymphopenia (10, 40). Also, some forms of exercise are anti-inflammatory, and if regularly repeated throughout life, there is a lower morbidity and mortality from diseases with an immunological and inflammatory etiology (11).

Many aspects of immune function change with age, and some of these changes can be restored temporarily by exercise. A recent theory proposed that exercise can trigger preventive and / or restorative mechanisms of T-cell immunosenescence (28, 34) involving a three steps process. Firstly, as shown by many studies (4, 32, 33), cells in a late differentiated stage are mobilized to the peripheral blood during exercise. Secondly, these cells extravasate from the blood to peripheral and / or inflamed tissues 1-2 h after exercising (6, 43, 44). At these sites, it is thought that T-cells are exposed to a variety of pro-apoptotic stimuli (e.g., reactive oxygen species, glucocorticoids, cytokines) that may cause apoptosis of these cells (15). The third and final phase of this hypothesis, proposes that the naïve T-cell repertoire is then able to expand in response to the "immune space" that has been created, initiated by a hypothetical negative feedback loop that governs the number of naïve and memory cells (12, 28).

If exercise can reverse the immunosenescent phenotype by causing selective apoptosis (28) is still a topic of debate (30, 40). For example, exercise could also prevent accumulation of terminally differentiated/exhausted T-cells with age by preventing CMV reactivation (31). Moreover, there is evidence for and against this model, including the idea suggesting that not all cells bearing a so-called "senescent" phenotype are actually senescent (40). More recent advances showed that many CMV-specific $CD8^+$ T cells, identified using MHC-class I tetramers, are multifunctional, producing IFN-γ, IL-2 and TNF-α and exhibiting potent cytotoxic activity (25). In addition, these cells have telomeres of intermediate length, despite ''senescent'' cell-surface characteristics (e.g., $CD45RA^+CD27^-$) (25).

Although, many studies indicate that exercise can slow human biological aging, the effects of long-term exercise on T cell function are not well known (5, 19, 23, 39). Therefore, the aim of this study was to evaluate the effects of training throughout life on the senescence of T cell and on the mobilization of senescent T cell subsets in response to a maximal acute exercise test.

The hypothesis of increasing the frequency of terminally differentiated T-cells in the blood after acute exercise suggests that the selective mobilization and death of these cells by acute and regular exercise could eventually allow naïve T-cells to occupy vacant immune space and increase the T cell repertoire (32). The analysis of this hypothesis in master athletes is original and was tested in this study.

MATERIALS AND METHODS

Participants

Nineteen master athletes volunteered to participate in this study. The subjects (n=15 men and n=4 women) were swimmers, judo and track and field athletes over 40 years old who had participated in training and competitions for more than 20 years throughout their life. The study also included a healthy, non-smokers, body mass and age-matched control group of 10 subjects (n=7 men and n=3 women) who performed no regular physical training in the last 20 years (Table 1). The inclusion criteria for masters were a minimum of twenty years of regular training and competition participation and being currently participating in regular training and competition. The inclusion criteria for the control group, was to not have performed regular physical training in the last 20 years. Exclusion criteria for both groups included smokers, any known cardiovascular, musculoskeletal or neurological disease and the use of any medication or supplementation. The master athlete sports history was accessed through a self-reported regarding the beginning of athletic life, the modalities practiced and the duration dedicated to each category, including the starting and finishing year, the hours per week and the months per year in the mentioned sport and the episodes of injury. A second questionnaire regarding the planning of the training period, including the development of the activities, namely type of activity, category, hours / week, classification of effort intensity (easy, moderate, intense or very intense), competitions in which the athlete participated and the results obtained, was also applied.

All subjects completed a medical and health questionnaire and gave their written informed consent to participate in this study. The experimental procedure was approved by the Ethics and Human Subjects Review Board of the Faculty of

Sports Science and Physical Education, University of Coimbra.

Table 1. Participants characteristics

	Masters	Control
Age (years)	53.5 ± 8.94	53.7 ± 6.04
Height (cm)	171.5 ± 5.86	169.7 ± 8.23
Body mass (kg)	74.7 ± 15.17	70.45 ± 13.71
BMI ($kg.m^{-2}$)	25.7 ± 4.65	24.6 ± 3.18
VO_{2max} (L.min-1)	2859.5 ± 697.22	2094.55 ± 4.14 **a**
VO_{2max} (mL.kg-1.min-1)	40.36 ± 11.55	29.29 ± 697.22 **b**

Values are Mean ± Standard Deviation (SD). N= Masters (19), Control (10). BMI= Body Mass Index; VO_{2max} = Maximal Oxygen Consumption. **a**= P<0.01 compared to masters; **b**= P<0.001 compared to masters.

Experimental design

All participants agreed to refrain from caffeine and alcohol in the previous 24 h and avoided strenuous exercise 72 h prior to the laboratory procedures. Participants arrived at the laboratory at 09:00am. First, they completed a comprehensive health-screening questionnaire. All subjects completed a maximal oxygen uptake (VO_{2max}) test on an electro-magnetically braked cycle ergometer (Lode Excalibur Sport V4.67, Groningen, The Netherlands). Participants began cycling at 75W and the power output was increased by 25W every 3 min until volitional fatigue. Oxygen uptake (breath-by-breath) was measured using an 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 at each 3-min stage and at the end of the test. Intravenous blood samples were collected in 6ml vacuum tubes containing EDTA as an anticoagulant (Becton-Dickinson, Oxford, UK) before (Pre), immediately after (Post), and 1 h after exercise.

Peripheral Blood Cell Counts

Total leukocyte and lymphocyte counts were determined using an automated cell counter (Coulter ACT Diff, Beckham Coulter, USA).

Flow cytometry

Specific lymphocyte populations were identified by immunofluorescent antibody staining of whole blood by eight-colour flow cytometry (FACSCanto II, BD Bioscience, San Jose, CA, USA). The following monoclonal antibodies (mAbs) were used: 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 5MZ.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 (PBCs) 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 flow cytometer.

For acquisition, initially, 1.000.000 events, corresponding to all nucleated cells present in the sample, were collected and information stored. Blood lymphocytes were identified and electronically gated using the forward and side light-scatter mode. T lymphocytes were identified according to their positivity for CD3 and typical light scatter. Two parameter dot plots were generated from the gated lymphocyte cell population to identify $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T-cell subset populations. CD4+ and CD8+ T-cells were subsequently further differentiated into naïve, central memory (CM), effector memory (EM), and CD45RA expressing effector memory cells (EMRA) subsets by CD45RA expression in combination with CD27. Senescent T-cells were detected based on the expression of $KLRG1^+$. The flow cytometer 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).

Cell purification by fluorescence-activated cell sorting

$CD8^+$ naïve and EMRA T 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.

Analysis of mRNA expression in purified CD8 T-cells:

The content of sorted purified cells were transferred to a 1.5-mL *Eppendorf* tube and centrifuged for 5 min at 300 g, and the pellet ressuspended in 350 µL of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen) in accordance with the manufacturer instructions. Total RNA was eluted in a 20-µL volume of RNase-free water and was reverse-transcribed with Tetra cDNA Synthesis (Bioline, London, UK) in accordance with the manufacturer instructions. 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 (CCR7: QT00025718; FAS-L: QT00041685, Qiagen) in a final volume of 10 µL. The reactions were performed by using the following thermal profile: one cycle of 10 min at 95°C, 50

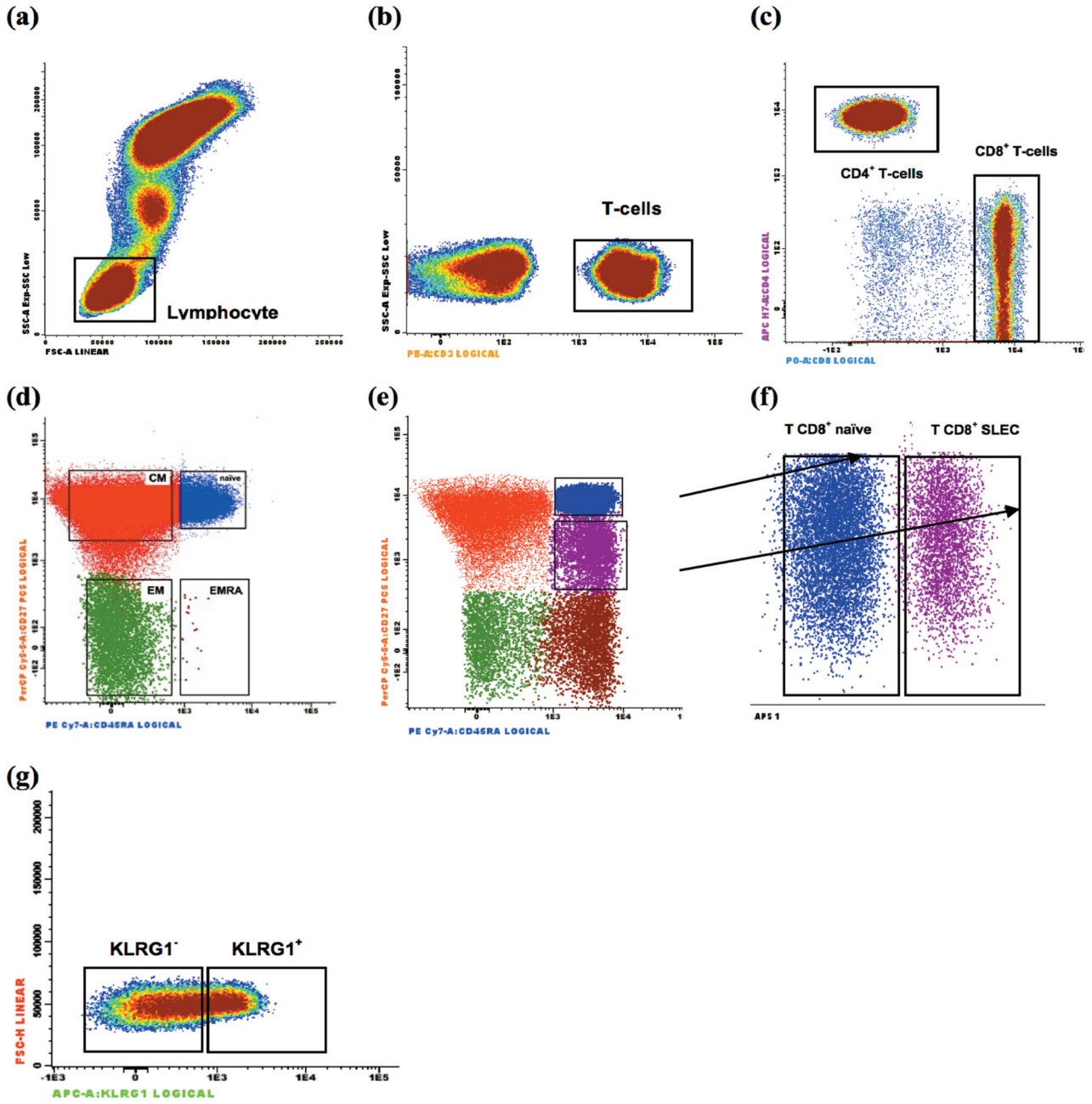

Figure 1. Representative analysis strategy used to define the different CD4+ and CD8+ T-cells populations and the % of these cells expressing the marker KLRG1.
The lymphocyte population was defined based on their low scatter *(side scatter)* and their small size *(forward scatter)* (Figure 1a). T lymphocytes were identified according to their CD3 positivity (Figure 1b). CD4+ and CD8+ T-cells were defined based on their positivity for CD4 and CD8, respectively (Figure 1c). Each subpopulation of $CD4^+$ and $CD8^+$ T-cells was divided into naïve ($CD27^+CD45RA^+$), central memory (CM; $CD27^+CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$) and CD45RA expressing effector memory cells (EMRA; $CD27^-CD45RA^+$) (Figure 1d, e). $CD8^+$ T-cells were further subdivided into another population, short-lived effector cells (SLEC, $CD27^{-/low}CD45RA^{++}$) (Figure 1f). All populations were analysed for their positivity for the KLRG1 marker (Figure 1g).

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 (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.

Statistical analysis

Descriptive results are presented as means ± standard deviation (SD). The Gaussian distribution for each parameter was assessed by a Shapiro-Wilk test. Alpha level was set at 0.05. All these routine analyses were assessed using SPSS software v19.0 (IBM Company, NY, USA).

For the analyses of change, accounting for the multilevel design of the study [level 1 units (intra-individual) within each level 2 unit (individuals of different groups)], hierarchical random effects models (REM) were constructed using a multilevel modelling approach (MLwiN v2.26, Center for Multilevel Modelling, University of Bristol, Bristol, UK) (3).

Analysis models that contained variables measured at different levels of a hierarchy are known as multilevel regression models. The following additive polynomial multilevel model was adopted to describe the changes in response to a maximum effort protocol:

$$yij = (\alpha + \mu j) + (\beta + \nu j)\, xij + (z1ij + z2ij + \cdots + znij) + \varepsilon ij$$

This equation is an example of REM in which level 1 regression coefficients are treated as random variables at level 2. In this example, the minutes from start (x) is in both the fixed and random parts of the model. This is seen clearly when equation 1 is rearranged into fixed and random parts:

$$yij = (\alpha + \beta j\, xij) + (z1ij + z2ij + \cdots + znij) + (\mu j + \nu jxij + \varepsilon ij)$$

where y is the %Total Ly, %Total LyT, %LyT, %Total CD4$^+$, %CD4$^+$, %Total CD8$^+$ and %CD8$^+$; naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) of CD4$^+$ T-cells (%CD4); naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) of CD8$^+$ T-cells (%CD8); LyT/KLRG1$^+$, %CD4$^+$/KLRG1$^+$, %CD4$^+$/naïve/KLRG1$^+$, %CD4$^+$/CM/KLRG1$^+$ and %CD4$^+$/EM/KLRG1$^+$, %CD4$^+$/EMRA/KLRG1$^+$ on measurement occasion i in the j^{th} individual, α is the constant for each j^{th} individual, $\beta_j\, x_{ij}$ is the slope for the dependent parameters over time (i.e., minute of measurement) for the j^{th} individual; and z_1 to z_n were the coefficients of explanatory variables (e.g., age, sex, VO_{2max} etc.) at assessment occasion i in the j^{th} individual. These were the fixed parameters in the model.

Both μj, $v_j x_{ij}$ and ε_{ij} formed the random parameters in the model. They were assumed to be independent and follow a normal distribution, with means equal to zero and variance σ^2. $\varepsilon_{ij} \sim N[0,\mathrm{var}(\varepsilon_{ij})]$ was the level 1 residual (within-individual variance) for the i^{th} assessment of %Total Ly, %Total LyT, %LyT, %Total CD4$^+$, %CD4$^+$, %Total CD8$^+$ or %CD8$^+$; naïve, central memory (CM), effector memory (EM) or CD45RA expressing effector memory cells (EMRA) of CD4$^+$ T-cells (%CD4); naïve, central memory (CM), effector memory (EM) or CD45RA expressing effector memory cells (EMRA) of CD8$^+$ T-cells (%CD8); LyT/KLRG1$^+$, %CD4$^+$/KLRG1$^+$, %CD4$^+$/naïve/KLRG1$^+$, %CD4$^+$/CM/KLRG1$^+$ or %CD4$^+$/EM/KLRG1$^+$, %CD4$^+$/EMRA/KLRG1$^+$ in the j^{th} individual. Also, $\mu_j \sim N[0,\mathrm{var}(\mu)]$ was the between individuals intercept variance and $v_{ij}x_{ij} \sim N[0,\mathrm{var}(v_{ij}x_{ij})]$ was the between individuals slope variance; thus, being used as the level 2 residuals (between subjects) variances for the j^{th} individual. The equation $\mu_j \times v_{ij}x_{ij} \sim N[0,\mathrm{var}(\mu_j \times v_{ij}x_{ij})]$ explained the intercept-slope covariance relationships among the intercepts and slopes in the model (3, 13).

Models were built using a stepwise procedure, i.e. predictor variables (z fixed effects) were added one at a time, and likelihood ratio statistics were used to judge the statistical fit of the model (3). Predictor variables (z) were accepted as significant if the estimated mean coefficient was greater than twice the standard error of the estimate. If the retention criterion was not met, the predictor variable was discarded. Minute of measurement power functions were introduced into the linear models to allow for the nonlinearity of changes in the dependent parameters. The following variables were introduced as predictors in the multilevel models: minute of measurement, minute of measurment^2, age, height, body mass and VO_{2max}. Dummy variables were created for sex and sample groups with female participants and controls, respectively, as reference categories. A total of twenty-eight independent multilevel REMs was constructed.

RESULTS

Participants anthropometric and physiologic characteristics are shown in table 1. Data from the questionnaires on training history and training habits showed that on average, master athletes had a practice of history of 24.6 ± 1.8 years and trained 10.3 ± 0.2 months per year with approximately 5 hours per week (5.5 ± 0.4 hours) per month trained.

The results obtained for the different cell subpopulations after analysis using multilevel models are summarized in tables 2, 3, 4, 5 and 6.

Table 2 shows the results from multilevel models for the proportion of total Ly, total LyT, total CD4 and CD4 T cells, total CD8 and CD8 T-cells. For the six models, the random effects are significant within individuals, indicating that %Total Ly, %Total LyT, %Total CD4, %CD4, %Total CD8 and %CD8 T-cells are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05], except for proportion of total CD4$^+$ cells (P<0.05). Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05], except proportion of total CD8$^+$cells. The variance between individuals is not, therefore, different at different minutes of measurement, except for %Total CD4 and %Total CD8. The fixed effects that significantly predicted %Total Ly, %Total LyT, %Total CD4, %CD4, %Total CD8 and %CD8 indicates that once the time of measurement is controlled (1 minute predicts 0.4%, 0.1, -0.5, -0.05, -0.6, 0.1 and 0.4, respectively). Once time of measurement is accounted for no significant effect of sports participation (controls *vs* masters) was noted for all variables. VO2max had an independent, significant and negative association with proportion of total Ly.

Table 3 summarizes the results from multilevel models for the proportion of CD4 naïve, CM, EM and EMRA. For the four models, the random effects are significant within individuals. The proportion of CD4 naïve, CM, EM and EMRA are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts (P<0.05) but not in terms of the slopes of their lines (P>0.05). Also, the variance of these intercepts and slopes are not significantly correlated (P>0.05). The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted %CD4 naïve, %CD4 CM, %CD4 EM and %CD4 EMRA indicate that once the time of measurement is controlled (1 minute predicts -0.01%,

Table 2. Multilevel regression models for the proportion of Total lymphocytes, Total T-lymphocytes, T-lymphocytes, Total $CD4^+$, $CD4^+$, Total $CD8^+$ and $CD8^+$ T-cells.

	%Total Ly	%Total LyT	%LyT	%Total CD4	%CD4	%Total CD8	%CD8
Fixed effects							
Constant	31.3845±4.0097	18.1119±1.0056	75.4511±2.9632	13.1916±1.0026	59.0956±1.6833	6.0599±0.5640	32.5100±1.5327
Minute of measurement	0.3709±0.0756	0.0930±0.0339	-0.5072±0.0838	-0.0470±0.0190	-0.6316±0.0675	0.1038±0.0238	0.3606±0.0578
Minute of measurement ^{2}	-0.0075±0.0012	-0.0023±0.0008	0.0092±0.0013	0.0005±0.0002	0.0112±0.0011	-0.0021±0.0004	-0.0066±0.0009
Age	NS	NS	NS	NS	NS	NS	NS
Females *vs* males	NS	NS	-6.4202±3.1680	-3.4520±1.1493	NS	NS	NS
Controls *vs* masters	NS	NS	NS	NS	NS	NS	NS
Height	NS	NS	NS	NS	NS	NS	NS
Body mass	NS	NS	NS	NS	NS	NS	NS
VO_{2max}	-0.023±0.0010	NS	NS	NS	NS	NS	NS
Random effects							
Level 1							
Constant (ε_{ij})	10.8641±2.0350	5.5379±1.0374	13.3507±2.5010	0.6474±0.1702	8.6572±1.6217	1.0756±0.2820	6.3568±1.1908
Level 2							
Constant (μ_j)	26.6720±7.9999	24.0956±6.8292	55.4478±15.7697	6.6531±1.8609	73.9934±20.2087	8.2070±2.3434	62.1141±16.8800
CA centered ($v_j x_{ij}$)	NS	NS	NS	0.0005±0.0002	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	-0.0344±0.0169	NS

Abbreviations: %Total Ly, percentage of lymphocytes to the total number of white blood cells; %Total LyT, percentage of T-lymphocytes to the total lymphocytes; %LyT, T-lymphocytes percentage; %Total $CD4^+$, percentage of $CD4^+$ T-lymphocytes to the total lymphocytes; %CD4, $CD4^+$ T-lymphocytes percentage; %Total $CD8^+$, percentage of $CD8^+$ T-lymphocytes to the total lymphocytes; %CD8, $CD8^+$ T-lymphocytes percentage; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of Total Ly, Total LyT, LyT, Total CD4, CD4, Total CD8 and CD8 in %. Random effects values are presented as estimated mean variance ± SEE (Total Ly, Total LyT, LyT, Total CD4, CD4, Total CD8 and CD8 in $\%^2$).

Table 3. Multilevel regression models for naïve, central memory (CM), effector memory (EM) and CD45RA expressing effector memory cells (EMRA) $CD4^+$ T-cells.

	%CD4			
	Naïve	CM	EM	EMRA
Fixed effects				
Constant	116.9764±48.9146	50.9481±2.9232	-78.6447±38.3960	1.2080±0.5510
Minute of measurement	-0.0061±0.0140	-0.4028±0.0789	0.2614±0.0515	-0.0097±0.0073
Minute of measurement ^{2}	NS	0.0071±0.0012	-0.0046±0.0008	NS
Age	NS	NS	0.2820±0.1317	NS
Females *vs* males	NS	9.1919±3.2184	-11.8384±3.5127	NS
Controls *vs* masters	NS	NS	NS	NS
Height	-0.6979±0.3126	NS	NS	NS
Body mass	NS	NS	NS	NS
VO_{2max}	0.0599±0.0024	NS	NS	NS
Random effects				
Level 1				
Constant (ε_{ij})	10.8009±2.0115	11.6590±3.0609	5.0347±0.9431	6.8678±2.0783
Level 2				
Constant (μ_j)	78.9011±21.7285	65.4753±19.2889	34.9337±9.6265	2.9954±0.5611
CA centered ($v_j x_{ij}$)	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS

Abbreviations: %CD4, $CD4^+$ T-lymphocytes percentage; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of naïve, CM, EM and EMRA in %. Random effects values are presented as estimated mean variance ± SEE (naïve, CM, EM and EMRA in $\%^2$).

Table 4. Multilevel regression models for naïve, central memory (CM), effector memory (EM) CD45RA expressing effector memory cells (EMRA) $CD8^+$ T-cells.

	%CD8				
	Naïve	SLEC	CM	EM	EMRA
Fixed effects					
Constant	59.3528±12.6252	-52.4484±17.0325	36.0371±2.1766	14.2907±1.4504	23.8093±2.9579
Minute of measurement	-0.4232±0.06554	0.1254±0.0259	-0.2643±0.0707	NS	0.4659±0.0876
Minute of measurement ^{2}	0.0074±0.0010	-0.0023±0.0004	0.0052±0.0011	NS	-0.0085±0.0014
Age	-0.5964±0.2192	0.1840±0.0774	NS	NS	NS
Females *vs* males	-8.9289±4.0912	NS	NS	NS	NS
Controls *vs* masters	NS	NS	NS	NS	NS
Height	NS	0.2856±0.0969	NS	NS	NS
Body mass	NS	NS	NS	NS	NS
VO_{2max}	NS	NS	NS	NS	NS
Random effects					
Level 1					
Constant (ε_{ij})	7.6713±1.4370	1.2250±0.3217	9.5113±1.7816	8.3741±1.5687	14.4887±3.8030
Level 2					
Constant (μ_j)	85.4878±23.1379	22.7971±6.1739	128.3961±34.5698	55.5906±15.3509	240.0121±65.5292
CA centered ($v_j x_{ij}$)	NS	0.0007±0.0003	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	-0.1136±0.0410	NS	NS	NS

Abbreviations: %CD8, $CD8^+$ T-lymphocytes percentage; SLEC: short-lived effector cells; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of naïve, CM, EM and EMRA in %. Random effects values are presented as estimated mean variance ± SEE (naïve, CM, EM and EMRA in $\%^2$).

-0.4, 0.3 and -0.01, respectively) no significant effect of sports participation (controls vs masters) was noted for all variables. VO_{2max} had an independent, significant and positive association with the proportion of CD4 naïve T cells.

Table 4 summarizes the results from multilevel models for the proportion of naïve, SLEC, CM, EM and EMRA CD8+ T-cells. For the five models, the random effects are significant within individuals. The proportion of naïve, SLEC, CM, EM and EMRA $CD8^+$ T-cells are increasing significantly at each minute of measurement within individuals ($P<0.05$). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts ($P<0.05$) but not in terms of the slopes of their lines ($P>0.05$), except for the proportion of $CD8^+$ SLEC T-cells ($P>0.05$). Also, the variance of these intercepts and slopes are not significantly correlated ($P<0.05$). The variance between individuals is not, therefore, different at different minutes of measurement, except for the proportion of $CD8^+$ SLEC T-cells. The fixed effects that significantly predicted naïve, SLEC, CM and EMRA $CD4^+$ SLEC T-cells indicate that once the time of measurement is controlled (1 minute predicts -0.4%, 0.1, -0.3 and 0.5, respectively) no significant effect of sports participation (controls *vs* masters) was noted for all variables.

The effects of exercise and age on total T-lymphocytes, $CD4^+$ and $CD8^+$ T-cells and the KLRG1 expression are shown in Table 5. Total $CD3^+$, $CD4^+$ and $CD8^+$ T-cells percentages did not increase with exercise. Senescent $CD4^+$ and $CD8^+$ T-cells were elevated in control compared to masters ($p < 0.05$) before and 1 h after exercise (Table 5).

Table 5. Values (%) of cells $CD3^+$, $CD3^+$ T-cells and $CD3^+CD4^+/CD8^+$ T-cells and KLRG1 expression in response to a maximum effort protocol.

	Pre		Post		1h	
%	**Masters**	**Control**	**Masters**	**Control**	**Masters**	**Control**
$CD3^+$	66.28±10.45	70.99±6.94	62.46±9.06	66.38±7.66	67.34±12.10	72.16±10.13
$CD3^+KLRG1^+$	6.58±4.08	9.19±4.92	9.70±5.77	12.23±6.09	8.68±5.33	13.69±7.57
$CD3^+CD4^+$	58.66±8.30	58.73±9.68	51.95±10.70	53.15±10.20	60.44±8.53	60.67±9.58
$CD3^+CD4^+KLRG1^+$	8.05±5.06*	13.49±8.17	11.35±6.79	17.07±9.80	7.31±4.97*	12.78±6.17
$CD3^+CD8^+$	30.64±8.20	31.93±8.18	35.63±10.24	35.26±8.49	28.37±9.77	30.73±7.14
	25.22±12.94	36.54±14.9			22.14±12.83	
$CD3^+CD8^+KLRG1^+$	*	1	32.32±15.90	42.63±12.58	*	37.90±11.80

Values are Mean ± SD. N= 9 (Control); N= 19 (Masters). * $P<0.05$ compared to control.

Table 6 summarizes the results from multilevel models for the proportion of LyT, $CD4^+$, $CD4^+$ naïve, central memory, effector-memory and EMRA expressing KLRG1. The random effects describe the two levels of variance [within individuals (Level 1 of the hierarchy) and between individuals (Level 2 of the hierarchy)]. For the six models, the random effects are significant within individuals, indicating that proportion of senescent LyT, $CD4^+$, naïve, CM, EM and EMRA $CD4^+$ T-cells are increasing significantly at each minute of measurement within individuals

Table 6. Multilevel regression models for the proportion of T-lymphocytes, $CD4^+$ and naïve, central memory, effector memory, CD45RA expressing effector memory $CD4^+$ T-cells expressing KLRG1.

			%$CD4^+$			
	%LyT/$KLRG1^+$	%$CD4^+$/$KLRG1^+$	naïve/$KLRG1^+$	CM/$KLRG1^+$	EM/$KLRG1^+$	EMRA/$KLRG1^+$
Fixed effects						
Constant	23.7922±2.9145	13.8351±2.0220	10.9632±1.9997	8.8069±1.5148	NS	NS
Minute of measurement	0.4936±0.0856	0.3158±0.0583	0.1216±0.0496	0.1227±0.0507	0.4557±0.1047	0.3514±0.1577
Minute of measurement ^{2}	-0.0090±0.0013	-0.0055±0.0009	-0.0019±0.0008	-0.0022±0.0008	-0.0087±0.0016	-0.0076±0.0028
Age	NS	NS	NS	NS	NS	NS
Controls *vs* masters	-6.7829±3.0608	-5.8145±2.4227	-5.3182±2.5761	-4.3505±1.9356	NS	NS
VO_{2max}	NS	NS	NS	NS	NS	NS
Random effects						
Level 1						
Constant (ε_{ij})	14.0597±2.6338	6.0097±1.1357	3.5199±0.7511	3.6377±0.7756	18.3809±3.6048	34.3770±12.1479
Level 2						
Constant (μ_j)	69.5709±19.5392	33.8227±9.5824	34.0991±10.6367	18.6913±6.0067	183.2635±52.5409	349.0878±131.5839
Minute of measurement ($v_j x_{ij}$)	NS	NS	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	NS

Abbreviations: %LyT, T-Lymphocytes percentage; %CD4, $CD4^+$ T-cell percentage; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of LyT/$KLRG1^+$, $CD4^+$/$KLRG1^+$, $CD4^+$/naïve/$KLRG1^+$, $CD4^+$/CM/$KLRG1^+$, $CD4^+$/EM/$KLRG1^+$, $CD4^+$/EMRA/$KLRG1^+$ in %. Random effects values are presented as estimated mean variance ± SEE (LyT/$KLRG1^+$, $CD4^+$/$KLRG1^+$, $CD4^+$/naïve/$KLRG1^+$, $CD4^+$/CM/$KLRG1^+$, $CD4^+$/EM/$KLRG1^+$, $CD4^+$/EMRA/$KLRG1^+$ in %^{2}).

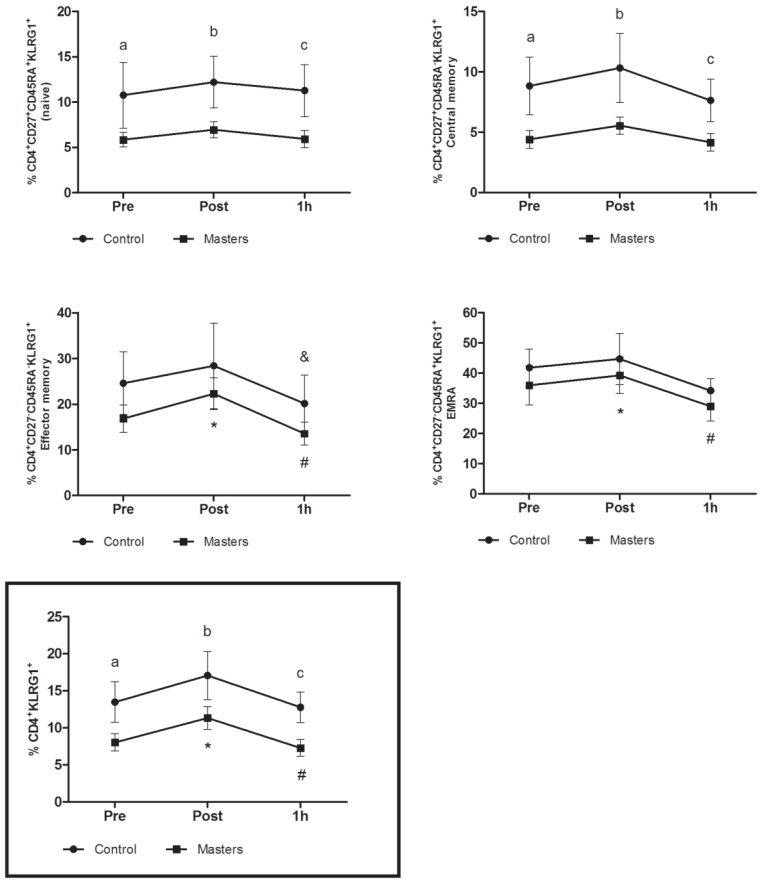

Figure 2: Proportion of naïve, central memory, effector memory and CD45RA expressing effector memory $CD4^+$ T cells expressing KLRG1 before and in response to a maximum effort protocol for control and master athlete groups. The values are presented as the mean ± standard deviation. a P<0.05 compared to master athletes in Pre. b P <0.05 compared to master athletes in Post. c P<0.05 compared to the master athletes in 1h. * P <0.05 Post compared to Pre. # P<0.05 1h compared to Post. & P<0.05 1h compared to Pre. Abbreviations: %$CD4^+KLRG1^+$ = percentage of $CD4^+$ T-cells expressing KLRG1. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

(P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05]. Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05]. The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted %LyT/$KLRG1^+$, %$CD4^+$/$KLRG1^+$, %$CD4^+$/naïve/$KLRG1^+$, %$CD4^+$/CM/$KLRG1^+$ indicate that once the time of measurement is controlled (1 minute predicts 0.5%, 0.3, 0.1 and 0.1, respectively), *masters* had significant less proportion of senescent LyT (-6.7829±3.0608%), $CD4^+$ T-cells (-5.8145±2.4227), naïve (-5.3182±2.5761) and effector memory (-4.3505±1.9356%) $CD4^+$ senescent T-cells than controls, while no significant effect of sports participation (controls vs masters) was noted for the proportion of effector memory and EMRA senescent $CD4^+$ T-cells.

Considering that the proportion of senescent $CD4^+$ T-cells and subsets of naïve, CM, EM and EMRA $CD4^+$ T-cells increases with each minute of measurement, we compared the mean values of the proportion of senescent $CD4^+$ T-cells and subsets at Pre, Post and 1h using ANOVA for repeated measures. The results of this analysis are presented in Figure 2. Senescent $CD4^+$ T-cells are mobilized by exercise only in the master athletes group, as shown by the significant increase in senescent $CD4^+$ T-cells at Post (Figure 2), corroborating the results of the multilevel analysis (Table 6). The proportion of

Table 7. Multilevel regression models for the proportion of $CD8^+$, naïve, short-lived effector cell, central memory, effector memory and CD45RA expressing effector memory $CD8^+$ T-cells expressing KLRG1.

		%$CD8^+$				
	%$CD8^+$/$KLRG1^+$	naïve/$KLRG1^+$	SLEC/$KLRG1^+$	CM/$KLRG1^+$	EM/$KLRG1^+$	EMRA/$KLRG1^+$
Fixed effects						
Constant	NS	15.8408±7.023	53.0178±3.6679	25.4383±3.3874	40.3917±5.7790	42.8580±4.2705
Minute of measurement	0.6729±0.1108	0.1058±0.0538	0.6824±0.1846	0.6063±0.0877	0.4867±0.1173	0.2627±0.1247
Minute of measurement ^{2}	-0.0117±0.0017	-0.0019±0.0008	-0.0120±0.0029	-0.0106±0.0014	-0.0091±0.0018	-0.0056±0.0019
Age	NS	NS	NS	NS	NS	NS
Controls *vs* masters	-13.0661±4.9568	-9.3624±2.0992	NS	-7.0282±3.0654	-12.0034±5.4968	NS
VO_{2max}	NS	NS	NS	NS	NS	NS
Random effects						
Level 1						
Constant (ε_{ij})	23.3417±4.3484	5.3239±1.0110	64.9409±12.1082	14.3256±2.7075	26.0568±6.8358	27.2463±7.4122
Level 2						
Constant (μ_j)	114.6349±40.0272	25.0755±7.1753	328.8600±92.0476	85.0265±24.0171	344.0468±94.8657	466.6415±131.9003
Minute of measurement						
($v_j x_{ij}$)	NS	NS	NS	NS	NS	NS
$\mu_j \times v_j x_{ij}$	NS	NS	NS	NS	NS	NS

Abbreviations: %LyT, T-Lymphocytes percentage; %CD8, $CD8^+$ T-Lymphocytes percentage; SLEC, short-lived effector cells; CM, central memory; EM, effector memory; EMRA, CD45RA expressing effector memory cells; VO_{2max}, maximal oxygen consumption.
Fixed effect values are presented as estimated mean coefficients ± SEE (standard error of estimate) of LyT/$KLRG1^+$, $CD8^+$/$KLRG1^+$, $CD8^+$/naïve/$KLRG1^+$, $CD8^+$/CM/$KLRG1^+$, $CD8^+$/EM/$KLRG1^+$, $CD8^+$/EMRA/$KLRG1^+$ in %.
Random effects values are presented as estimated mean variance ± SEE (LyT/$KLRG1^+$, $CD8^+$/$KLRG1^+$, $CD8^+$/naïve/$KLRG1^+$, $CD8^+$/CM/$KLRG1^+$, $CD8^+$/EM/$KLRG1^+$, $CD8^+$/EMRA/$KLRG1^+$ in $\%^2$).

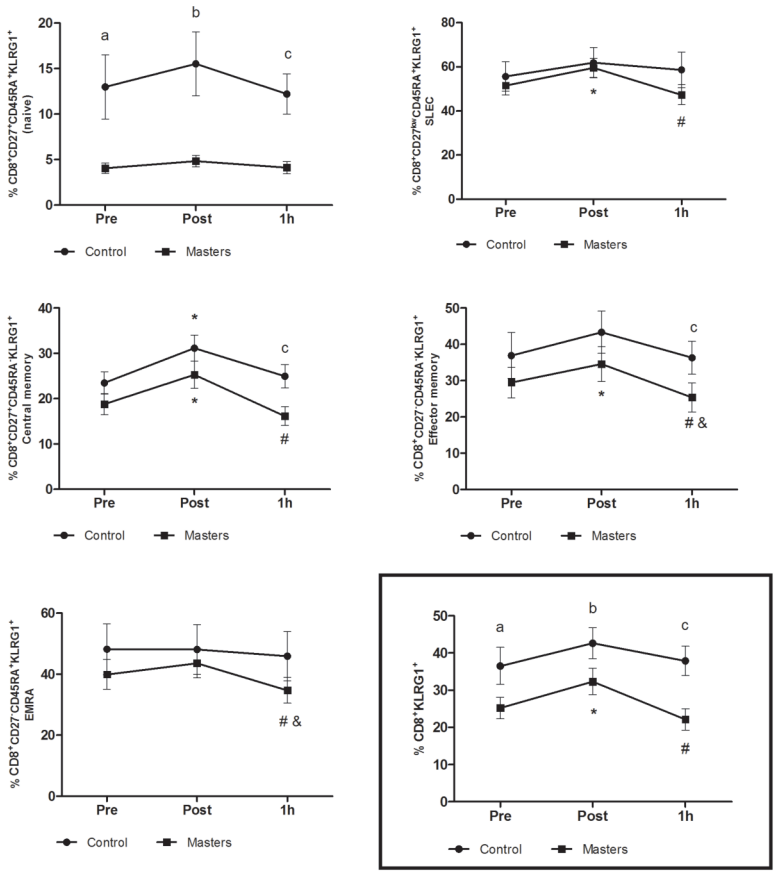

Figure 3: Proportion of senescent naïve, central memory, effector memory and CD45RA expressing effector memory $CD8^+$ T-cells before and in response to a maximum effort protocol for control and master athletes groups. The values are presented as the mean ± standard deviation. a P<0.05 compared to master athletes in Pre. b P <0.05 compared to master athletes in Post. c P<0.05 compared to the master athletes in 1h. * P <0.05 Post compared to Pre. # P<0.05 1h compared to Post. & P<0.05 1h compared to Pre. Abbreviations: %$CD8^+$/$KLRG1^+$ = percentage of $CD8^+$ T-cells expressing KLRG1. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

senescent $CD4^+$ T-cells return to Pre-values in 1h (Figure 2). The results indicated that the proportions of senescent naïve and CM $CD4^+$ T-cells are not different between the two groups. The senescent EM and EMRA $CD4^+$ T-cells increased in Post for master athletes and return to the baseline values in 1h. In the control group, the only difference observed was the proportion of the senescent $CD4^+$ EM T-cells, with lower values at 1h when compared to Pre.

Table 7 summarizes the results from multilevel models for the proportion of senescent $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells. The random effects are significant within individuals, indicating that proportion of $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells expressing $KLRG1^+$ are increasing significantly at each minute of measurement within individuals (P<0.05). The between individuals variance matrix for each model indicates that individuals have significantly different curves in terms of their intercepts [constant/constant ($\mu_j \times \mu_j$), P<0.05] but not in terms of the slopes of their lines [minute of measurement/minute of measurement ($v_j x_{ij} \times v_j x_{ij}$), P>0.05]. Also, the variance of these intercepts and slopes are not significantly correlated [constant/age ($\mu_j \times v_j x_{ij}$), P>0.05]. The variance between individuals is not, therefore, different at different minutes of measurement. The fixed effects that significantly predicted the proportion of senescent $CD8^+$, naïve, SLEC, CM, EM e EMRA $CD8^+$ T-cells indicates that once the time of measurement is controlled

(1 minute predicts 0.7, 0.1, 0.7, 0.6 and 0.5 and 0.3 respectively), master athletes had significant less senescent $CD8^+$ T-cells (-13.0661±4.9568), $CD8^+$ naïve (-9.3624±2.0992), central memory (-7.0282±3.0654) and effector memory (-12.0034±5.4968) senescent T-cells than controls.

Senescent $CD8^+$ T-lymphocytes and subsets increased in response to each minute of measurement of the test protocol. The senescent $CD8^+$ T-cells are mobilized by the exercise and return to the pre-exercise values at 1h in the master athletes group (Figure 3). The senescent $CD8^+$ T-cells and the senescent SLEC, CM, EM $CD8^+$ T-cells are also mobilized by exercise in the master athletes group. The values returned to Pre-values 1h after the end of the protocol. In the control group, only the senescent $CD8^+$ CM T-cells increased at Post and the values remained elevated after 1h. The senescent $CD8^+$ EMRA T-cells did not increase at Post but decreased in 1h to values below those observed at Post and Pre in the master athletes group ($P < 0.05$).

The mRNA expression of the CCR7 for $CD8^+$ naïve T-cells and the Fas-L for $CD8^+$ EMRA T-cells was not different between masters and controls. The mRNA expression for both genes did not change in response to the maximal effort test (Figure 4).

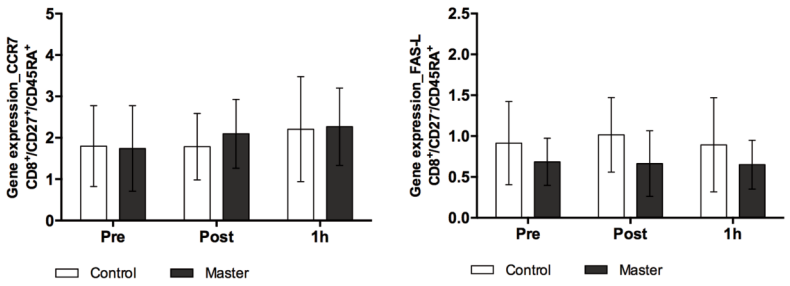

Figure 4. Effect of acute exercise on expression of CCR7 genes in $CD8^+$ naïve and Fas-L in $CD8^+$ EMRA T-cells.
Data are presented as mean ± SD. Pre (before), Post (10 min after test), 1 h (1 h after test). Control (N = 9); Masters (N = 19).

DISCUSSION

Differences between masters and controls, suggesting lifelong training effects, were observed for senescent $CD4^+$ T cells and naïve and CM $CD4^+$ T-cells subsets, and for senescent $CD8^+$ T-cells and naïve, CM, EM $CD8^+$ T-cells subsets. The proportion of senescent $CD4^+$ and $CD8^+$ EMRA cells did not differ between conditions. The results also indicate that the proportion of $CD4^+$ and $CD8^+$ T-cells and their subsets are not different between masters and controls at all times of measurement (Tables 2, 3 and 4). Age had a negative effect on the proportion of $CD8^+$ naïve T-cells and a positive effect on the proportion of $CD8^+$ SLEC T-cells. VO_{2max} negatively influenced the proportion of total lymphocytes and showed a positive effect on the proportion of $CD4^+$ naïve T-cells.

In general, studies in both mice and humans, have showed a gradual reduction of naïve $CD4^+$ and $CD8^+$ T-cell numbers with aging, a consequence of the decreased production of these cells by the thymus. Despite this decrease in thymic production from puberty to old age, it is known that humans have a remarkable ability to maintain relatively constant numbers of lymphocytes over many decades through the homeostatic proliferation of peripheral T-cells in healthy adults (1). However, there are important differences between the $CD4^+$ and $CD8^+$ T-cells populations. It has been demonstrated, in the elderly, that the low thymus functionality shows a thymocyte production tendentious for $CD4^+$ T-cells (7). In fact, all subjects with a very low thymic function, defined as the percentage of double positive thymocytes below 10%, had CD4/CD8 ratios higher than 2 (7). This suggests a dysregulation of homeostasis, mainly affecting the subgroup of $CD8^+$ naïve T-cells. $CD8^+$ T-cells undergo major expansion after activation and can establish a stable set of highly differentiated memory cells at rest. In contrast, the ability of $CD4^+$ T-cells to expand and survive appears to be lower, so that the vast majority of activated $CD4^+$ T-cells can undergo apoptosis quickly. Furthermore, the total $CD4^+$ T-cell repertoire appears to be less likely to have late-differentiated compared to $CD8^+$ T lymphocytes (1). We observed a negative influence of age on $CD8^+$ naïve T-cells, and a positive effect of VO_{2max} on $CD4^+$ naïve T-cells. This suggests that the effect of age is greater than training on $CD8^+$ naïve T-cells, whereas individuals with a better physical condition (higher VO_{2max} values), tend to have higher numbers of $CD4^+$ naïve T-cells (Table 2).

On the other hand, the results showed a positive effect of age on $CD8^+$ SLEC T-cells. $CD8^+$ SLEC T-cells are cells that still express CD45RA but lose expression of CD27 (Figure 1e) and have high expression of KLRG1 (Figure 3). When visualized by APS (Automatic Population Separator) these were shown to be a distinct population of the $CD8^+$ naïve T-cells (Figure 1f). We classified these cells based on the definition found in the literature for short-lived effector cells (SLEC) cells (46). Yuzefpolskiy and colleagues found that cells with high expression of KLRG1 ($KLRG1^{hi}$) represent the SLEC population. In addition, it appears that SLEC cells have a preferential localization at non-lymphoid sites, suggesting that they are continuously encountering antigens at peripheral sites, thus undergoing a terminal differentiation process (46). We suggest that future studies should develop new methodologic approaches for a better characterization of this population.

Changes with age have been observed in gene expression of several genes in the naïve compartment of $CD4^+$ T and $CD8^+$ T-cells (Mirza et al., 2011). Taking this into account, we quantified the expression of CCR7 mRNA in $CD8^+$ naïve T-cells. CCR7 participates in both regulation of trafficking and homing of leukocytes to lymph nodes (8). T-cells become progressively differentiated, suffering loss of the surface molecules, initially CD45RA undergoes downregulation, followed by loss of CCR7 expression and ultimately a decrease in CD28 and CD27 expression (24). The CCR7 gene expression was not modified by the maximal exercise test and was the same for masters and controls (Figure 4). This suggests that neither exercise nor age seem to affect the homing capacity of these cells.

Exercise induces leukocytosis, but the response of major leukocyte subtypes is not uniform. According to Simpson and colleagues (2015) there are 3 main characteristics that are shared by all leukocyte subsets that are preferentially redistributed after a single exercise session, namely: 1) the cells mobilized by the exercise tend to have an increased cytotoxic/effector function and have a mature/differentiated phenotype; 2) these cells redistributed with exercise tend to present phenotypes associated with tissue migration; 3) leukocyte subtypes which are preferentially redistributed with exercise show high expression of adrenergic receptors (β2-ARS) and glucocorticoid receptors, and are therefore highly sensitive to catecholamines and cortisol (35). This is an important factor to consider since aging is associated with profound declines in immunity and older individuals (> 50 years) having a diminished ability to redistribute certain leukocyte subtypes in response to a single exercise bout (37). Spielmann and colleagues (2014) showed that this was due to a lower mobilization of naïve T-cells, possibly due to the reduction in the thymic output. Thus, senescent T-cells accumulate with age, reducing the repertoire of naïve T-cells and increasing the risk of infection in the host. Senescent T-lymphocytes are cells that have already encountered an antigen, and express the KLRG1 and/or CD57 receptor, do not expand clonally after antigenic restimulation and prevail in the blood of older adults. Because this response is likely to be influenced by certain lifestyle factors, we examined the association between aerobic fitness (VO_{2max}) and age-related senescent T-cell accumulation. We found no interactions between VO_{2max} or age in any of the subsets of KLRG1$^+$ cells, contrary to previous studies (38). However, master athletes, which had higher aerobic capacity values than controls, showed lower values of senescent CD4$^+$, CD8$^+$ T-cells and their subsets when compared to controls. The exception was observed for senescent CD4$^+$ and CD8$^+$ EMRA T-cells. This suggests that repetitions of lifelong exercise avoid accumulating of senescent T-cells, corroborating the theory of immune space proposed by Simpson (28). Therefore, we postulate that maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells, enhancing the beneficial effects of maintaining a physically active lifestyle in the immune system of adult subjects. If it is possible to maintain an adequate number of naïve T-cells, the immune system will be better able to recognize and respond to new pathogens (38).

The theory of the immune space indicates that exercise can make space by the mobilization of senescent or terminally differentiated T-cells and death of this cells by apoptosis as a result of the apoptotic signals produced by exercise (28). We are able to confirm the mobilization of senescent CD4$^+$ and CD8$^+$ T-cells by exercise (Fig. 2 and 3). Regarding cells of a late-stage differentiated phenotype (EMRA), the results showed that only EMRA CD4$^+$ T-cells (not CD8$^+$ T-cells) were mobilized during exercise. Because the 'post' sample was collected 10-minutes after exercise, is it possible that we have missed the peak cell mobilization (29). A recent study showed that the mobilization and apoptosis of low and highly differentiated T-cell subsets immediately after exercise was dependent on the kind of exercise performed with continuous exercise deleting mainly low differentiated T-cells (14). We assessed an apoptotic marker on the sorted CD8$^+$ EMRA T-cells. The Fas-L gene expression in these cells was not different between the groups and between the measurement points. Fas and Fas-L are two molecules involved in the regulation of cell death, and their interaction leads to apoptosis of thymocytes that fail to properly rearrange TCR genes and those that recognize autoantigens, a process called negative selection. In addition, the Fas and Fas-L interaction leads to activation-induced cell death, a form of apoptosis by repeated TCR stimulation, responsible for the peripheral deletion of activated T-cells (42). Since the gene expression of Fas-L was not different between the groups, it is possible that cellular homing markers are involved (16). We suggest that future research should include the determination of cellular homing markers and other markers of apoptosis such as caspase-3 and anti-apoptotic marker like Bcl-2 to refute or corroborate this hypothesis. The results of a study similar to ours showed that in athletes (65-85 years old) who underwent intense training, there was less apoptosis of CD45RO$^+$ and CD45RA$^+$ T-cells than in athletes with moderate training routine or untrained people, as demonstrated by a higher expression of Bcl-2 and a lower expression of caspase-3 (27). However, these were not measured after acute exercise. Therefore, it is necessary to determine which mechanisms are responsible for the lower number of senescent T-cells in master adult athletes and if this response is maintained in elderly master athletes. The secretion of IL-7, that is produced by skeletal muscle and regulates Bcl-2 expression, could explain why athletes would be protected from spontaneous T cell apoptosis (27). Not all cells bearing the classic 'senescent' phenotype are senescent and there are new markers of senescence (i.e. P16ink4a) and markers of exhaustion (i.e. PD-1) that should be looked at on these cell types.

CONCLUSION

CD8$^+$ naïve T-cells decreased and CD8$^+$ SLEC T-cell increased with age while VO_{2max} positively influenced the proportion of CD4$^+$ naïve T-cell, suggesting that individuals with better physical condition are better at preserving their CD4$^+$ naïve T-cell population. However similar values of total naïve CD4$^+$ and CD8$^+$ T-cells and their memory subsets were found for the master and control groups. The main differences between groups were found in the KLRG1 senescence marker expression. Lifelong training decreased the percentage of the senescent naïve, CM and EM CD8$^+$ T-cells and senescent naïve and CM CD4$^+$ T-cells. In both CD4$^+$ and CD8$^+$ T-cell subsets, the percentage of senescent EMRA T cells was also lower in the master athletes. This suggests that lifelong training induces the death of these cells and corroborates the immune space hypothesis proposed by Simpson and colleagues (27). In addition, we postulate that maintaining high levels of aerobic fitness during the natural course of aging may help preserve the proportion of CD4$^+$ naïve cells and prevent the accumulation of senescent T-cells, hallmarks of a "younger" immune system.

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