Moderate and intense exercise lifestyles attenuate the effects of aging on telomere length and the survival and composition of T cell subpopulations

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Abstract Studies indicate that exercise might delay human biological aging, but the effects of long-term exercise on T cell function are not well known. We tested the hypothesis that moderate or intense exercise lifestyle may attenuate the effects of aging on the telomere length and the survival and composition of T cell subpopulations. Elderly (65–85 years) with intense training lifestyle (IT, $n = 15$), moderate training lifestyle (MT, $n = 16$), and who never trained (NT, $n = 15$) were studied. Although the three groups presented the age-associated contraction of the TCD4⁺/TCD8⁺ naïve compartments and expansion of the memory compartments, both training modalities were associated with lower proportion of terminally differentiated

(CD45RA⁺CCR7^{neg}) TCD4⁺ and TCD8⁺ cells, although among the latter cells, the reduction reached statistical significance only with IT. MT was associated with higher proportion of central memory TCD4⁺ cells, while IT was associated with higher proportion of effector memory TCD8⁺ cells. However, both training lifestyles were unable to modify the proportion of senescent (CD28^{neg}) TCD8⁺ cells. Telomeres were longer in T cells in both training groups; with IT, telomere length increased mainly in TCD8⁺ cells, whereas with MT, a modest increase in telomere length was observed in both TCD8⁺ and TCD4⁺ cells. Reduced commitment to apoptosis of resting T cells, as assessed by caspase-3 and Bcl-2 expression, was seen predominantly with IT.

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Measurement of pro-inflammatory cytokines in serum and peripheral blood mononuclear cell (PBMC)'s supernatants did not show chronic low-grade inflammation in any of the groups. In conclusion, MT and IT lifestyles attenuated some of the effects of aging on the immune system.

Keyword Aging · Exercise · Telomere · T lymphocytes · Apoptosis

Introduction

Aging-related changes that affect the competence of the immune system are defined by the term immunosenescence (Pawelec 2014). The decline of the immune system with age is reflected by increased frequencies of infection, cancer, cardiovascular disease, and neurodegenerative disease (Sarkar and Fisher 2006; Targonski et al. 2007; Yu et al. 2015). Both innate and adaptive immune responses are affected by the aging process; however, the adaptive response seems to be especially affected by age-related changes in the immune system. Loss of CD28 expression, telomere shortening, and the re-expression of CD45RA are phenotypic changes in T cells that lead to functional alterations such as low proliferative responses, insufficient IL-2 synthesis, and apoptosis resistance (Weng et al. 1995; Spaulding et al. 1999; Topp et al. 2003; Saule et al. 2006; Parish et al. 2010). Another common feature of immunosenescence is the loss of naïve T cells and accumulation of memory T cells in the periphery as a result of naïve cells differentiating into memory cells over time by contact with pathogens (Arnold et al. 2011). Immunosenescence is also related to the emergence of an immune risk profile. This immune risk profile is characterized by latent CMV infection, inversion of the CD4/CD8 ratio, and expansion of terminally differentiated memory effector T cells, which are T cells that re-express the CD45RA molecule and have a shorter lifespan. This profile was associated with increased mortality within 2 years among older adults (men and women) (Ferguson et al. 1995; Strindhall et al. 2013). Additionally, aged individuals tend to exhibit a chronic low-grade inflammatory state that has been implicated in the pathogenesis of many age-related diseases (atherosclerosis, Alzheimer's disease, osteoporosis, and diabetes) (Michaud et al. 2013).

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 physical activity, as it presents fewer risks compared with immunotherapeutic procedures (e.g., gene therapy, cytokine therapy, monoclonal antibody therapy) and is associated with increased longevity and a lower risk of chronic diseases and infections (Lynch et al. 1996; Evenson et al. 2003; Barlow et al. 2006; Kostka et al. 2008). Although the impact of exercise on the immune system is an area of extensive research, most studies have focused on the responses to acute exercise. These studies have shown that the immune response to acute exercise is transient and variable, being influenced by a range of factors such as the intensity, duration and mode of the exercise, concentrations of hormones during exercise, changes in body temperature, blood flow, hydration status, and body position (vertical vs. horizontal) [Nieman and Nehlsen-Cannarella 1991; Shepard and Shek 1996; Nieman 1997]. Findings from randomized controlled trials examining the effects of exercise training on specific aspects of immunosenescence are inconsistent, possibly due to differences in experimental design among these studies (Simpson et al. 2012; de Araújo et al. 2013). However, few studies have addressed the immune system response to long-term physical training in the elderly (Simpson et al. 2012). These studies indicated that exercise may delay the aging of the immune system, but the effects of long-term exercise training and its intensity variations on specific T cell subsets are unclear.

The goal of the present study was to test the hypothesis that a moderate or intense exercise lifestyle may attenuate the effects of aging on telomere length and the survival and composition of T cell subsets.

Materials and methods

Participants

The participants of the present study were recalled from a larger cohort of 61 elderly individuals (65–85 years) who participated in our previous study on the effect of training on vaccination responses (de Araújo et al. 2015). These individuals were recruited mainly from runner associations, sports clubs, community-based exercise programs for the elderly, outpatient services, and

our institution's association of former employees. Of the 61 individuals, 15 who never trained, 16 moderately trained subjects (i.e., who participated in volleyball, basketball, or running less than 6 km two to three times/week), and 15 intensely trained subjects (i.e., who trained ≥ 5 days/week (>50 km/week) accepted to participate in the present study. The trained elderly had participated in regular training for at least 5 years. Exclusion criteria included co-morbidities that could interfere with the immune system (e.g., AIDS, cancer, rheumatoid arthritis, and uncontrolled diabetes mellitus), use of immunosuppressive drugs (e.g., corticosteroids), smoking, and alcohol abuse. Participants underwent clinical evaluation by a physician and answered the following questionnaires: Global Depression Score (Yesavage et al. 1982), Mini Nutrition Assessment (Vellas et al. 1999), Mini-Mental State Examination [MMSE] (Brucki et al. 2003), and Quality of Life SF-36 (Ciconelli et al. 1999). The study was approved by the Hospital das Clínicas Review Board (#0135/11), and all subjects signed an informed consent form. Blood sampling in the trained groups was conducted ≥ 48 h after the last regular training session and at least 1 week after participation in any competition. Fitness status was assessed through the International Physical Activity Questionnaire (IPAQ) and VO_2 max consumption, as previously described (de Araújo et al. 2015).

Surface immunophenotyping of naïve and memory T cells by flow cytometry

Seventy microliters of whole blood was stained with a mix of the following labeled monoclonal antibodies for 20 min at room temperature: anti-CD3 V500, anti-CD4 V450, anti-CD8 APC-Cy7, anti-CD45RA fluorescein isothiocyanate (FITC), and anti-CCR7 PE-Cy7 (all from BD Biosciences, San Diego, CA, USA). Samples were depleted of erythrocytes with OptiLyse C (Beckman Coulter, CA, USA), washed and suspended in BD FACSFlow solution (BD Biosciences, CA, USA), and acquired on a FACSFortessa with FACSDiva software (BD Biosciences, CA, USA). Cells were analyzed with FlowJo software, version 7.4 (Tree Star, San Carlos, CA). Fluorescence voltages were determined using matched unstained cells. Compensation was carried out with single-stained CompBeads (BD Biosciences, CA, USA). Samples were acquired until at least 200,000 events were collected in a live lymphocyte gate.

Telomere length measurement by FlowFish

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized blood by density gradient centrifugation over Ficoll-Paque Plus (GE Healthcare Life Sciences, Little Chalfont, UK). CD3^+ cells and CD4^+ and CD8^+ T cell subsets were negatively selected using magnetic microbeads and MACS Cell Separation Reagents (Miltenyi Biotec, Germany) according to the manufacturer's instructions. A further purification step with CD45RO and CD28 microbeads (Miltenyi Biotec, Germany) was performed to obtain CD45RO^+ or $\text{CD45RO}^{\text{neg}}$ CD4^+ T cells and CD28^+ or CD28^{neg} CD8^+ T cells. Telomeres were analyzed in these subpopulations using the Telomere peptide nucleic acid (PNA) kit/FITC (Dako, CO, UK) according to the manufacturer's instructions and as previously described (Derradji et al. 2005). Briefly, DNA from these cells or control cells (the cell line 1301) was denatured for 10 min at 82°C in hybridization solution with or without a FITC-conjugated PNA telomere probe and then hybridized overnight in the dark at room temperature. Hybridization was followed by two 10-min washes with a Wash Solution at 40°C . Then, the sample was suspended in DNA staining solution for identification of cells in G0/1 phase. Samples were acquired on a FACSFortessa with FACSDiva software (BD Biosciences, CA, USA) and analyzed with FlowJo software, version 7.4 (Tree Star, San Carlo, CA). Relative telomere length (RTL) was calculated as the ratio of the telomere signals (FITC fluorescence) of the sample and the control 1301 cell line cells, with correction for the DNA index of G0/1 cells, and converted to kilobase pairs using the telomere length of the 1301 cell line (23,480 bp) (Derradji et al. 2005).

Expression of caspase-3 and Bcl-2

PBMCs were seeded into 24-well plates (Costar, Cambridge, MA) with RPMI-1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 20 mM HEPES (GIBCO-BRL), 2 mM glutamine (GIBCO-BRL), 0.1 mM sodium pyruvate (GIBCO-BRL), and 10 % heat-inactivated human AB serum. The PBMCs were cultured for 3 days with or without $10\ \mu\text{g/mL}$ phytohemagglutinin (PHA, Sigma) in 5 % CO_2 at 37°C . Expression of caspase-3 and Bcl-2 in T cell subsets was measured by incubating 10^6 cells with anti-CD3 V500, anti-CD4 V450, anti-CD8 APC-Cy7, anti-CD45RO

PE-Cy5, and anti-CD28 APC (BD Biosciences, San Diego, CA) for 30 min at 4 °C and then fixing and permeabilizing the cells with BD Cytofix/Cytoperm (BD Biosciences, San Diego, CA) and incubating the cells with anti-caspase-3 PE and anti-Bcl-2 FITC (BD Biosciences, San Diego, CA). Samples were acquired on a FACSFortessa with FACSDiva software (BD Biosciences, CA, USA) and analyzed with FlowJo software, version 7.4 (Tree Star, San Carlo, CA).

Cytokine quantification

Supernatants obtained from PBMC cultures were assayed for tumor necrosis factor (TNF), interferon gamma (IFN- γ), IL-10, IL-6, IL-4, and IL-2 levels using the Human Th1/Th2 Cytometric Bead Array Kit (BD Biosciences, CA, USA). Cytokine levels in serum (IL-10, IFN- γ , IL-8, IL-1 β , IL-12p70, IL-6, and TNF) were measured using enhanced sensitivity flex (BD Biosciences, San Diego, CA, USA). Samples were analyzed by flow cytometry (LSRFortessa, BD Biosciences, CA, USA). The detection limits in the supernatant assays were (pg/mL) as follows: TNF, 2.8; IFN- γ , 7.1; IL-10, 2.8; IL-6, 3.0; IL-4, 2.6; and IL-2, 2.6, and in the serum assays, they were (fg/mL) as follows: IL-10, 13.7; IFN- γ , 66.7; IL-8, 69.9; IL-1 β , 48.4; IL-12p70, 12.6; IL-6, 68.4; and TNF, 67.3.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). The Kruskal-Wallis analysis with Dunn's post-test was used to compare non-parametric data from three groups, whereas ANOVA with Newman-Keuls post-test was used for parametric data. Significance was set at $p < 0.05$.

Results

Characteristics of the study population

Clinical and demographic characteristics from the 46 subjects distributed in the three study groups are shown in Table 1. There were no statistically significant differences in age or body mass index among the groups. None of the subjects was malnourished or at risk of malnutrition. Data from the MMSE questionnaire

showed that all three groups had preserved and comparable cognitive functions. However, as expected from their lifestyles, the IT group exhibited significantly higher IPAQ and VO_{2max} scores than did the NT and MT groups. In addition, the IPAQ and VO_{2max} scores were also significantly higher in the MT group than in the NT group (Fig. 1a, b). We found no statistically significant differences in clinical status among the groups according to the SF-36 Health Survey (Table 1). Thus, although our three groups could be classified as moderately, intensely, and not trained, their clinical and demographic characteristics were comparable. Accordingly, laboratory tests showed no statistically significant differences among the groups with regard to leukocyte counts; fasting glucose levels; parameters of liver and kidney function; and levels of hemoglobin, sex hormones, and total cholesterol (Table 1). All individuals except one in the NT group, two in the MT group, and one in the IT group were seropositive for CMV.

T lymphocyte subpopulations

The percentages of T cells are shown in Table 2. The proportion of total T cells was within the normal range (median ≥ 60 %) in all groups. The CD4/CD8 ratio averaged 2 in the three groups; there were rare individuals who exhibited an inverted ratio (< 1.0), one of the parameters of the immune risk profile (Ferguson et al. 1995; Strindhall et al. 2013). Other age-related changes include the decrease in the pool of antigen-inexperienced (naïve) T cells and the concomitant increase in the number of antigen-experienced (memory) T cells, which has been linked to the loss of immunological efficiency of the aged immune system (Pawelec 2014). Antigen-experienced cells can be further divided into central memory (T_{CM}), effector memory (T_{EM}), and effector memory cells that re-express CD45RA (T_{EMRA}) according to their expression of the tyrosine phosphatase isoform CD45RA and the chemokine receptor CCR7 (Akbar and Fletcher 2005). T_{EMRA} cells are the most antigen-experienced, exhibiting a reduced proliferative capacity and shorter lifespan (Fletcher et al. 2005; Hamann et al. 1997). Thus, we determined whether a moderate or intense exercise lifestyle influences the differentiation status of T cell subpopulations. Regarding CD4⁺ T cells, the most striking finding was the twofold lower proportion of T_{EMRA} cells in the training groups compared with the non-trained group (NT vs. MT, $p < 0.001$ and NT vs. IT, $p < 0.01$) (Fig. 2a). In all

Table 1 Baseline characteristics of participants

Variable	NT <i>n</i> = 15	MT <i>n</i> = 16	IT <i>n</i> = 15	<i>P</i> value
Clinicodemographic data				
Age	70 (68–78)	69 (67–72.8)	73 (70–76)	NS
BMI (kg/m ²)	25.95 (22.6–28.1)	25.2 (23.8–26.6)	23.3 (22.2–24.8)	NS
Time training (years)	NA	10 (6.75–20)	21 (10–30)	<0.05; IT vs MT
Mild suspected depression, GDS-15	1/15	0/16	0/15	NS
Nutrition, MNA	25.5 (25–28)	25.75 (24.1–28.6)	26.5 (24.8–27.6)	NS
Mental status, MMSE	27.5 (26.5–29)	29 (27.2–30)	28 (27–29)	NS
Physical functioning, SF-36	90 (75–95)	95 (87.5–100)	97.5 (88.75–100)	NS
Bodily pain, SF-36	74 (51–100)	81 (61.75–96.5)	92 (62–100)	NS
General health perceptions, SF-36	77 (67–87)	81 (61.75–96.5)	87 (70.75–100)	NS
Vitality, SF-36	75 (70–90)	83.75 (71.25–90)	77.5 (62.5–86.25)	NS
Social role functioning, SF-36	100 (62.5–100)	100 (78.13–100)	100 (72.63–100)	NS
Emotional role functioning, SF-36	83.35 (33.3–100)	100 (100–100)	100 (33.3–100)	NS
Mental health, SF-36	80 (72–96)	84 (77–91)	82 (67–93)	NS
Laboratory data				
Glycemia (80–115 mg/dL)	96 (73–122)	88 (75–102)	87 (79.5–98.5)	NS
Hemoglobin (13–18 g/dL)	14.65 (14.25–15.9)	15.4 (14.35–15.9)	14.4 (14–15.4)	NS
Leukocytes (4–11 10 ³ /mm ³)	6.3 (5.5–6.9)	6.6 (5.0–8.0)	5.5 (4.5–6.1)	NS
Liver function				
ALT (<41 U/L)	20.5 (15.5–25.25)	22 (19–26.5)	25 (22.5–30.5)	NS
ALP (49–129 U/L)	57 (48–67.5)	63 (48–101)	63 (49–72)	NS
AST (<37 U/L)	21 (16.75–23.5)	22.5 (19–26.75)	22 (17–29)	NS
GGT (8–61 U/L)	20 (18–30.25)	27 (20–36)	26 (23–40.5)	NS
Kidney function				
Urea (10–50 mg/dL)	36 (31–43)	41.5 (32.75–47.25)	36.5 (33–44.5)	NS
Creatinine (0.7–1.2 mg/dL)	0.98 (0.89–1.0)	1.0 (0.92–1.18)	0.95 (0.87–1.1)	NS
Hormones				
TSH (0.27–4.2 U/I/mL)	2.9 (1.35–4.47)	2.08 (1.62–3.46)	1.71 (1.36–2.87)	NS
Free T4 (0.93–1.70 ng/dL)	1.2 (1.0–1.35)	1.12 (0.96–1.24)	1.09 (1.0–1.25)	NS
PTH (16–87 pg/mL)	43 (20.25–61)	47 (44–76.25)	56.5 (32.25–85.5)	NS
Testosterone, free (131–640 pmol/mL)	198 (172–293)	268.5 (201–370.3)	270 (189–412.5)	NS

Table 1 (continued)

Variable	NT <i>n</i> = 15	MT <i>n</i> = 16	IT <i>n</i> = 15	<i>P</i> value
Cholesterol				
LDL (low risk <130 mg/dL; moderate risk 130–159 mg/dL; high risk >159 mg/dL)	113 (93–131)	120 (90–141)	100 (83.25–122.8)	NS
Triglycerides (optimal <150 mg/dL; borderline 150–200 mg/dL; high 200–499 mg/dL; very high >500 mg/dL)	87 (63–183)	108 (93–173)	76 (71–108)	NS

Data represented as median (25–75 percentile). Mini Mental State examination (MMSE) score: 25–30 (normal), 21–24 (mild cognitive decline), and 0–10 (severe cognitive decline). Mini Nutritional Assessment MNA score: 24–30 (normal nutritional status), 17–23.5 (at risk of malnutrition), and <17 (malnourished). Geriatric Depression Scale (GDS) score: 0–4 (normal), 5–9 (mild depression), and 10–15 (more severe depression)

ALT alanine aminotransferase, *ALP* alkaline phosphatase, *AST* aspartate aminotransferase, *GTT* gamma glutamyl transpeptidase, *TSH* thyroid-stimulating hormone, *T4* free thyroid, *PTH* parathyroid hormone, *LDL* low-density lipoprotein, *NT* not applicable, *NS* not significant, *NT* nontrained, *MT* moderately trained, *IT* intensely trained

three groups, naïve cells accounted for less than 30 % of all CD4⁺ T cells; the MT group exhibited the lowest percentage, as well as a significant increase in the number of T_{CM} cells. In all three groups, T_{EM} cells accounted for approximately 20 % of all CD4⁺ cells.

The profiles of the CD8⁺ T cells are shown in Fig. 2b. The proportion of CD8⁺ T_{EMRA} cells was lower in the training groups than the non-trained group, similar to CD4⁺ T_{EMRA} cells, although in this case, the difference reached statistical significance only between the IT and NT groups. Of note was the threefold higher proportion of CD8⁺ T_{EMRA} cells compared with CD4⁺ T_{EMRA} cells, indicating a higher attrition with aging in CD8⁺ T cells than in CD4⁺ T cells. Interestingly, the IT group had a significantly higher proportion of CD8⁺ T_{EMRA} cells than did the NT and MT groups (*p* < 0.05). In all three groups, naïve cells accounted for approximately 30 % of all CD8⁺ T cells, and similar to CD4⁺ T cells, T_{CM} cells represented the largest CD8⁺ T cell subpopulation (>40 %).

Another important feature of immunosenescence is the progressive loss of CD28 expression by CD8⁺ T cells. This molecule, which is a member of the TNF family, interacts with CD80 and/or CD86 on activated antigen-presenting cells and provides a costimulatory signal to achieve full T cell activation; CD8⁺CD28^{neg} T cells are therefore thought to be senescent. As expected for elderly individuals, half of the CD8⁺ T cells no longer expressed CD28. However, no significant differences in the percentage of CD8⁺CD28^{neg} T cells were found among the groups (Table 2), suggesting that training did not affect the loss of CD28.

Telomere length

Telomeres are repetitive DNA-protein complexes that protect the ends of linear chromosomes and maintain genomic stability (Blasco 2005). Telomere shortening has been considered a marker of immunosenescence. The impact of regular exercise on telomere length remains controversial, with studies showing either no effect (Shin et al. 2008; Woo et al. 2008; Laine et al. 2015) or a protective effect (Cherkas et al. 2008; Ludlow et al. 2008; Du et al. 2012). Therefore, we measured and compared telomere length in total T cells [CD3⁺], CD4⁺CD45RO⁺ T cells, CD4⁺CD45RO^{neg} T cells, CD8⁺CD28⁺ T cells, and CD8⁺CD28^{neg} T cells among the groups. T cell telomere length was significantly longer in the trained groups than in the NT group

Fig. 1 Fitness status of elderly individuals. **a** Weekly caloric expenditure assessed by International Physical Activity Questionnaire (IPAQ) and measured in metabolic equivalents (METs). **b**

VO₂ max consumption scores. Bars represent mean $\pm$ SEM from the non-trained (NT, $n = 15$), moderately trained (MT, $n = 16$), and intensely trained (IT, $n = 15$). *** $p < 0.0001$, * $p < 0.05$

(Fig. 3a). We then investigated whether this difference was due to longer telomeres in the CD4⁺ and/or CD8⁺ T cells. Figure 3b shows a trend for longer telomeres in both CD4⁺ and CD8⁺ T cells of the trained elderly; however, the differences were significant only for the CD8⁺ T cells of the IT group. The same result was found when the CD4⁺ and CD8⁺ subsets were analyzed (Fig. 3c, d); there was a trend for longer telomere length in both the CD45RO⁺ and CD45RO^{neg} subsets of CD4⁺ T cells and in both the senescent and non-senescent

subsets of CD8⁺ T cells in the trained groups compared with the NT group. However, the differences were statistically significant only for the senescent CD8⁺ T cell subset of the IT group. Interestingly, in the three groups, the telomeres were longer in CD4⁺CD45RO^{neg} T cells than in CD4⁺CD45RO⁺ T cells, which is consistent with the notion that the CD45RO^{neg} subset is predominantly composed of naïve cells (Fig. 3c). Similarly, in the three groups, the telomeres were significantly longer in CD8⁺CD28⁺ T cells than in CD8⁺CD28^{neg} T

Table 2 Proportions, number, and ratio of T cell subsets

	NT $n = 15$	MT $n = 16$	IT $n = 15$	<i>P</i> value
%CD3 ⁺	63.9 (56.1–72.9)	67.1 (62.6–70.4)	66.0 (53.8–78.1)	NS
Cells/mm ³	1800 (1320–2010)	1650 (1350–1825)	1600 (1400–1900)	NS
%CD4 ⁺	33.8 (29.4–40.3)	35 (27.2–42.9)	37.8 (30.8–44.5)	NS
%CD8 ⁺	17 (12.8–29.2)	17.6 (14–22.2)	18.5 (14.5–22)	NS
CD4 ⁺ /CD8 ⁺	1.85 (1.1–2.9)	1.9 (1.4–2.4)	2.0 (1.0–3.2)	NS
n , CD4 ⁺ /CD8 ⁺ < 1	3.0	1.0	3.0	NS
%CD8 ⁺ CD28 ⁺	47.3 (23.4–56.95)	49.3 (40.1–59)	42.5 (35.6–59.9)	NS
%CD8 ⁺ CD28 ^{neg}	52.6 (42.9–76.6)	50.7 (41.5–59.8)	57.1 (40.1–64.4)	NS

Data represented as median (25–75 percentile)

NT non-trained, MT moderately trained, IT intensely trained, NS not significant

Fig. 2 A comparison of the frequency of naive ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^{neg}$), effector memory ($CCR7^{neg}CD45RA^{neg}$), and effector memory RA ($CCR7^{neg}CD45RA^+$) cells in $CD4^+$ (a) and $CD8^+$ (b) T cells. Bars

represent mean $\pm$ SEM from the non-trained (NT, $n=15$), moderately trained (MT, $n=16$), and intensely trained (IT, $n=15$). *** $p<0.0001$, ** $p<0.001$, * $p<0.05$

(senescent) cells, as expected (Fig. 3d). Overall, our data suggest that a training lifestyle is associated with lower telomere attrition.

Apoptosis markers

Previous studies have shown increased apoptosis of $CD45RO^+$ and $CD45RA^+$ $CD4^+$ T cells and $CD8^+$ T cells in aging humans (Aggarwal and Gupta 1998, 1999). As we detected reduced proportions of T_{EMRA} (terminally differentiated) cells, which have a shorter lifespan, in the trained elderly, we asked whether regular physical activity could have an impact on the number of T cells that are committed to apoptosis. We analyzed caspase-3 and Bcl-2 expression in $CD4^+CD45RO^+$ T cells, $CD4^+CD45RO^{neg}$ T cells, $CD8^+CD28^+$ T cells, and $CD8^+CD28^{neg}$ T cells. Caspase-3 is a specialized protease that initiates apoptosis by cleaving a variety of proteins that are critical for cellular integrity and activating enzymes that promote the death of the cell. Bcl-2 is an anti-apoptotic protein that binds to the mitochondrial membrane and blocks the release of cytochrome c, avoiding apoptosis by the intrinsic pathway. As shown in Fig. 4a, caspase-3 expression was detected in a sizable fraction of $CD45RO^+$ and $CD45RO^{neg}$ resting $CD4^+$ T cells in the NT group, while it was lower in the MT group and virtually absent in the IT group; the differences between the IT and NT groups, but not between the NT and MT groups, were statistically significant ($p<0.001$). As expected, the number of $CD45RO^+$ and $CD45RO^{neg}$ $CD4^+$ T cells expressing caspase-3 increased after stimulation with PHA in a similar fashion in the three groups. As for $CD4^+$ T cells,

the three groups exhibited differential caspase-3 expression on senescent and non-senescent $CD8^+$ T cells, with the MT showing again a slight reduction while the IT group contained the lowest number of caspase-3 $^+$ cells. After PHA exposure, caspase-3 was substantially expressed by the $CD28^+$ and $CD28^{neg}$ $CD8^+$ T subsets in the three groups. The results of Bcl-2 mirrored those of caspase-3. As expected, most $CD4^+$ T cells and $CD8^+$ T cells expressed Bcl-2 (Fig. 4c, d); however, the percentages of $CD45RO^+$ and $CD45RO^{neg}$ $CD4^+$ T cells expressing Bcl-2 were significantly higher in the IT group than in the MT and NT groups ($CD45RO^{neg}$, $p<0.001$; $CD45RO^+$, $p<0.01$). Similarly, higher percentages of $CD28^+$ and $CD28^{neg}$ $CD8^+$ T cells expressing Bcl-2 were found in the IT group than in the MT ($p<0.01$) and NT groups ($p<0.05$). There were no differences in Bcl-2 expression between the MT and NT groups. Consistent with the higher expression of caspase-3 in PHA-stimulated cells, Bcl-2 expression was decreased in $CD4^+$ and $CD8^+$ T cells in a similar manner in the three groups upon PHA stimulation. In summary, the data demonstrate that elderly with a training lifestyle, especially those with intense training, have a reduced commitment to apoptosis of their peripheral blood resting T cells.

Cytokine levels

Physiological aging has been associated with a chronic subclinical systemic inflammatory state, termed "inflamm-aging," which is characterized by elevated serum levels of pro-inflammatory cytokines and reduced levels of anti-inflammatory cytokines (e.g., IL-

Fig. 3 Comparison of the telomere length among the elderly groups of various T cell compartments. Total T cells (CD3⁺) (a), CD4⁺ and CD8⁺ T cells (b), TCD4RO⁺/RO^{neg} (c), and

TCD8CD28⁺/CD28^{neg} cells (d). Bars represent mean $\pm$ SEM from the non-trained (NT, $n = 15$), moderately trained (MT, $n = 16$), and intensely trained (IT, $n = 15$). * $p < 0.05$

10) (Franceschi et al. 2000). It has also been reported that T cells from elderly donors secrete higher levels of several pro-inflammatory cytokines after stimulation than do T cells from young donors (Fagiolo et al. 1993; McNerlan et al. 2002). We detected substantial serum levels of IL-8 and IL-6 in all groups, with no significant differences among the groups. IL-10 was barely detectable in the three groups, whereas IFN- γ , IL-1 β , IL-12p70, and TNF were undetectable (Table 3). We also quantified cytokine levels in PBMC culture supernatants. Spontaneous cytokine secretion (i.e., from unstimulated cells) was always below the limit of detection of the assay (data not shown). Upon PHA stimulation, the PBMCs from the three groups secreted high levels of IL-2, followed by IL-6, TNF, IFN- γ , and IL-10 (Table 3). IL-4 was undetectable (data not shown). There were no differences in cytokine levels among

the three groups. Taken together, our data do not suggest a chronic low-grade inflammation in any of the elderly groups.

Discussion

The increased proportion of memory T cells in aged humans exemplifies the complex mechanisms that underlie many of the age-related immune alterations (Pawelec 2014). The shift from a population of predominantly naïve T cells to a population of predominantly memory T cells reflects the influence of cumulative exposure to foreign antigens/pathogens over time. As expected, our data showed this shift in all three groups, but moderate and intense training attenuated some of the effects of aging on memory T cells. In fact, memory T

Fig. 4 Survival capacity in resting and PHA-stimulated TCD4RO^{neg}/RO⁺ and TCD8CD28⁺/CD28^{neg} cells. PBMCs were isolated and cultured in absence (unstimulated) and the presence of PHA, and apoptosis markers were analyzed. Expressions of pro-apoptotic molecule caspase-3 and anti-apoptotic molecule Bcl-2 were determined on TCD4CD45RO⁺/RO^{neg} (a, c) and

TCD8CD28⁺/CD28^{neg} (b, d) cells. Bars represent mean $\pm$ SEM from the non-trained (NT, $n = 15$), moderately trained (MT, $n = 16$), and intensely trained (IT, $n = 15$). PHA phytohemagglutinin, PBMCs peripheral blood mononuclear cells, Unstim unstimulated. *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$

cells are not homogenous, comprising functionally distinct populations that can be identified by the differential expression of cell surface markers, such as the tyrosine phosphatase isoform CD45RA and the chemokine receptor CCR7. Using these markers, T cells were subdivided into naïve (CD45RA⁺CCR7⁺), T_{CM} (CD45RA^{neg}CCR7⁺), T_{EM} (CD45RA^{neg}CCR7^{neg}), and T_{EMRA} cells that re-express CD45RA (T_{EMRA}; CD45RA⁺CCR7^{neg}). Functionally, T_{CM} cells produce more IL-2 and exhibit a higher proliferative capacity than do T_{EM} cells, whereas T_{EM} cells produce higher amounts of IFN- γ and TNF- α (Sallusto et al. 2004). CD45RA⁺ memory cells (T_{EMRA}) have lost the expression of CD28, CD27, and CCR7 and exhibit a low proliferative capacity, a high susceptibility to apoptosis, short telomeres, and high levels of perforin and Fas ligand; thus, T_{EMRA} cells represent the most differentiated type of memory cell (Hamann et al. 1997; Geginat et al. 2003; Fritsch et al. 2005). This age-associated shift

has been reported to occur more intensely in the CD8⁺ cell compartment than the CD4⁺ T cell compartment (Czesnikiewicz-Guzik et al. 2008). In fact, in our non-trained elderly, T_{EMRA} cells accounted for ~15 % of the CD8⁺ T cells and only ~5 % of the CD4⁺ T cells. We show here that moderate and intense exercise lifestyles attenuated some of these aging effects on the composition of T cell subpopulations. The intense training lifestyle was associated with a marked reduction in T_{EMRA} cells among CD4⁺ and CD8⁺ T cells whereas the effect of the moderate training lifestyle was more evident in CD4⁺ T_{EMRA} cells than in CD8⁺ T_{EMRA} cells. In addition, intense training was associated with a higher proportion of CD8⁺ T_{EM} cells. These effects may translate into better immune responses in the trained elderly since (a) T_{EMRA} cells have a short lifespan and a narrow range of functions, mainly cytotoxicity, and (b) T_{EM} cells not only respond quickly but also still have the capacity to proliferate and to amplify further the immune response

Table 3 Cytokine quantification in supernatants and serum

Groups	Supernatants (pg/mL)				Serum (fg/mL)					
	IL-2	IL-6	TNF	IFN	IL-10	P value	IL-8	IL-6	IL-10	P value
NT (<i>n</i> = 15)	18,558 (1219–73,048)	9293 (6587–14,553)	7175 (1782–12,050)	1138 (280–1138)	744 (602–1560)	NS	1184 (1046–1720)	245 (95.2–441)	46.2 (23.5–98.8)	NS
MT (<i>n</i> = 16)	10,461 (18.6–73,048)	9409 (6391–15,593)	4417 (1946–14,886)	2387 (179–5647)	1292 (710–1593)	NS	1648 (967.5–3296)	282.4 (128–439)	55.4 (38.9–91.3)	NS
IT (<i>n</i> = 15)	30,530 (2783–200,535)	11,675 (9383–16,404)	12,152 (3644–26,253)	2128 (412–7345)	1016 (640–1621)	NS	1428 (971–1958)	308.5 (158–418)	49.3 (34.8–91.1)	NS

Data represented as median (25–75 percentile).

NT non-trained, MT moderately trained, IT intensely trained, NS not significant

through the secretion of pro-inflammatory cytokines. While there is a large body of evidence on the beneficial effects of chronic aerobic exercise on the aged immune system (de Araújo et al. 2013), there are only two reports addressing specifically the effect of chronic exercise on the composition of the memory T cell compartments, which yielded opposite results. Spielmann et al. showed that aerobic fitness was associated with lower percentages of the most differentiated CD4⁺ and CD8⁺ T cells; however, the study did not analyze elderly individuals, with the oldest group being 52–61 years old (Spielmann et al. 2011). In contrast, Moro-Garcia et al. studied subjects with a similar age range to our elderly but found opposite results: their athletes had significantly increased numbers of CD4⁺ T_{EMRA} cells compared with non-athletes, as well as a trend toward increased numbers of CD8⁺ T_{EMRA} cells (Moro-Garcia et al. 2014). In addition, the authors found an increased proportion of CD8⁺ T cells and a decreased proportion of CD4⁺ T cells, with a consequent reduction in the CD4/CD8 ratio. We found very few trained or non-trained subjects with an inverted CD4/CD8 ratio. Differences in the characteristics of the training between the two studies, such as the types of exercise, could possibly account for the different results. Whereas their older athletes were involved in mixed types of aerobic/non-aerobic exercise, such as stretching, endurance, resistance and core body exercises, our intense training subjects were typically runners who ran over 50 km per week. We speculate that different impacts on the immune system may arise from high volume mixed aerobic/anaerobic exercises compared with regular aerobic exercises (Unal et al. 2005; Saygin et al. 2006). Thus, our study indicated that elderly people with an aerobic training lifestyle have a more preserved memory compartment with proportionally fewer T_{EMRA} cells and expanded T_{CM} or T_{EM} cell compartments.

An important aging-related event is the loss of expression of the co-stimulatory molecule CD28 on CD8⁺ T cells. As in previous studies (Kapasi et al. 2003; Raso et al. 2007), we did not detect an influence of training on the proportion of CD8⁺CD28^{neg} T cells. Thus, a training lifestyle seems not to exert a major influence in the aging-associated loss of CD28 expression despite modifying the composition of the memory T cell compartment. However, the findings of the present study, as well as those of a parallel study showing better influenza vaccine responses in the IT and MT groups, corroborate the beneficial effect of a training lifestyle (de Araújo

et al. 2015). A possible connection between the changes in the memory T cell compartment promoted by an exercise lifestyle and stronger vaccine responses has recently been suggested by Derhovanessian et al., who showed that accumulation of late-differentiated CD4⁺ T cells was associated with poorer influenza vaccine responses (Derhovanessian et al. 2013). The proposed mechanism was based on an in vitro model of T cell aging where “old” CD45RA re-expressing memory CD4⁺ T cells lose their original functional properties to gain suppressive function, including the suppression of immunoglobulin synthesis by B cells (Derhovanessian et al. 2013).

Another significant finding from the present study was that both moderate and intense trainings were associated with better-preserved telomeres in T cells: telomeres from these groups were, on average, 200 bp longer than those of the NT group. This trend toward longer telomeres was detected not only in the whole CD4⁺ and CD8⁺ T cell compartments but also in their respective CD45RO⁺/CD45RO^{neg} and CD28⁺/CD28^{neg} subsets, although statistical significance was observed only in the total CD8⁺ and CD8⁺CD28^{neg} compartments between the IT and NT groups. These data are particularly important because previous studies on telomere length and physical activity showed conflicting results (Cherkas et al. 2008; Ludlow et al. 2008; Shin et al. 2008; Woo et al. 2008; Du et al. 2012; Laine et al. 2015). Comparisons among these studies are limited by differences in study design, including the immunological parameters assessed and the cohorts analyzed, which differed not only in age and gender but also in the type, intensity, and regularity of physical activity. Our results are consistent with those of Werner et al. who elegantly showed that middle-aged track-and-field athletes had reduced telomere erosion compared with non-athletes and that the probable mechanism involved exercise-associated increases in both the expression of telomere-stabilizing proteins and the activity of telomerase (Werner et al. 2009). We hypothesize that the longer TL of the TCD8⁺ compartment in our trained elderly involved similar mechanisms. These mechanisms also help to explain why it was the CD8⁺CD28^{neg} T cell subset, and not the CD8⁺CD28⁺ T cell subset, that accounted for the increase in TL: telomerase-associated protective effect of exercise can be more readily seen in cell subsets that undergo higher number of cell divisions, as is the case of CD8⁺CD28^{neg} T cells, which have a much longer replicative history than

CD8⁺CD28⁺ T cells (Weng et al. 1995). In contrast, Puterman et al. showed no direct effect of physical activity on telomere length in “stressed” female caregivers who were 54–82 years old but did show that physical activity moderated telomere erosion caused by stress (Puterman et al. 2010). Recent studies stressed the importance of telomere length in the quality of the immune response. Najarro et al. demonstrated that more robust antibody responses to an influenza vaccine were positively associated with lymphocyte telomere length (Najarro et al. 2015), reinforcing a connection between our own findings of longer telomeres and stronger and more sustained antibody responses to an influenza vaccine in trained lifestyle individuals. The authors also showed that individuals with longer telomeres exhibited stronger and more sustained influenza-specific CD8 T cell responses in vitro (Najarro et al. 2015). The authors did not find, however, an association between telomere length and monocyte antigen-presenting cell function, suggesting that the adaptive immune system is more susceptible to age-associated effects than the innate immune system and is consequently a better target for measures aiming to moderate the pace of immunosenescence. Recent in vivo data has given further support to the concept that longer telomeres are associated with better immune function. Cohen et al. showed that, among healthy individuals, those who had PBMC with longer telomeres were less susceptible to an experimental infection with the common cold virus than those with shorter telomeres; the longer telomeres of the CD8⁺CD28^{neg} T cell compartment primarily accounted for this effect (Cohen et al. 2013). In addition, the individuals at lower risk for clinical illness were those whose CD8⁺CD28^{neg} T cells had longer telomeres (Cohen et al. 2013). Thus, although the precise immunological mechanisms connecting telomere length and immune response are not elucidated, it is clear that preserved telomere length has a positive impact on the quality of the immune response. As previous studies on the pace of telomere erosion have estimated that telomeres lose 52 bp/year in total lymphocytes (Derradji et al. 2005), our training lifestyle individuals, whose total T cells had telomeres ~200 bp longer than those in non-trained subjects, have retarded their age-associated telomere erosion by ~4 years.

Early studies have shown that aging humans have increased susceptibility to apoptosis of both CD4⁺ and CD8⁺ T cells and their CD45RO⁺ and CD45RA⁺ subsets (Aggarwal and Gupta 1998, 1999). However, there

are conflicting data about susceptibility to apoptosis: studies using in vitro models of aging with T cells exposed to repeated stimulation have yielded conflicting results, showing increased, decreased, or unaffected susceptibility to apoptosis (Borthwick et al. 2000; Parish et al. 2009; Spaulding et al. 1999). Our data on the expression of the anti-apoptotic molecule Bcl-2 and the pro-apoptotic molecule caspase-3 in CD4⁺ and CD8⁺ T cells stimulated with a mitogen (a supra-physiological stimulus) showed considerable intra-group variability and no significant differences among the three groups. In contrast, there were significant differences between the groups when spontaneous expression by resting T cells was examined. Both the CD4⁺ and CD8⁺ T cells in the IT group were significantly less prone to undergo apoptosis than were the cells in the MT and NT groups, as demonstrated by a higher expression of Bcl-2 and a lower expression of caspase-3. These results are in agreement with those of Aggarwal and Gupta, who showed that increased caspase-3 expression and decreased Bcl-2 expression in CD45RA⁺ and CD45RO⁺ T cells of aging humans mediated an increased susceptibility to apoptosis (Aggarwal and Gupta 1998, 1999). A possible explanation for the age-associated proneness to spontaneous apoptosis is the decreases in both IL-7 levels and IL-7 receptor with aging (Kim et al. 2006; Kang et al. 2004). The IL-7 pathway is necessary for the development and survival of memory T cells by upregulating Bcl-2 pro-survival proteins (Chetoui et al. 2010). It would thus be interesting to know whether a training lifestyle can lead to preservation of this pathway, particularly because IL-7 is also secreted by human skeletal muscle cells, and this secretion can be modulated by exercise (Haugen et al. 2010). This might explain why intense but not moderate training lifestyle was associated with a reduction in the susceptibility to apoptosis of resting peripheral blood T cells.

IL-6 is a pro-inflammatory cytokine whose serum level increases with age and is one of the components of the aging inflammatory background known as inflamm-aging (Franceschi et al. 2000). Stimulation with the mitogen PHA induced a high production of this cytokine in our three groups, consistent with studies showing that stimulated T cells from elderly individuals secrete higher IL-6 levels than do those from young donors (Fagiolo et al. 1993; McNerlan et al. 2002). In contrast, serum IL-6 levels were low in all three groups. The single pro-inflammatory cytokine detected in higher levels in the serum of the elderly was IL-8.

However, a recent study applied a multivariate analysis to determine the relationship between markers of inflammation, age, chronic illness, and mortality in old adults (68 ± 16 years) and concluded that inflamm-aging is not only an increase in inflammatory markers but also a balance between pro- and anti-inflammatory markers (Morrisette-Thomas et al. 2014).

This study had limitations, including the disadvantages inherent in cross-sectional studies. However, our study addressed a more natural, uncontrolled setting than do controlled interventional studies, as discussed previously (Rohrbach et al. 2006). Another issue was the sample size, which was relatively small and may have reduced the number of statistically significant differences and resulted, in some instances where the differences were statistically significant, that these differences were smaller than the coefficient of variation of the groups. Our main focus was the selection of highly comparable groups of elderly individuals, a goal that has been achieved according to the demographic, clinical, and laboratory data depicted in Table 1, even though some parameters related to lifestyle such as diet, prescribed and over the counter medications, and supplements could not be controlled. This preoccupation consequently resulted in the formation of smaller groups; therefore, studies with larger cohorts are warranted to confirm the present findings. Another peculiarity of our study lies in the characteristics of our non-trained elderly group, which was composed of independent-living elderly who maintained a high quality level of life, which was likely higher than those of healthy, physically fit elderly living in nursing homes, the usual control group in most studies in this field. We speculate that this may have resulted in the finding of no evident inflammatory background in our control group. We also acknowledge that the T_{EMRA} cell compartment may comprise cells with different degrees of differentiation, as has been shown by studies that used additional cell surface markers, such as KLRG1 and CD27 (Spielmann et al. 2011; Moro-García et al. 2014). It would thus be interesting to see whether these late differentiation markers would be expressed by T_{EMRA} cells at lower levels in trained individuals. Another important point would be the assessment of the impact of CMV infection on the training effects; unfortunately, this is not feasible in our setting because almost all Brazilian elderly are exposed throughout life to CMV.

In conclusion, we showed that moderate and intense exercise lifestyles attenuated some of the effects of

aging on telomere length and the composition and survival of T cell subpopulations.

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Author contributions Léia CR Silva, Adriana L de Araújo, and Juliana R Fernandes contributed to the recruitment of the participants, questionnaire application, blood sampling, and processing of the data. Manuella ST Matias and Luiz EG Leme contributed with the recruitment and clinical evaluation of the participants. Paulo Roberto Silva was responsible for the physical evaluation of the participants and for the treadmill VO₂ max consumption test. Gil Benard and Luiz EG Leme designed and coordinated the study. Gil Benard, Luiz EG Leme, Léia CR Silva, and Adriana L de Araújo wrote the paper.

Compliance with ethical standards

Conflict of interest All authors declare that they have no financial or any other kind of personal conflicts with this paper.

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