Senescent phenotypes and telomere lengths of peripheral blood T-cells mobilized by acute exercise in humans

Richard J. Simpson[1, 2], Cormac Cosgrove[2] Meng M. Chee[3], Brian K. McFarlin[1], David B. Bartlett[1, 2], Guillaume Spielmann[1, 2], Daniel P. O'Connor[1], Hanspeter Pircher[4] and Paul G. Shiels[3]

[1] Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, 3855 Holman Street, Houston, Texas 77204, USA.
[2] Biomedicine and Sports Science Research Group, School of Life Sciences, Edinburgh Napier University, 10 Colinton Road, Edinburgh EH10 5DT, Scotland UK.
[3] Division of Cancer Sciences and Molecular Pathology, Department of Surgery, University of Glasgow, Queen Elizabeth Building, Glasgow Royal Infirmary, 10 Alexandra Parade, Glasgow G31 2ER, Scotland UK
[4] Institute for Medical Microbiology and Hygiene, Department of Immunology, University of Freiburg, Freiburg, Germany

ABSTRACT

Acute bouts of aerobic exercise are known to mobilize antigen-experienced CD8+ T-cells expressing the cell surface marker of senescence, KLRG1, into the blood. It is not known; however, if this is due to a selective mobilization of terminally differentiated T-cells (i.e. KLRG1+/CD28-/CD57+) or a population of effector memory T-cells (i.e. KLRG1+/CD28+/CD57-) that have not reached terminal differentiation. The aim of this study was to further characterize KLRG1+ T-cells mobilized by acute exercise by assessing the co-expression of KLRG1 with CD28 or CD57 and to determine telomere lengths in the CD4+ and CD8+ T-cell subsets. Nine moderately trained male subjects completed an exhaustive treadmill running protocol at 80% . Blood lymphocytes isolated before, immediately after and 1h after exercise were labelled with antibodies against KLRG1, CD28 or CD57, CD4 or CD8 and CD3 for 4-color flow cytometry analysis. Telomere

Corresponding Author
Dr Richard J. Simpson BSc, PhD
Assistant Professor in Exercise Physiology/Immunology
Laboratory of Integrated Physiology
Department of Health and Human Performance
University of Houston, Houston, Texas, USA 77204
Phone: (713) 743-9270, Fax: (713) 743-9860, Email: rjsimpson@uh.edu

lengths in CD3+, CD4+ and CD8+ T-cells were determined using Q-PCR. The relative proportion of KLRG1+ cells among the CD8+ T-cells increased by 40% immediately after exercise, returning to baseline 1h later. This was due to a mobilization of KLRG1+/CD28- (61% increase), KLRG1+/CD57+ (56% increase) and to a lesser extent, KLRG1+/CD57- cells (24% increase). Telomeres in CD8+ T-cells displayed an increased relative length immediately after exercise, whereas no change occurred for CD4+ or the overall CD3+ T-cells. In conclusion, the increased frequency of KLRG1+/CD8+ T-cells in blood after acute exercise is predominantly due to a selective mobilization of terminally differentiated T-cells. The increased relative telomere length in CD8+ T-cells after exercise might indicate that KLRG1+ cells mobilized by exercise are under stress or aberrant signaling-induced senescence (STASIS). We postulate that a frequent mobilization of these cells by acute exercise might eventually allow naïve T-cells to occupy the "vacant" immune space and increase the naïve T-cell repertoire.

Keywords: Killer cell lectin-like receptor G1 (KLRG1); CD28; CD57; immunosenescence; effector memory T-cells; exercise immunology; STASIS

INTRODUCTION

Immunosenescence is the term used to describe the biological aging of the immune system, which is characterized by poor vaccine efficacy and an increased susceptibility to infection (8, 25, 43). A hallmark of immunosenescence is the progressive accumulation of antigen-experienced oligoclonal T-cells, which occupy the so-called "immune space", increasing infection risk by reducing the naïve T-cell repertoire and compromising the adaptive immune response to novel pathogens (13, 14, 24, 26, 27). Due to their apparent increased resistance to apoptosis (39), it has been suggested that therapeutic vaccination, monoclonal antibody therapy, or cytokine therapy, might be required to remove expanded clones of these antigen-experienced effector and memory T-cells that exhibit a late differentiation phenotype (13, 14, 24).

As a possible and less-riskier alternative to these immunological interventions, we have proposed that a frequent mobilization of antigen-experienced T-cells with exercise, moving them from the peripheral tissues into the blood compartment, might create "vacant space" where naïve T-cells could eventually take occupancy (37). Acute bouts of aerobic exercise are known to mobilize T-cells from the peripheral tissue into the blood compartment, transiently increasing the blood lymphocyte number (1, 5, 6, 21, 33-36). The origin of the mobilized T-cells is subject to debate, but may come from secondary lymphoid tissue, such as the spleen, Peyer's patches and mucosal epithelium of the gastrointestinal and pulmonary tracts (4, 15, 16, 23), as well as peripheral non-lymphoid sites that preferentially localize effector memory T-cells (20). As both lymphoid and non-lymphoid tissues appear to be susceptible to oligoclonal T-cell "overcrowding", this prompted us to suggest that the removal and subsequent deletion of these cells with regular bouts of acute exercise could be advantageous (37). Moreover, it is known that CD8+ T-cells are more susceptible to senescence and oligoclonality than CD4+ T-cells (14) and acute exercise elicits the mobilization of CD8+ T-

cells to a greater extent than CD4+ T-cells, with many of these mobilized cells exhibiting phenotypes that are characteristic of antigen-experienced and effector memory T-cells (1, 6, 33, 34).

The main surface marker we have used to identify senescent T-cells (i.e. incapable of further cellular division) is the killer-cell lectin-like receptor G1 (KLRG1) (33, 34), which ligates with E-cadherin (17, 29) and is known to inhibit clonal expansion of CD8+ T-cells (10). T-cells expressing KLRG1 fail to proliferate in response to mitogenic stimulation, despite some of these cells retaining expression of the co-stimulatory molecule CD28 (24, 46, 47). Although we have shown that acute aerobic exercise mobilizes KLRG1+, CD57+ and CD28- T-cells (particularly within the CD8+ population) into the peripheral blood compartment in both young and older adults (33, 34), a limitation of this work was the failure to document the combined expression of KLRG1 with either CD57 (a marker of replicative senescence) or CD28; as Ibegbu et al. (12) reported that it is more useful to identify combinations of KLRG1 and CD57 expression during the functional characterization of CD8+ T-cells. Although most CD8+ T-cells expressing CD57 also express KLRG1, many KLRG1+ cells do not express CD57 (KLRG1+/CD57-) and might represent a "memory" T-cell phenotype as these cells also express CD27, CD28 and CCR7 (12). Conversely, the CD57 expressing KLRG1+ T-cells do not express these cell surface receptors, indicating that this is an effector T-cell population that is terminally differentiated (12).

T-cells that have reached terminal differentiation are known to have overly eroded and critically short telomeres, which is believed to underpin their proliferative arrest. Telomeres, which are DNA nucleoprotein complexes that form the physical ends of linear eukaryotic chromosomes, function to protect chromosome ends from degradation and end-to-end fusion that could potentially lead to chromosomal translocations, perturbations of cell growth and malignancy (9, 40, 41). Shortened telomeres have been associated with CD57 expression on the surface of T-cells (3), however, the effects of acute exercise on T-cell telomere length has not been fully elucidated. To our knowledge, only one previous study has examined changes in lymphocyte telomere length following acute exercise (4).

The aim of this study was to determine if the increased proportion of KLRG1+ T-cells in blood immediately after acute exercise was due to a selective mobilization of terminally differentiated cells with a senescent phenotype (i.e. KLRG1+/CD57+ and KLRG1+/CD28-), or a population of the so-called antigen-experienced cells with a "memory" phenotype (i.e. KLRG1+/CD57-; KLRG1+/CD28+). A second aim was to determine telomere lengths of pan CD3+ T-cells and the CD4+ and CD8+ T-cell subsets in blood following an acute bout of exercise.

METHODS

Subjects: Nine moderately trained male subjects (mean ± SD age: 26.4 ± 6.7 y; height: 181 ± 5.4 cm; mass: 73.7 ± 6.1 kg; $\dot{V}O_{2max}$: 56.9 ± 5.1 ml·kg^{-1}·min^{-1}) participated in this study. Subjects were healthy non-smokers who were taking no medication or supplementation and were free from any infectious illness for 6-weeks prior to testing. After receiving oral and written information pertaining to

the risks and demands of the study, each subject signed an informed consent document. A local ethics committee for human subject research at Edinburgh Napier University granted approval for the study.

Experimental Design: All subjects completed a test of maximal oxygen uptake ($\dot{V}O_{2max}$) and an intensive treadmill-running protocol at a speed corresponding to 80% of the $\dot{V}O_{2max}$. Subjects were asked to refrain from physical activity for 24 h prior to each test, which began at 09:00. The $\dot{V}O_{2max}$ of each subject was determined following the incremental treadmill-running protocol described by Simpson *et al*. (36). Oxygen uptake (breath by breath) was measured using online gas analysis (CPX, Medical Graphics Corporation, Oldham, UK) and heart rate was monitored by short-range telemetry (Polar S610, Polar Electro, Kempele, Finland) throughout each test. Approximately one week after the initial $\dot{V}O_{2max}$ test, each subject completed an intensive running protocol on a motorized treadmill (Woodway, Ergo ELG 55, Germany) at a speed corresponding to 80% of the pre-determined $\dot{V}O_{2max}$. Subjects were asked to maintain this running speed until volitional exhaustion (mean running time 33:59 ± 3:52 min:sec). Intravenous blood samples were collected in 6 ml vacuum tubes containing EDTA as an anticoagulant (Becton-Dickinson, Oxford, UK) before, immediately after, and 1 h after exercise.

Peripheral Blood Cell Counts: Total Leukocyte and lymphocyte counts were determined using a Sysmex XS automated haematology analyzer (Minnesota, USA). Absolute cell concentrations of the lymphocyte subset populations were determined by multiplying the percentage of all lymphocytes expressing CD3/CD4 or CD3/CD8 (as determined by flow cytometry) by the total lymphocyte count.

Peripheral Blood Mononuclear Cell Separation: Whole blood was mixed with an equal volume of 0.9% NaCl and a 6 ml volume of the diluted blood was layered over 3 ml of Lymphoprep (Axis-Shield, Oslo, Norway). Samples were centrifuged at 800 g at room temperature for 30 min. After centrifugation, the peripheral blood mononuclear cells (PBMCs) at the sample/medium interface were removed and washed twice for 10 minutes, first with 0.9% NaCl then with phosphate-buffered saline + 1% bovine serum albumin + 0.02% sodium azide (PBS-BSA). The viability of each sample was >98% as determined by trypan blue exclusion.

Labelling of Cell Surface Antigens: Aliquots of 1.0×10^6 cells were labelled with an AlexaFluor-488 conjugated anti-KLRG1 (IgG2a, clone: 13F12F2) monoclonal antibody (mAb) (18), an APC conjugated anti-CD3 mAb, a PE-Cy-7 conjugated anti-CD4 or anti-CD8 mAb, and either an anti-CD28 (all purchased from Immunottols, Friesoythe, Germany) or CD57 mAb conjugated to PE (Abcam, Cambridge, MA, USA) in a four-colour direct immunofluorescence procedure. Cells were also labelled with each individual mAb in a single-colour procedure to serve as colour compensation controls during flow cytometry analysis. Cells were incubated with 0.1 ml of each pre-diluted mAb for 45 min at room temperature. After incubation, cells were re-suspended in 0.5 ml of PBS-BSA and analysed by flow cytometry.

Flow Cytometry: Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on a FACSCalibur flow cytometer equipped with a 15 mW argon ion laser emitting light at a fixed wavelength of 488 nm and a red

diode laser emitting at a fixed wavelength of 635 nm (BD Biosciences, San Jose, CA, USA). The initial set-up of the instrument was performed using CELLQuest Pro software (BD Biosciences, San Jose, CA, USA). Blood lymphocytes were identified and electronically gated using the forward and side light-scatter mode. Fluorescent signals were collected in logarithmic mode (4 decade logarithmic amplifier) and cell numbers per channel in linear mode. AlexaFluor-488 fluorescence was detected in the FL1 filter centered at 530 nm with a 30nm half-peak bandpass and PE fluorescence was detected in the FL2 filter centered at 578 nm with a 28nm bandpass. PE-Cy7 fluorescence was detected in the FL3 filter with a 670 nm longpass and APC fluorescence was detected in the FL4 filter centered at 670 nm. Appropriately conjugated isotype controls were used in each assay to account for non-specific binding of Ig and to set the voltages for each fluorescence detector filter, with the peak of the negative cell population being centred in the first logarithmic decade of the fluorescent amplifier. The fluorescence of each directly conjugated mAb was also analysed in isolation using one-colour analysis to control for spectral overlap among the detector filters. For each sample, 20,000 of the gated CD3+ lymphocytes were acquired for analysis.

Following sample processing, FCS files from CELLQuest Pro were transferred to FCS Express Version 3 (De Novo Software, Los Angeles, CA, USA) for analysis. Two parameter dotplots were generated from the gated lymphocyte cell population to identify CD3+/CD4+ or CD3+/CD8+ T-cell subset populations. The expression of KLRG1, CD28, CD57 and the co-expression of KLRG1 with CD57 or CD28 was then assessed on the CD3+/CD4+ and CD3+/CD8+ T-cell populations by generating appropriate histograms and dotplots. Electronic colour compensation (using the files from the one-colour assays) was performed before analysis to exclude any overlapping emission spectra among the detector filters. The percentage of all CD3+/CD4+ and CD3+/CD8+ T-cells expressing the cell surface markers of interest were tabulated for statistical analysis.

Negative Isolation of CD3+, CD4+ and CD8+ T-cell Subset Populations: T-cell fractions were negatively sorted from the isolated PBMCs using CD3+, CD3+/CD4+ and CD3+/CD8+ negative enrichment kits (BD Biosciences, Oxford, UK). PBMCs at a concentration of 10 $x10^6$ $cells.ml^{-1}$ in IMag buffer were incubated with 50µl of the appropriate biotinylated enrichment monoclonal antibody cocktail for 20 min at room temperature. The enriched T-cell fraction was labelled with the antibody cocktail for a second time to maximize cell purity. The purity of the enriched CD3+, CD4+ and CD8+ T-cell fractions was >95%, >95% and >92% respectively for eight of the nine subjects as determined by CD3+, CD3+/CD4+ and CD3+/CD8+ co-expression using two-colour flow cytometry. The cell purity for one subject was slightly lower at >92%, 91% and 86% for CD3+, CD4+ and CD8+ T-cell fractions respectively; however, we did not exclude this data as the measured telomere lengths for this subject were not outliers. One million cells from each enriched T-cell and T-cell subset fraction were centrifuged at 300 g for 7 min and the supernatant was fully aspirated. The cells were then resuspended, dropwise, in foetal calf serum (Gibco, Invitrogen, Scotland, UK) and 7% dimethyl sulfoxide (Sigma-Aldrich, Germany) on ice before transferring to a cryo tube. The cryo tube containing the cell fraction was placed in a polystyrene container and stored overnight at -80°C, before being submerged in liquid N2 for long-term storage prior to telomere length analysis.

Telomere Length Analysis: The mean telomere lengths of the CD3+, CD4+ and CD8+ enriched T-cell samples were determined by quantitative PCR (Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, CA) following the methods described by Cawthon et al. (7). Briefly, DNA was extracted from the enriched T-cell subsets following standard procedures. The relative telomere to single copy gene (T/S) ratio were validated against control sample DNA, which had mean terminal restriction fragment lengths (mTRF) previously determined by southern blotting procedures, allowing for the conversion of T/S ratio values to kilobase pairs (9). All telomere length analysis of the enriched T-cell and T-cell subset fractions were performed within 6-weeks of sample storage.

Statistical Analysis: All statistical analyses were performed using SPSS version 17 for Mac statistical analysis software (Chicago, IL, USA). The effects of exercise over time (i.e. pre, immediately after and 1 h after exercise) on expression of each surface receptor on each of the T-cell subsets were tested using separate restricted maximum likelihood linear mixed models (LMM). The LMM method was used to fit a covariance matrix for the residuals to account for dependency of the repeated measures, allowing different variances for each time point and different covariances between time points. When the time main effect was significant, post hoc paired t-tests with Bonferroni adjustment were used to test the immediately after and 1h time points against the pre-exercise value. Paired sample t-tests were also used to compare relative changes fro pre to immediately post-exercise between cell types in response to exercise. Statistical significance was accepted at $p<0.05$.

RESULTS

Changes in Leukocyte, Lymphocyte and T-cell Subset Counts: The total leukocyte, lymphocyte, CD3+, CD4+ and CD8+ T-cells counts in response to the exercise protocol are presented in Table 1. The stereotypical leukocytosis was observed in response to exercise with an initial increase in the total lymphocyte count immediately after exercise ($p<0.01$), followed by a reduced lymphocyte concentration

Table 1. Total cell numbers ($x10^9$/l) of leukocytes, lymphocytes, pan CD3+ T-cells and the CD3+/CD4+ and CD3+/CD8+ T-cell subsets in peripheral blood following an acute bout of high-intensity exercise. Statistically significant difference from pre-exercise indicated by * ($p<0.05$) and ** ($p<0.01$).

	Pre Exercise	Post Exercise	1h Post Exercise
Leukocytes	5.02 ± 0.73	9.22 ± 2.28**	10.17 ± 3.74*
Lymphocytes	1.63 ± 0.34	2.94 ± 0.86**	1.21 ± 0.40*
CD3+	1.06 ± 0.22	1.46 ± 0.46*	0.83 ± 0.28*
CD3+/CD4+	0.60 ± 0.13	0.75 ± 0.23	0.56 ± 0.18
CD3+/CD8+	0.41 ± 0.08	0.76 ± 0.21**	0.27 ± 0.09*

(relative to pre-exercise) 1 h later ($p<0.05$). The exercise bout elicited a change the concentration of pan-CD3+ T-cells and CD8+ T-cells but not CD4+ T-cells.

Expression of KLRG1, CD28 and CD57 on T-cell Subsets: The percentage of all CD3+ T-cells, CD3+/CD4+ T-cells and CD3+/CD8+ T-cells expressing

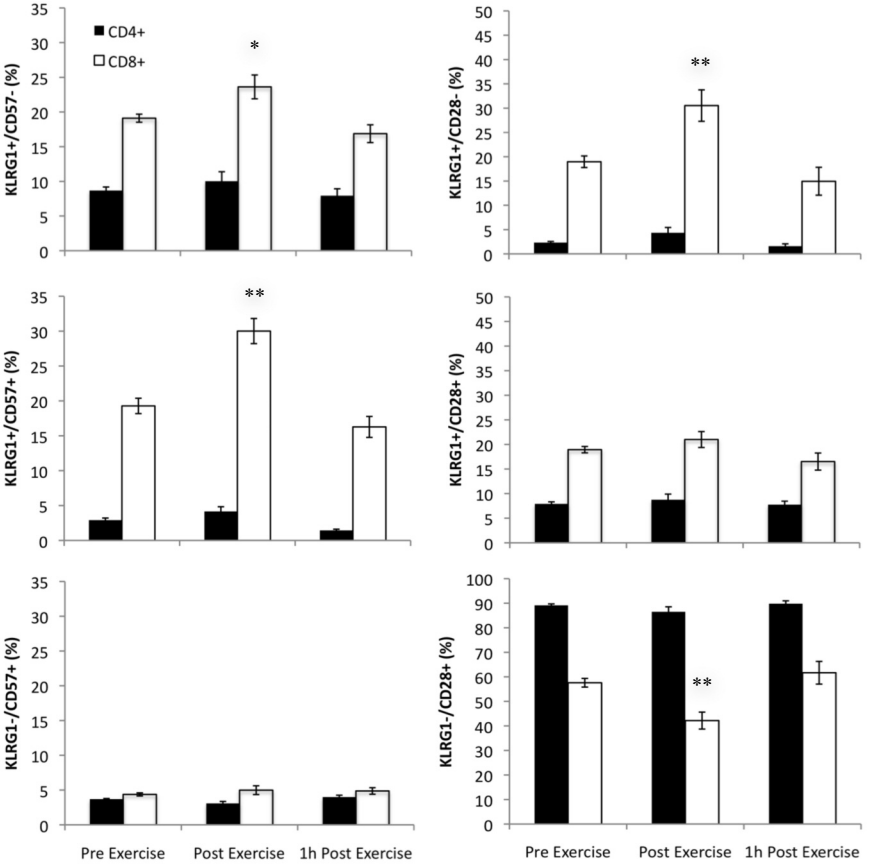

Figure 1. The percentage of all CD3+/CD4+ and CD3+/CD8+ T-cell subsets expressing combinations of KLRG1 and CD28 or combinations of KLRG1 and CD57 in response to an acute bout of high-intensity exercise. Statistically significant difference from pre-exercise indicated by * ($p<0.05$) and ** ($p<0.01$).

KLRG1, CD28 or CD57 in response to the exercise protocol are shown in Table 2. The percentage of all CD3+ T-cells expressing KLRG1 or CD57 was elevated immediately after exercise ($p<0.01$), which was due to an increased expression on CD8+ T-cells ($p<0.05$) and not the CD4+ subset ($p>0.05$). Similarly, the percentage of all CD3+ T-cells expressing CD28 was lower immediately after exercise ($p<0.05$), which was due to a reduction in the percentage of CD8+ T-cells expressing CD28 ($p<0.01$) and not the CD4+ subset ($p>0.05$).

The Co-expression of KLRG1 with CD28 or CD57 on T-cell Subsets: The percentage of all CD4+ and CD8+ T-cells expressing KLRG1 but not CD28 (KLRG1+/CD28-), CD28 but not KLRG1 (KLRG1-/CD28+) and both KLRG1 and CD28 (KLRG1+/CD28+) are shown in Figure 1. On the CD8+ subset, the frequency of KLRG1+/CD28- cells increased immediately after exercise ($p<0.01$). Conversely, the proportion of KLRG1-/CD28+ cells was lowered

immediately after exercise ($p<0.01$). The percentage of KLRG1+/CD28+ cells within the CD8+ subset remained unchanged ($p>0.05$). No phenotypic changes, with regards to KLRG1 and CD28 expression, were found on the CD4+ T-cells in response to the exercise protocol ($p>0.05$). Immediately after exercise, the proportion of cells expressing KLRG1 and CD57 (KLRG1+/CD57+) and KLRG1 but not CD57 (KLRG1+/CD57-) increased within the CD8+ T-cell subset ($p<0.05$) (Figure 1). No statistically significant changes were found for KLRG1 and CD57 expression combinations on CD4+ T-cells ($p>0.05$). Representative flow cytometry plots showing the expression of KLRG1 with CD28 or CD57 are shown in Figure 2 (next page).

Relative Change in the Proportion of KLRG1+/CD57+ and KLRG1+/CD57- cells within the CD8+ T-cell subset: Due to the statistically significant increases for both KLRG1+/CD57+ and KLRG1+/CD57- cells within the CD8+ T-cell subset, we compared the relative change in cell proportions in response to exercise between these two phenotypes. The percentage values obtained immediately after exercise were divided by the corresponding pre-exercise values for each subject. A pair-wise t-test showed the mean fold-increase of 1.9 ± 0.6 for the KLRG1+/CD57+ phenotype to be significantly greater ($p<0.05$) than the 1.3 ± 0.4 fold increase for the KLRG1+/CD57- phenotype (data not shown).

Telomere Lengths of T-cell Subsets: Mean telomere lengths for the negatively sorted CD3+, CD4+ and CD8+ T-cells in response to the exercise protocol are shown in Figure 3 (next page). A significant increase in relative telomere length was observed in CD8+ T-cells immediately after exercise ($p<0.05$), while exercise did not significantly affect telomere length in CD4+ T-cells or the total CD3+ cell population ($p>0.05$).

Table 2. The percentage of pan CD3+ T-cells and the CD3+/CD4+ and CD3+/CD8+ T-cell subsets expressing KLRG1, CD28 or CD57 in response to an acute bout of high-intensity exercise. Statistically significant difference from pre-exercise indicated by * ($p<0.05$) and ** ($p<0.01$).

	Pre Exercise	Post Exercise	1h Post Exercise
CD3+ T-cells			
KLRG1+	21.8 ± 11.7	32.8 ± 12.7**	18.0 ± 10.7
CD28+	87.0 ± 10.0	78.5 ± 11.3*	90.3 ± 7.8
CD57+	13.4 ± 8.9	19.2 ± 10.3**	11.3 ± 7.1
CD3+/CD4+ T-cells			
KLRG1+	9.1 ± 4.9	14.5 ± 8.7	8.8 ± 4.0
CD28+	96.1 ± 3.2	93.6 ± 3.6	97.7 ± 1.2
CD57+	5.2 ± 1.6	6.5 ± 2.9	4.4 ± 1.4
CD3+/CD8+ T-cells			
KLRG1+	35.6 ± 15.0	49.9 ± 13.5*	30.2 ± 17.2
CD28+	76.7 ± 14.8	63.5 ± 15.3**	78.5 ± 12.3
CD57+	22.6 ± 12.5	32.8 ± 14.8±*	19.7 ± 13.2

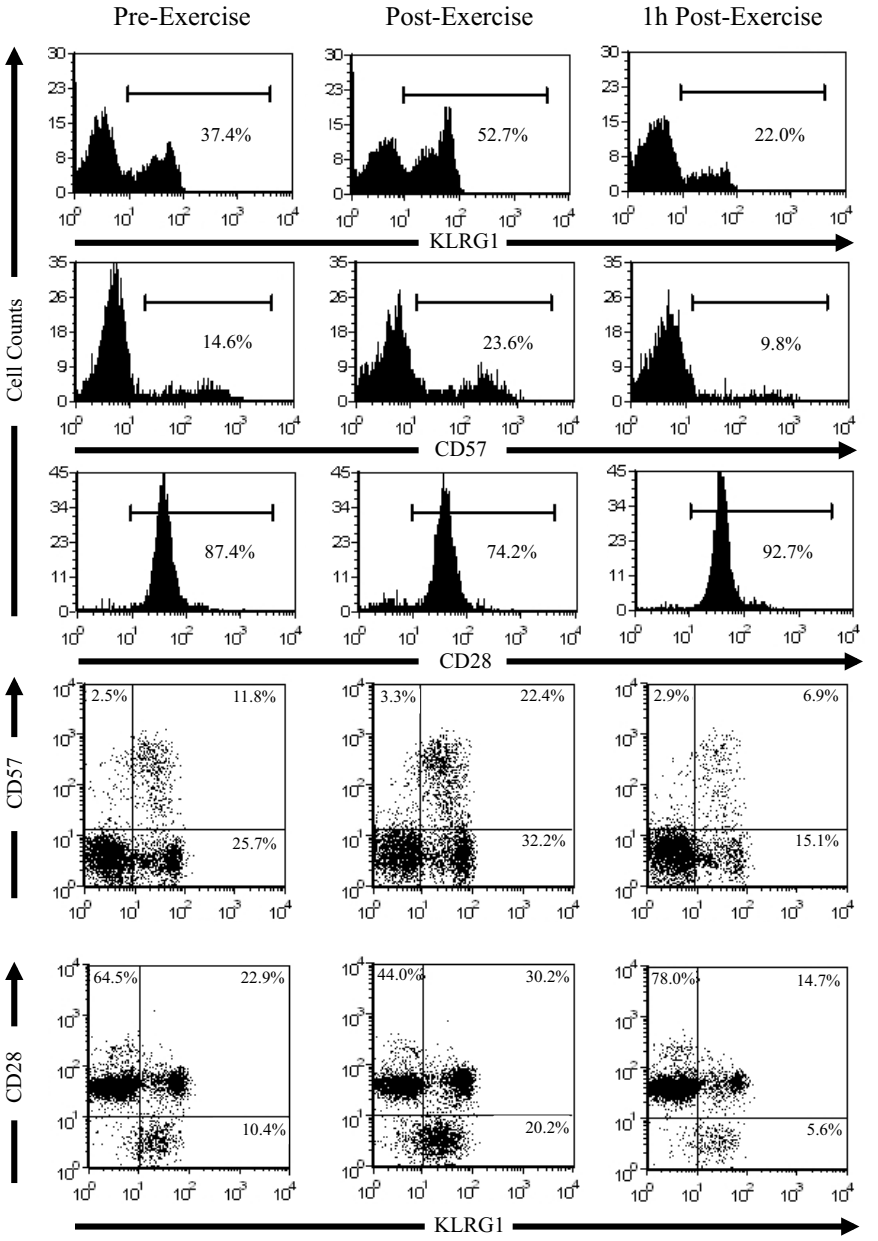

Figure 2. Representative flow cytometry histograms and dotplots from a single subject showing the expression of KLRG1, CD57 and CD28 on CD3+/CD8+ T-cells in response to an acute bout of high-intensity exercise. Similar plots were generated to determine the expression of these cell surface receptors on CD3+/CD4+ T-cells (data not shown). Values are the percentage of all CD3+/CD8+ T-cells expressing the markers of interest.

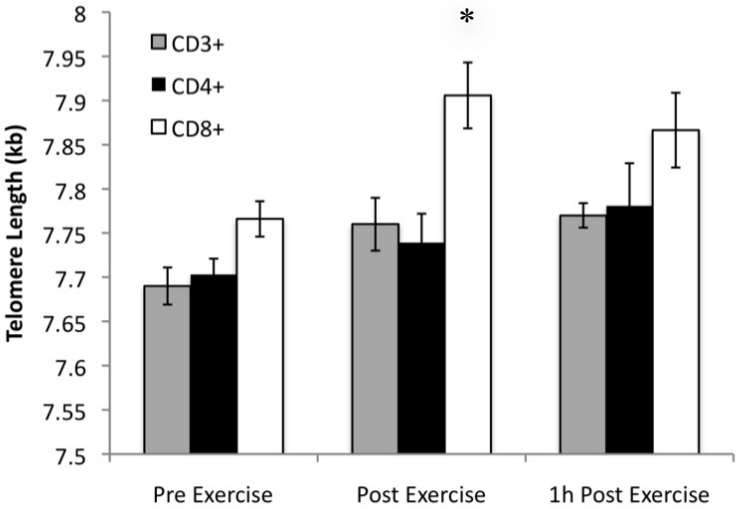

Figure 3. Mean telomere length of negatively sorted CD3+, CD4+ and CD8+ T-cells in response to an acute bout of high-intensity exercise. Statistically significant difference from pre-exercise indicated by * ($p<0.05$)

DISCUSSION

This study reports the effects of an acute bout of high-intensity aerobic exercise on the mobilization of T-cell subsets with a senescent phenotype and their telomere lengths. We sought to determine if the increased proportion of CD8+ T-cells expressing the cellular senescence marker KLRG1 that we reported previously (33, 34), was due to an influx of terminally differentiated T-cells (i.e. KLRG1+/CD57+ and/or KLRG1+/CD28- phenotype) and/or a cell population exhibiting phenotype characteristics of effector memory T-cells that have not reached terminal differentiation (i.e. KLRG1+/CD57- and/or KLRG1+/CD28+) (12). We show that the increased proportion of KLRG1+ cells within the CD8+ T-cell subset after exercise is due mostly to a selective mobilization of highly differentiated T-cells exhibiting a KLRG1+/CD57+ and KLRG1+/CD28- cell surface phenotype, which quickly leave the peripheral blood compartment within 1h of exercise cessation. We have also shown that relative telomere lengths of negatively sorted CD8+ T-cells was longer immediately after exercise, although this was contrary to what we expected. The current study supports the contention that acute aerobic exercise results in a selective mobilization of highly differentiated T-cells into the blood compartment.

The increased frequency of KLRG1+, CD57+ and CD28- cells among the CD8+ T-cell population immediately after exercise is consistent with our previous studies; however, in this early work, we did not explore the relationship between KLRG1 and CD57 or KLRG1 and CD28 expression (33, 34). Combinations of KLRG1, CD28 and CD57 expression have been used to determine the fate and functional properties of CD8+ T-cells (12, 47). It has been postulated that

KLRG1+/CD57- T-cells are effector cells destined to become long-lived memory cells due to their expression of the IL-7 receptor CD127 (12). Conversely, KLRG1+/CD57+ cells are still capable of pro-inflammatory cytokine secretion and expression of cytolytic granules (i.e. perforin and Granzyme B) but do not express CD127, CCR7 or the co-stimulatory molecules CD27 and CD28, indicating that these are terminally differentiated effector T-cells that are unable to proliferate but still maintain immediate effector cell properties (12). In the present study, the increased proportion of CD8+ T-cells expressing KLRG1 in blood immediately after acute exercise was due to a preferential mobilization of these terminally differentiated T-cells.

Exercise only transiently increased the proportion of KLRG1+/CD57+/CD28- CD8+ T-cells in blood, with the immediate post-exercise response reverting back to resting levels by 1-h post exercise. Two pertinent questions emerge from this observation. Firstly, do the antigen-experienced T-cells mobilized by acute exercise return to their pre-exercise destinations or do they migrate to other tissues in the body; and secondly, what is the fate of these mobilized T-cells after they leave the peripheral blood compartment. For instance, it is not known if specific T-cell populations leave the blood to sequester in other tissues, to subsequently undergo activation-induced cell death, or to circulate among the peripheral tissues before eventually returning to their pre-exercise destinations. Although lymphocytes mobilized by acute exercise do not appear to undergo apoptosis in the bloodstream (28, 35, 42), lymphocytes that enter and exit the blood with exercise might be more susceptible to apoptosis following extravasation to the tissues due to their heightened expression of the cell surface death receptor CD95 (Fas/Apo-1) (22, 35, 44) and/or the increased milieu of apoptotic signals known to change with acute exercise such as glucocorticoids and reactive oxygen species (31, 38, 42). Previous studies using murine exercise models have shown that T-cells mobilized by acute exercise preferentially migrate to intestinal Peyer's patches (15) and that acute exercise, in turn, increases apoptotic cell death of intestinal lymphocytes (11). Furthermore, as shown in the present study and in our previous work (33, 34), CD8+ T-cells expressing CD57 are preferentially mobilized and subsequently removed from the blood in response to an acute bout of exercise. CD8+ T-cells expressing CD57 have, in turn, been shown to be more susceptible to apoptosis *in vitro* (3, 32, 45) than CD8+ T-cells that do not express CD57. It is possible therefore that some CD8+ T-cells with a senescent phenotype might undergo apoptosis in other tissues (i.e. intestinal Peyer's patches) following their extravasation from the peripheral blood compartment in response to acute exercise. Future research should attempt to investigate the apoptosis susceptibility of antigen-experienced CD8+ T-cells mobilized by acute exercise and the relationship between this potential susceptibility and their phenotype characteristics.

To our knowledge, this is only the second study to measure changes in telomere length of T-cell subsets in response to an acute bout of exercise, but the first to use negatively sorted "untouched" lymphocytes and Q-PCR in this context. Telomere length has been used extensively as a marker of biological age and, because they erode progressively with each round of cell division (i.e. during the clonal expansion of T-lymphocytes in response to an antigenic stimulus), critically shortened telomeres trigger mechanisms for senescence causing the cell to undergo proliferative arrest (9, 40, 41). As such, T-cells with a senescent pheno-

type (i.e CD57+/CD28-) are known to have shortened telomeres and are associated with persistent viral infections in humans (27). Due to the increased frequency of senescent CD8+ T-cells in the peripheral blood immediately after acute exercise, our finding that the mean telomere length of CD8+ T-cells was longer after the exercise bout was not expected. However, although there was a preferential mobilization of senescent CD8+ T-cells in response to the exercise bout, it is important to note that a large number of naïve non-senescent T-cells (i.e. cells with a KLRG1- and/or a CD45RA+/CCR7+ phenotype) are also mobilized into the blood in response to acute exercise (6, 33). It is possible then that the naïve T-cells in the peripheral lymphoid tissues that enter the blood in response to acute exercise have longer telomeres than those resident in blood, resulting in the greater mean telomere length of CD8+ T-cells that we observed immediately after exercise. As such, measuring mean telomere length of CD8+ T-cells in response to acute exercise might not provide an accurate indication of the biological age of the mobilized cells due to the altered composition of CD8+ cell types with varying degrees of replicative history. Furthermore, due to the greater relative telomere length in CD8+ T-cells that was accompanied by an increased proportion of KLRG1+ cells after exercise, it might be argued that the KLRG1+ T-cells mobilized into blood with exercise are under stress or aberrant signaling-induced senescence (STASIS) as opposed to replicative senescence (30).

Our telomere length findings are contrary to those of Bruunsgaard et al. (4), who reported shorter telomere lengths in CD8+ T-cells isolated after acute exercise in young subjects. The discrepancy between these studies could be due to technical differences in the isolation of T-cell subsets, particularly within the CD8+ T-cell population, or to differences in methodology. Because a subset of NK-cells expressing CD8 are preferentially mobilized into the blood following acute exercise (5), by positively sorting CD8+ cells, Bruunsgaard et al. (4) will have inadvertently analyzed mean telomere length on a cell population with an increased CD8+ NK-cell/CD8+ T-cell ratio immediately post-exercise compared to the resting condition. This is problematic, as the rate of telomere attrition in NK-cells and CD8+ T-cells are known to differ (19). Although we used negatively sorted "untouched" T-cell subset populations to quantify telomere length in response to exercise, our own technique is not without caveats. The antibody cocktail in the negative isolation kit contains an anti-CD56 mAb to remove NK-cells, however, some CD8+ T-cells are known to express CD56 (5). This is likely to have resulted in a disproportionate loss of some phenotypically distinct CD8+ T-cells prior to telomere length measurement in the present study.

A notable weakness of the present study is that we failed to screen our participants for latent viral infections. Reactivating latent herpes viruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are major contributors of oligoclonal T-cell accumulation (13, 14, 26, 27), and future work should determine if the exercise-induced mobilization of terminally differentiated T-cells differs between CMV or EBV infected and non-infected participants. Further, identifying whether these highly differentiated T-cells that enter the blood with exercise are specific to any particular latent viruses would also be illuminating (37). Indeed, acute psychological stress is known to mobilize CMV and EBV-specific T-cells and effector T-cells into the blood (2), therefore It would be expected that physical stress would elicit a similar response.

In conclusion, we have shown that an acute bout of high-intensity aerobic exercise preferentially mobilizes a population of terminally differentiated CD8+ T-cells exhibiting a senescent phenotype into the peripheral blood compartment. This extends our previous findings (33, 34) by showing that the mobilization of antigen-experienced CD8+ T-cells expressing the cellular senescence marker KLRG1 is mostly due to a population of terminally differentiated effector T-cells that express CD57 but not CD28. We postulate that a frequent mobilization of these cells with habitual exercise might blunt the age-induced diminution of the naïve T-cell repertoire, as newly generated naïve T-cells might eventually take occupancy within the "immune space" vacated by these senescent T-cells. Future research should attempt to determine the fate and destination of these terminally-differentiated T-cells after their extravasation from the bloodstream; establish the effects of regular exercise on the frequency of antigen-specific T-cells with a senescent phenotype in resting blood; and determine if frequent cell shifts with acute exercise influence the positive changes in adaptive immune function that are known to occur in response to chronic exercise.

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