A 10km Time Trial Running Bout Acutely Increases the Number of Angiogenic T-Cells in the Peripheral Blood Compartment of Healthy Males

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Subject Area: Human/environmental and exercise physiology

New Findings:

What is the central question of the study?

- Are $CD31^+$ angiogenic T-cells (T_{ANG}) preferentially mobilised in response to acute exercise?

What is the main finding and its importance?

- Our study reveals that T_{ANG} cells are redistributed into the circulation in response to acute strenuous exercise, but to a lesser extent than $CD31^-$ T-cells. Of the T_{ANG} cells mobilised, T_{ANG} cells expressing CXCR4 show greater redistribution compared to $CXCR4^-$ T_{ANG} cells. SDF-1α does not appear to play a role in the redistribution of T_{ANG} cells expressing CXCR4. The results suggest that a single bout of strenuous exercise may provide a short vasculogenic window which could offer benefit to the vascular system via the redistributing $CD31^+$ angiogenic T-cells.

Abstract:

$CD31^+$ T-cells have been documented to possess vasculogenic properties, and have been termed 'angiogenic T-cells' (T_{ANG}). No study to date has fully characterised the effect of acute exercise on T_{ANG} cells. Twelve male participants aged 24-45yrs undertook a running 10km time trial, with peripheral blood samples taken pre-, immediately post- and 1-hour post-exercise for quantification of T_{ANG} cells and subsequent CXCR4 cell surface expression by flow cytometry. T_{ANG} cells demonstrated a 102% increase in number in peripheral circulation immediately post-exercise compared to pre-exercise levels, followed by a large egress (50%) from the circulation in total T_{ANG} cells 1-hour post-exercise. This was due to both $CD4^+$ and $CD8^+$ T_{ANG} changes, with $CD8^+$ T_{ANG} cells displaying greater ingress (123%) and egress (52%) compared to $CD4^+$ T_{ANG} cells (ingress: 83%, egress: 37%). CXCR4 cell surface expression intensity was only affected on $CD8^+$ T_{ANG} cells, with a significant increase in cell surface expression immediately post-exercise vs. pre-exercise levels. $CD31^-$ T-cells displayed greater redistribution than $CD31^+$ T_{ANG} cells (119% vs. 102%). CXCR4-expressing T_{ANG} cells showed greater response to acute exercise than $CXCR4^-$ cells, which was accompanied by large changes in CXCR4 ligand SDF-1α. The results show that acute exercise increases T_{ANG} cells in the circulation in response to an acute exercise stressor. Additionally, CXCR4 cell surface expression appears to be increased in response to exercise, which may result from the direct upregulation of CXCR4 on the T-cell surface, or could be due to $CD31^+$ T-cells being redistributed into the blood expressing greater levels of CXCR4.

1. Introduction:

Cardiovascular disease (CVD) is often characterised by endothelial dysfunction (Gokce *et al.*, 2003; Manganaro *et al.*, 2014). The endothelium plays important roles in cardiovascular health by regulating blood vessel diameter, adhesion of leukocytes to the vascular wall, and the transport of substances across the vessel wall. Exercise training results in improved endothelial function (Black *et al.*, 2008; Black *et al.*, 2009; Ashor *et al.*, 2015), partly attributable to shear stress-related mechanisms (Birk *et al.*, 2012); however recent evidence suggest that exercise may promote endothelial health by mobilising cells termed circulating angiogenic cells (CAC) into the circulation which act to promote endothelial growth and repair (Van Craenenbroeck *et al.*, 2010; Ross *et al.*, 2014).

Hur *et al.* (2007) discovered that CD31-expressing T-cells displayed significant vasculogenic properties, distinct from $CD31^-$ T-cells. These $CD31^+$ T-cells consisted of both $CD4^+$ and $CD8^+$ T-cells These $CD31^+$ 'angiogenic T-cells' (T_{ANG}) were required for optimal growth of endothelial progenitor cells (EPCs) *in vitro* and had the ability to secrete various pro-angiogenic growth factors (VEGF and IL-8) to support endothelial cell growth and tube formation (Hur *et al.*, 2007; Kushner *et al.*, 2010). However, it is unknown whether $CD4^+CD31^+$ T-cells and $CD8^+CD31^+$ T-cells possess the same vasculogenic functional ability, or if there are any differences between these phentoypes. Indeed total circulating T_{ANG} cells ($CD3^+CD31^+$) are positively associated with endothelial function (Weil *et al.*, 2011) and are reduced in those with vascular disease or vascular risk factors compared to healthy counterparts (Hur *et al.*, 2007; Rouhl *et al.*, 2012), suggesting a role for T_{ANG} cells in cardiovascular disease risk management. T_{ANG} cells have been included in the group of CACs which also includes $CD34^+$ progenitor cells, EPCs ($CD34^+KDR^+$), and angiogenic monocytes ($Tie2^+$) (Forget *et al.*, 2014). CACs may play an important role in maintaining endothelial integrity, and therefore the effects of acute exercise and exercise training on some CACs has been investigated (Van

Craenenbroeck *et al.*, 2008; Jenkins *et al.*, 2011; Ross *et al.*, 2014). Previous studies have shown a beneficial impact of acute exercise on circulating number and function on some CAC subsets, such as mobilisation of EPCs into the circulation within 2 hours post-exercise (Rehman *et al.*, 2004; Van Craenenbroeck *et al.*, 2008; Ross *et al.*, 2014), however to date, no study has investigated the effects of an acute exercise bout on T_{ANG} cells, and $CD4^+$ and $CD8^+$ subsets.

T_{ANG} cells also express C-X-C chemokine receptor 4 (CXCR4) on their cell surface (Hur *et al.*, 2007), which is a receptor for the ligand stromal-derived factor-α (SDF-1α) (Yamaguchi *et al.*, 2003). The CXCR4:SDF-1α axis is crucial for EPC and T-cell migration to ischaemic tissue (Bryant *et al.*, 2012; Xia *et al.*, 2012; Adams *et al.*, 2013b; Mao *et al.*, 2014). Hur *et al.* (2007) demonstrated that T_{ANG} migration was significantly blunted by blocking CXCR4, although blocking of CXCR4 did not fully attenuate the T_{ANG} cell migration, indicating that CXCR4 is required for optimal cell migration, but not essential. Acute exercise results in the increase in circulating SDF-1α and is purported to be partly responsible for exercise-induced increase in CXCR4-expressing progenitors (Chang *et al.*, 2015). Additionally, systemic increases in SDF-1α reportedly stimulated the chemotaxis of $CXCR4^+$ progenitor cells into the circulation (Powell *et al.*, 2005; Prokoph *et al.*, 2012). Therefore, increases in SDF-1α may stimulate $CXCR4^+$ T_{ANG} cells to enter the circulation as a result of the exercise bout. It is not yet known if CXCR4 plays a role in exercise-induced redistribution of T-cells as a result of an acute bout of exercise, but there is some evidence to suggest that exercise, through elevated circulating cortisol, may enhance CXCR4 cell surface expression *in vitro* (Okutsu *et al.*, 2005). Indeed cortisol was found to stimulate an increase in CXCR4 cell surface expression via glucocorticoid receptors on naïve T-cells (Besedovsky *et al.*, 2014).

An acute bout of exercise is known to stimulate the redistribution of T-cells (Simpson *et al.*, 2007; Turner *et al.*, 2010; Witard *et al.*, 2012) which may result in increased immunosurveillance. However, circulating T_{ANG} cell changes to an acute bout of exercise is

not yet known. If these T_{ANG} cells are highly responsive to acute bouts of exercise, then this may provide a potential 'vasculogenic' window whereby some cardiovascular benefit may arise through increased exposure to the vascular endothelium, where they can secrete pro-angiogenic factors to stimulate endothelial repair.

The aims of the study were therefore: to characterise the effect of an acute bout of exercise on T_{ANG}, and their $CD4^+$ and $CD8^+$ subsets; in addition to investigating the effect of acute exercise on CXCR4 cell surface expression on these T_{ANG} cells. The response of $CD31^+$ T_{ANG} cells were compared to $CD31^-$ T-cells to investigate whether there is preferential redistribution of T_{ANG} cells in response to acute exercise. It was hypothesised that acute exercise would be associated significant elevation in $CD31^+$ T-cells, and $CD31^+$ T-cells would display a greater change with acute exercise than $CD31^-$ T-cells.

2. Materials and Methods

2.1 Ethical Approval

Ethical approval for the study was granted by the Edinburgh Napier University Research and Ethics Governance Committee. Written informed consent was obtained from all participants prior to commencement of the study. Study procedures conformed to the standards set by the Declaration of Helsinki.

2.2 Participants

Twelve physically active healthy adult males (mean ± SD) age: 32 ± 7 years; height: 182 ± 8 centimetres (cm); body mass: 76.6 ± 8.41 kilograms (kg), maximum oxygen consumption ($\dot{V}O_{2max}$): 58.3 ± 4.30 $ml \cdot kg \cdot min^{-1}$, volunteered to take part in the study. All participants were

non-obese (BMI<30), non-smokers, were not taking medication affecting the immune system, and were free from infection for 6 weeks prior to participation in the study and were accustomed to running 10km distances. Participants were advised not to partake in any strenuous exercise for 72 hours prior to the visits to the Human Performance Laboratory.

2.3 Assessment of $\dot{V}O_{2max}$

Participants firstly visited the Human Performance Laboratory having refrained from ingesting caffeine and alcohol 24h prior to the start of the trial. Blood pressure was measured after 5-minute rest in a supine position on the non-dominant arm.

Maximal oxygen uptake ($\dot{V}O_{2max}$) of each participant was measured by graded treadmill exercise test (GXT) to volitional exhaustion as described by Simpson *et al.* (2006). The test comprised of progressive 3-minute stages, starting at a speed of 10km·h^{-1}, with subsequent stages increasing in speed by 3km·h^{-1}. Upon completion of the 3rd stage at 16km·h^{-1}, the gradient increased by 2.5% every minute until the participant reached volitional exhaustion. Oxygen uptake was measured during the test using breath-by-breath online gas analysis (CPX; LABManager v5.3.0, Cardinal Health, Germany) and heart rate (HR) was recorded throughout the maximal graded exercise test by HR telemetry (Polar, Finland).

2.4 10km time-trial

Within one week of completion of the $\dot{V}O_{2max}$ test, participants returned to the Laboratory to undertake a 10km time-trial (TT). This particular mode of exercise was chosen as the popularity of 10km running challenges has increased in recent years, and thus the number of individuals taking part in such 10km running bouts has increased. It may be that such 10km running bouts

have a significant vasculogenic benefit and thus we wanted to investigate if this could be reflected by changes in circulating T_{ANG} cells.. Participants attended the laboratory at 8am after an overnight fast, and having refrained from caffeine and alcohol for 24 hours. Participants were asked to refrain from participating in strenuous exercise for 72 hours prior to the 10km TT. The exercise trial consisted of a self-paced 10km running effort on a treadmill ergometer (Woodway PPS Med, Woodway Inc, Germany). Participants were told to complete the 10km distance in the shortest time possible (mean ± SD): 43:01 ± 05:27 (mm:ss), and were told they could adjust the speed throughout the trial if necessary. They were however blinded from the actual speed shown on the treadmill. Participants were notified when they had 1km of the TT remaining. Participants were allowed to drink water *ab libitum*.

2.5 Blood Sampling and Lymphocyte Phenotyping

Blood was taken from participants in a supine position before, immediately post- and 1-hour post-exercise. Peripheral blood was drawn into 6mL vacutainers (BD Biosciences, UK), coated in either EDTA, or sodium citrate. Total blood differential leukocyte counts were determined using an automated haematology analyser (Sysmex, XS 1000i, UK). Sodium citrated whole blood was processed for analysis of SDF-1α. Sodium citrate whole blood was centrifuged at 1500g for 15 minutes at 21ºC. Plasma was removed, mixed and centrifuged again in 1.5mL tubes at 13000g for 2 minutes at 21ºC to obtain platelet-free plasma. Samples were then frozen at -80ºC until analysis. SDF-1α was quantified in platelet-free plasma by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) in accordance with manufacturer's instructions. ELISA plates were read on a plate reader at a wavelength of 450nm, with wave correction at 550nm, for SDF-1α analysis (Labtech LW5000, UK).

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA whole blood by density gradient centrifugation, following procedures previously described by Simpson *et al.* (2006). Aliquots of 0.5 x 10^6 isolated PBMCs, as counted using a haemocytometer, were labelled with monoclonal antibodies anti-CD3-APC (Clone SK7, IgG_1), anti-CD31-FITC (Clone WM59, IgG_1), anti-CD4 (Clone RPA-T4, IgG_1)/anti-CD8-PE (Clone HIT8a, IgG_1) and anti-CXCR4-PE-Cy5 (Clone 12G5, IgG_{2a}) (all BD Biosciences, UK) and were allowed to incubate for 45 minutes at 4ºC in the dark prior to flow cytometric analysis. Isotypes for CD31 (Clone MOPC-21, IgG_1) and CXCR4 (Clone G155-178, IgG_{2a}) were used in matched concentrations as controls to distinguish positive and negative events. Immediately prior to flow cytometric enumeration, 500µL (PBS-BSA) was added to the samples. Pre- and post-exercise samples were analysed after matched incubation times to standardise flow protocols. T_{ANG} cells were identified as $CD3^+CD31^+$ cells (Hur *et al.*, 2007; Kushner *et al.*, 2010), with sub-fraction analysis of $CD4^+$ ($CD3^+CD4^+CD31^+$) and $CD8^+$ ($CD3^+CD8^+CD31^+$) also performed. Sub-analysis of $CD4^+$ and $CD8^+$ T-cells were performed as these cells show differing levels of mobilisation to exercise (Ingram *et al.*, 2015). As yet it is unknown if these cell sub-fractions exhibit differing levels of pro-angiogenic activity. CXCR4 expression was quantified to investigate the role of SDF-1:CXCR4 axis on T_{ANG} cell mobilisation.

2.6 Flow cytometry

T-cell data was obtained using CELLQuest Pro software (BD Biosciences, USA) on a FACS Calibur four-colour flow cytometer equipped with a 15 mW argon ion laser emitting light at fixed wavelength of 488nm. A lymphocyte gate was identified using forward and side scatter plots. 100,000 gated lymphocytic events were measured per sample. Total numbers of T-cells were obtained by multiplying the percentage values obtained from the flow cytometer by the

corresponding total lymphocyte counts. Mean fluorescence intensity (MFI) of CXCR4 cell surface receptor expression was also obtained by flow cytometry. Gating strategy for $CD31^+$ T_{ANG} and $CD31^-$ T-cells are shown in Fig 1.

Changes in blood volume was accounted for by using known measures of haematocrit and haemoglobin obtained from automated haematology analysis (Sysmex, XS 1000i, UK) (Dill & Costill, 1974; Ingram *et al.*, 2015).

2.7 Statistical analysis

All data are presented as mean ± SEM unless otherwise stated. All data were assessed for normal distribution using Shapiro-Wilk test for normality. All data were normally distributed. To assess the response of total T_{ANG} cells, $CD4^+$ and $CD8^+$ T_{ANG}, $CXCR4^+$ T_{ANG} cells and CXCR4 cell surface expression (as quantified by mean fluorescence intensity [MFI]) to acute exercise, several repeated measures analysis of variance (ANOVA) were performed, including the three time points as dependent factor (pre-exercise, immediately post-exercise, and 1-hour post-exercise). To compare the effect of acute exercise between different phenotypes of T-cells enumerated ($CD31^{+/-}$ or $CXCR4^{+/-}$), two-way repeated measures ANOVA tests were performed, with time (pre-, immediately post-, and 1-hour post-exercise) and phenotype ($CD31^{+/-}$ or $CXCR4^{+/-}$) as independent factors. The ingress and egress of T-cells are expressed as percentage change (%Δ), and the different levels of ingress and egress of T-cells, depending upon phenotype ($CD31^{+/-}$ or $CXCR4^{+/-}$) were compared using paired samples *t*-tests, with effect sizes reported for paired comparisons calculated using Cohen's *d* (Cohen, 1992). Circulating changes in SDF-1α were assessed using one-way repeated measures ANOVA. The relationship between changes in SDF-1α and T_{ANG} cells in response to exercise was evaluated using Pearson's coefficient correlation. When significant differences were detected, Bonferroni post-

hoc tests were performed to determine the differences. Where significant interactions were observed, Eta squared (η^2) values were calculated for effect size. Data was analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA). Significance was set at $p<0.05$.

3. Results

3.1 Blood volume changes with acute exercise

The acute exercise bout resulted in changes in haematocrit (43.2±0.05% vs. 44.2±0.05%, vs. 43.0±0.05%, pre-, post- and 1 hour post-exercise respectively, $p<0.05$ pre- vs. post-exercise), haemoglobin (14.5+0.18 $g \cdot dL^{-1}$ vs. 14.92±0.20 $g \cdot dL^{-1}$ vs. 14.48±0.19$g \cdot dL^{-1}$, pre-, post- and 1 hour post-exercise respectively, $p<0.05$ for pre- vs. post-exercise, post- vs. 1 hour post-exercise) suggestive of significant blood volume changes. As a result, blood volume was found to decline from pre- to post-exercise by 3.03±0.85%, and from pre- to 1 hour post-exercise blood volume decreased by 0.13±0.75%. The percentage changes in blood volume were applied to flow cytometric T-cell data to reflect for such changes.

3.2 T-cell response to acute strenuous exercise

Table 1 illustrates the summary data for total $CD3^+$ T-cells and $CD4^+$ and $CD8^+$ T-cell subsets to the 10km TT effort. The 10km TT bout resulted in a lymphocytosis, with total T-cells ($p < 0.05$), $CD3^+CD4^+$ ($p < 0.05$) and $CD3^+CD8^+$ T-cells ($p < 0.05$) exhibiting large elevations from baseline (135%, 76% and 134% increase from baseline respectively). Total T-cells ($p < 0.05$), $CD4^+$ ($p < 0.05$) and $CD8^+$ ($p < 0.05$) subsets subsequently fell below baseline levels 1-hour post-exercise.

3.3 $CD31^+$ T-cell responses to acute exercise

Fig. 2 shows that T_{ANG} cells demonstrated a large ingress from pre- to immediately post-exercise ($p < 0.05$). The circulating level of these T_{ANG} cells returned to baseline levels in the hour post-exercise period ($p < 0.05$). $CD4^+$ T_{ANG} and $CD8^+$ T_{ANG} cells both exhibited a large ingress as a result of the exercise bout and subsequent egress 1-hour post-exercise (Fig. 2). $CD8^+$ T_{ANG} cells demonstrated a greater ingress ($t_{(11)} = 3.397$, $p < 0.05$, $d = 0.44$) and egress ($t_{(11)} = 3.222$, $p < 0.05$, $d = 0.67$) in response to acute exercise than $CD4^+$ T_{ANG} cells ($F_{(2,22)} = 11.687$; $p < 0.05$, $\eta^2 = 0.44$ for time x phenotype interaction).

3.4 $CD31^+$ vs. $CD31^-$ T-cell responses to acute exercise

$CD31^+$ T-cells and $CD31^-$ T-cells display marked pro-angiogenic differences, with $CD31^+$ T-cells displaying a significantly greater ability to stimulate endothelial cell proliferation and tube formation, as well as greater vasculogenic gene expression (Hur *et al.*, 2007). We wanted to investigate if these cells displayed differences in their redistribution in response to exercise which may be associated with differences in CD31 expression. Table 2 demonstrates the response of $CD31^+$ T_{ANG} and $CD31^-$ T-cells to acute exercise. Total $CD31^+$ T_{ANG} demonstrated a similar response to acute exercise to $CD31^-$ T-cells ($F_{(2,22)} = 0.451$, $p > 0.05$ for time x phenotype interaction), with a large ingress immediately post-exercise, returning to baseline 1 hour post-exercise. However, acute exercise had a greater effect on redistribution of $CD4^+CD31^-$ T-cells compared to $CD4^+$ T_{ANG} cells ($F_{(2,22)} = 9.059$, $p < 0.05$, $\eta^2 = 0.03$ for time x phenotype interaction), and this was also the case for $CD8^+$ T-cell subsets ($CD31^-$ vs. $CD31^+$) ($F_{(2,22)} = 38.584$, $p < 0.05$ $\eta^2 = 0.11$ for time x phenotype interaction). Responsiveness of $CD31^+$ vs. $CD31^-$ T-cells and their ingress and egress from the peripheral blood compartment due to

acute exercise is shown in Fig. 3. The %Δ of cells from pre- to post-exercise was greater in $CD31^-$ T-cells compared to $CD31^+$ T-cells, but only for total $CD3^+$ ($t_{(11)}$ = 2.312, $p < 0.05$, d = 0.26) and $CD8^+$ ($t_{(11)}$ = 3.493, $p < 0.05$, d = 0.87) T-cell subsets. No such difference was observed for $CD4^+$ T-cells.

3.5 CXCR4 cell surface expression

T_{ANG} cells expressing CXCR4 increased in peripheral blood post-exercise compared to baseline levels ($p < 0.05$) (Fig. 2). As with total T_{ANG} cells, this increase was subsequently demonstrated by both $CD4^+$ ($p < 0.05$) and $CD8^+$ ($p < 0.05$) T_{ANG} cells expressing CXCR4.

Table 3 shows that CXCR4 cell surface expression was unaffected by the exercise bout on total T_{ANG} cells ($F_{(2,22)}$ = 2.724, $p > 0.05$) and $CD4^+$ T_{ANG} cells ($F_{(2,22)}$ = 1.860, $p > 0.05$). However, CXCR4 cell surface expression was significantly increased on $CD8^+$ subset of T_{ANG} cells from pre- to post-exercise ($F_{(2,22)}$ = 14.127, $p < 0.05$, η^2 = 0.72), with cell surface expression returning to baseline 1 hour post-exercise.

3.6 The role of SDF-1α: CXCR4 axis in $CD31^+$ T-cell response to exercise

Circulating SDF-1α, a ligand for CXCR4, demonstrated a significant response to the exercise bout ($F_{(2,22)}$ = 42.217, $p < 0.05$, η^2 = 0.79), with significant elevation post-exercise compared to resting levels (2583 ± 102 vs. 1970 ± 79 pg·mL^{-1}, $p < 0.05$); followed by a decrease in the circulation immediately post-exercise to 1-hour post-exercise (2162 ± 86 vs. 2583 ± 102 pg·mL^{-1}, $p < 0.05$).

To investigate if the SDF-1:CXCR4 axis may play a role in redistribution of T_{ANG} cells, we compared the movement in and out of the circulation of $CXCR4^+$ T_{ANG} cells with $CXCR4^-$

T_{ANG} cells. Fig. 4 demonstrates that $CXCR4^+$ T_{ANG} cells demonstrated a greater level of ingress ($t_{(11)}$ = 3.467, $p < 0.05$, d = 1.36) and subsequent egress in response to the 10k TT exercise bout ($t_{(11)}$ = 2.867, $p < 0.05$, d = 0.29) compared to $CXCR4^-$ T_{ANG} cells. This was observed for total T_{ANG} cells, as well as $CD4^+$ (ingress: $t_{(11)}$ = 3.981, $p < 0.05$, d = 1.45; egress: $t_{(11)}$ = 7.320, $p < 0.05$, d = 1.24) and $CD8^+$ (ingress: $t_{(11)}$ = 2.998, $p < 0.05$, d = 0.84; egress: $t_{(11)}$ 1.612, $p < 0.05$, d = 0.40) subsets.

However, there was no significant relationship between %Δ SDF-1α and %Δ in $CXCR4^+$ T_{ANG} (total, $CD4^+$ or $CD8^+$).

4. Discussion

The main finding of this study is that T_{ANG} cells increase in the peripheral circulation in response to an acute bout of exercise, and subsequently leave the circulation during the early stages of exercise recovery, mirroring the classic biphasic response of T-cells to exercise (Simpson *et al.*, 2006; Turner *et al.*, 2010; Ingram *et al.*, 2015). This is in contrast to the study by Lansford *et al.* (2016) found no changes in T_{ANG} cells in response to acute exercise. However this study did not report circulating number changes, but proportional changes (% of $CD3^+$ T-cells). The data presented in our study also suggests that $CD8^+$ T_{ANG} cells are more responsive to exercise than $CD4^+$ T_{ANG} cells. However, $CD4^+CD31^-$ and $CD8^+CD31^-$ T-cells demonstrated a greater ingress and subsequent egress from the circulation compared to their $CD31^+$ counterparts. Past research has shown preferential redistribution of T-cells expressing adhesion molecules, such as integrins and CD56, in response to acute bouts of exercise (Goebel *et al.*, 2000; Shephard, 2003; Simpson *et al.*, 2006). However, the data presented in this study suggests that this is not the case for $CD4^+$ and $CD8^+$ T-cells expressing the adhesion molecule

CD31. The 10km TT protocol used for this study also resulted in a significant increase of $CXCR4^+$ T_{ANG} cells in the peripheral blood compartment.

Acute exercise is well known to stimulate the redistribution of T-cells into the circulation in humans (Simpson *et al.*, 2007; Turner *et al.*, 2010; Witard *et al.*, 2012). Most studies previously have utilised a controlled exercise bout (e.g. 60-70% $\dot{V}O_2$peak) whereas our study reported the changes in T-cells in response to a self-regulated exercise bout, a 10km time trial.. There also appears to be a preferential ingress of highly differentiated T-cells, and these cells also egress from the circulation to a greater extent than less differentiated naïve T-cells (Simpson *et al.*, 2007; Spielmann *et al.*, 2014) which may represent a greater immunosurveillance, with more cytotoxic, effector-phenotype T-cells being redistributed. $CD31^+$ T-cells are a subset of T-cells which have been previously shown to have vasculogenic properties such as being able to secrete significant quantities of pro-angiogenic growth factors VEGF and IL-8 (Hur *et al.*, 2007), and the *in vitro* function of these cells was demonstrated to be associated with endothelial function (Weil *et al.*, 2011). In addition, several studies have demonstrated an association between $CD31^+$ T-cell number and/or function and cardiovascular health outcomes (Hur *et al.*, 2007; Rouhl *et al.*, 2012). Our data suggest that exercise offers potential benefit to the vascular system through the large redistribution in T_{ANG} cells and CXCR4 cell surface expression post-exercise observed in this study. This redistribution may cause T_{ANG} cells to migrate to ischaemic tissue to stimulate vasculogenesis and endothelial cell proliferation. Additionally, increasing circulating T_{ANG} cell number in the peripheral circulation may assist in endothelial repair by increasing access of these T_{ANG} cells to areas of endothelial damage.

$CD8^+$ T_{ANG} cells demonstrated a greater redistribution due to the acute exercise bout than $CD4^+$ T_{ANG} cells. However, $CD8^+$ T_{ANG} cells showed a lesser ingress and egress (%Δ) than $CD8^+CD31^-$ cells. $CD4^+$ and $CD8^+$ T-cells expressing cell surface markers indicative of senescence and highly differentiated status, ingress and egress to a greater extent than less

differentiated and naïve T-cells (Simpson *et al.*, 2007; Campbell *et al.*, 2009; Turner *et al.*, 2010). Unfortunately, the cell surface marker panel used within this study did not contain cell surface markers for such differentiation status. The exact phenotypic definition of these T_{ANG} cells is yet to be elucidated, but evidence suggests that CD31 expression is lost upon T-cell activation (Zehnder *et al.*, 1992) and differentiation (Demeure *et al.*, 1996), suggesting that T-cells lacking CD31 are likely to be activated or highly differentiated T-cell subgroup, thus offering a potential explanation as to why T-cells lacking CD31 expression preferentially mobilise to acute exercise.

T-cell redistribution in response to exercise has been reported to be catecholamine-dependent (Schedlowski *et al.*, 1996; Simpson *et al.*, 2007; Dimitrov *et al.*, 2010; Witard *et al.*, 2012). However, exercise-induced increases in circulating SDF-1α may stimulate T-cells expressing CXCR4 to migrate into the circulation. SDF-1α is known to mobilise $CXCR4^+$ progenitor cells into the circulation (Powell *et al.*, 2005; Prokoph *et al.*, 2012), but no study to date has investigated if SDF-1α may be partly responsible for the T-cell ingress observed during exercise. In the current study, SDF-1α was observed to increase in the circulation of the participants in response to the exercise bout, and this was accompanied by preferential ingress of $CXCR4^+$ T_{ANG} cells compared to $CXCR4^-$ T_{ANG} cells. However, we failed to observe a significant relationship between SDF-1α release and $CXCR4^+$ T_{ANG} ingress into the circulation, suggesting that although $CXCR4^+$ T_{ANG} cells are redistributed to a greater extent than $CXCR4^-$ cells, there are likely to be other factors at play.

To the author's knowledge, this was the first study to demonstrate an increase in T-cell CXCR4 cell surface expression as a result of an acute bout of exercise. CXCR4 cell surface receptor expression was increased on $CD8^+$ T_{ANG} cells only, and this was not detected on $CD4^+$ T_{ANG} or total T_{ANG} cells. The reason for this selective increase in CXCR4 cell surface expression on

$CD8^+$ T-cells is not known, but this may be a potential mechanism by which we see greater ingress of $CD8^+$ T-cells into the circulation in response to exercise. Post-exercise concentrations of cortisol have been found to upregulate CXCR4 expression on T-cells *in vitro* (Okutsu *et al.*, 2005), but we believe that this is the first study to detect such increased CXCR4 expression *in vivo*. CXCR4 is implicated in migratory function of various cell lineages, such as $CD34^+$ progenitor cells and other various circulating angiogenic cells (CACs), such as endothelial progenitor cells (Adams *et al.*, 2013a; Sun *et al.*, 2013; Noels *et al.*, 2014). Migratory capacity of T_{ANG} cells has been shown to be positively associated with endothelial function (Weil *et al.*, 2011), and thus exercise, by upregulating CXCR4 expression, may promote endothelial function through a previously undiscovered mechanism. However, careful interpretation is advised, as acute exercise may simply stimulate the ingress of T-cells with an already heightened CXCR4 expression, as indicated by increased ingress of $CXCR4^+$ T_{ANG} cells compared to $CXCR4^-$ T_{ANG} cells, rather than any changes occurring at the individual cell level.

Limitations

There are several limitations which require mentioning. We did not measure circulating cortisol which may be a mechanism in any potential upregulation of CXCR4 cell surface expression on $CD31^+$ T-cells. In addition, major contributors to T-cell redistribution, epinephrine and nor-epinephrine, were not measured in plasma samples, however the mechanism of epinephrine-induced T-cell redistribution are well documented (Dimitrov *et al.*, 2010). Functional changes in these cells, such as migration and pro-angiogenic cytokine secretion, in response to acute exercise were not addressed and should be in future studies to determine the vasculogenic

potential of these angiogenic cells in response to exercise to understand the potential benefits of this exercise response on the vascular system.

There are distinct gender differences in as T-cell changes with acute exercise (Brown *et al.*, 2014). Our study did not include females, and so further research is warranted to investigate if there are any gender differences in T_{ANG} cell changes with acute bouts of exercise.

Additionally, the exercise bout utilised was of a self-regulated pace, a 10km time trial. There is likely to be an intensity-dependent effect with T-cell redistribution, and thus T_{ANG} cells also. Although our participants were well accustomed to a 10km running exercise bout, the variation in performance may result in a variation in the ingress and egress of these T-cells of interest. However, the applicability of a 10km running study has contextual impact, with our results suggesting a 10km running bout will significantly increase the number of T_{ANG} cells in the circulation, with potential for vasculogenic effects.

Conclusion

In summary, we have shown for the first time that one bout of strenuous exercise was effective in acutely increasing the circulating number of T_{ANG} cells, which are thought to contribute to endothelial homeostasis by contributing to endothelial repair mechanisms. This was primarily due to increase in numbers of $CD8^+$ T-cells expressing CD31, with a lesser increase in numbers of $CD4^+$ T-cells expressing CD31. In addition, the SDF-1α:CXCR4 axis may play a small role in stimulating the movement of T-cells into the circulation in response to exercise. These results demonstrate that exercise may contribute to improving endothelial health by acutely redistributing $CD31^+$ angiogenic T-cells (T_{ANG}) and increasing CXCR4 cell surface expression. Future research should be aimed at fully investigating the cell surface antigen profile of T_{ANG} cells, which may elucidate some of the findings within this study. In addition, further studies

investigating acute exercise and T_{ANG} cells should aim to investigate migratory function of these cells to assess if CXCR4 upregulation on T_{ANG} cells results in a functional improvement of these cells.

Additional Information:

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions:

M.R conceived and designed the research; M.R and P.T performed the experiments; M.R analysed data; M.R, R.S, L.A, E.M and G.F.J interpreted results of the experiments; M.R prepared figures. M.R drafted manuscript; all authors edited and revised the manuscript; all authors approved the final version of the manuscript.

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Figure Legends:

Figure 1. Flow cytometric quantification of $CD31^+$ T_{ANG} cells. Side scatter vs. forward scatter for identification of lymphocyte gate (A), $CD3^+$ gating for identification of T-cells (B), identification of $CD4^+$ (C) or $CD8^+$ (D) T-cells followed by identification of $CD31^+$ and $CD31^-$ subsets (E).

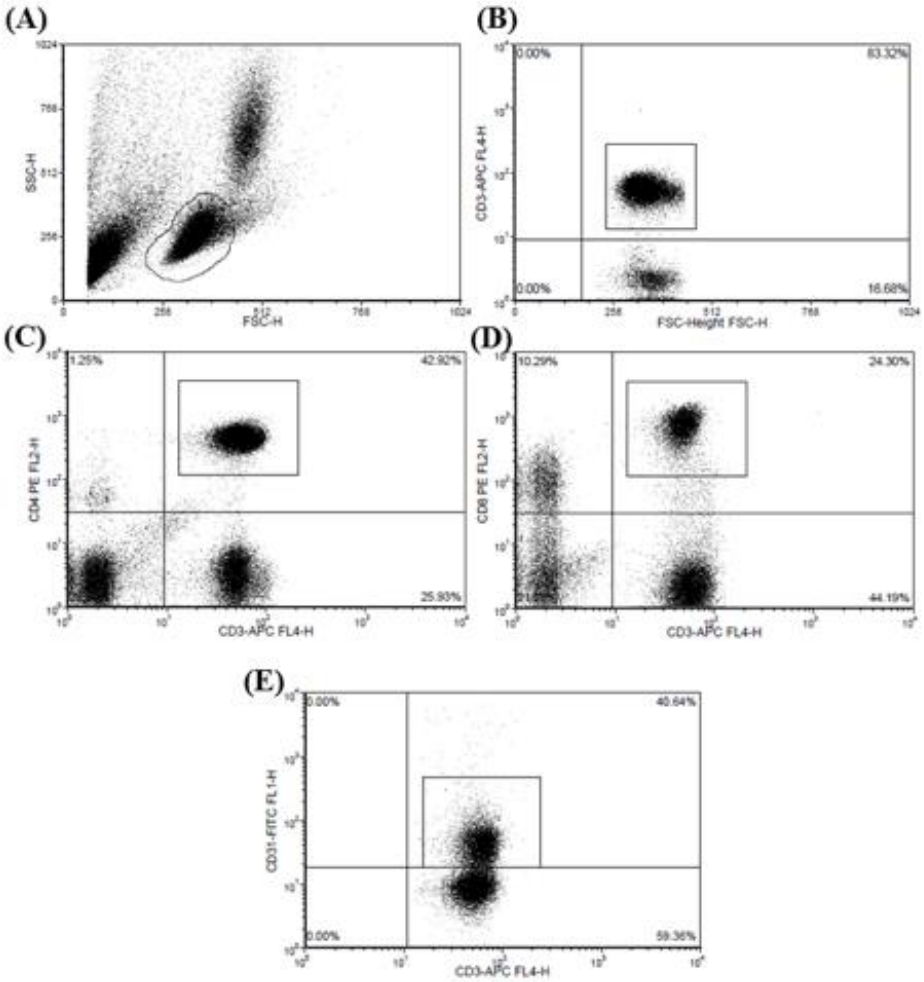


Figure 2. The effect of acute exercise on ingress (A) and egress (B) of T_{ANG} cells and subsets within the peripheral blood compartment. Values are %Δ ± SEM from (A) pre- to immediately post-exercise and (B) post- to 1-hour post-exercise. Statistical difference between phenotypes indicated by * $p < 0.05$.

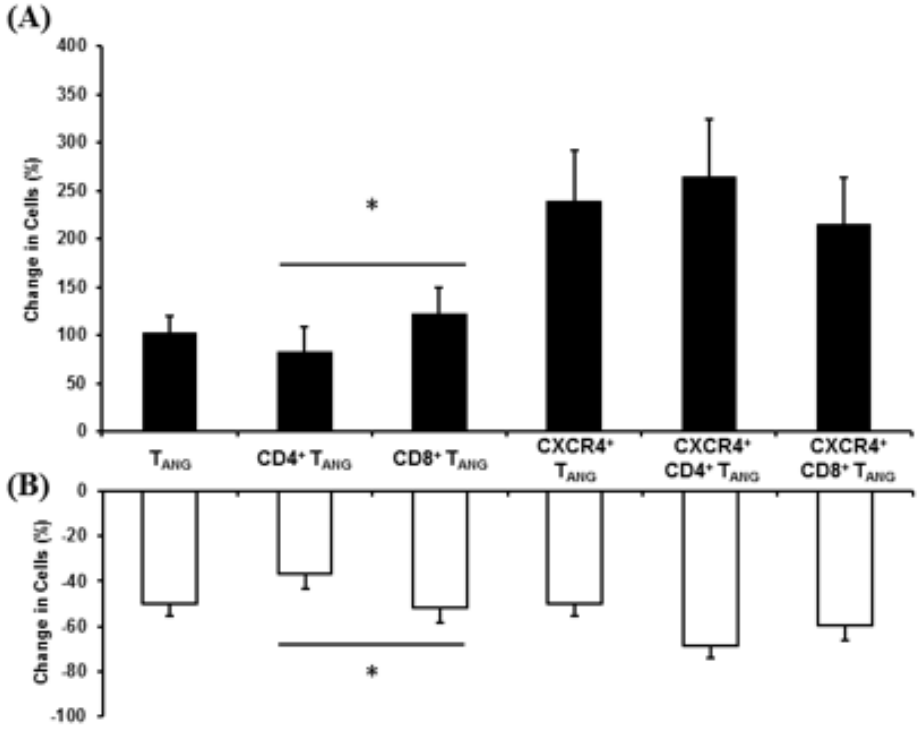


Figure 3. Percentage changes in $CD31^+$ and $CD31^-$ T-cells (A: ingress into the circulation; B: egress from circulation) with acute exercise. Values are %Δ ± SEM from (A) pre- to post-exercise and (B) post- to 1-hour post-exercise. Statistical difference between phenotypes indicated by * $p < 0.05$.

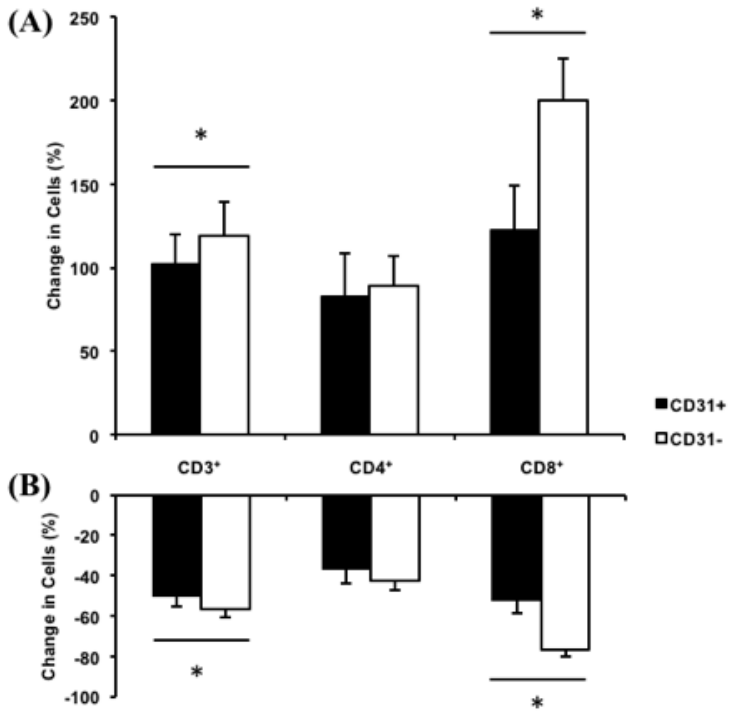
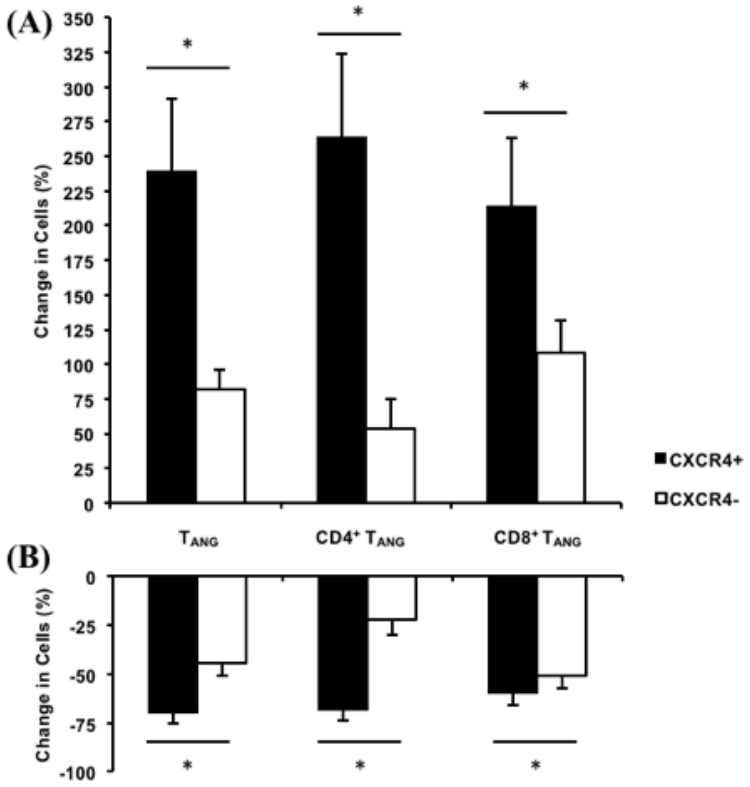


Figure 4. Percentage changes in $CXCR4^+$ and $CXCR4^-$ $CD31^+$ T cells (A: ingress into the circulation; B: egress from circulation) with acute exercise. Values are %Δ ± SEM from (A) pre- to post-exercise and (B) post- to 1-hour post-exercise. Statistical difference between phenotypes indicated by * *p<0.05.*



Tables

Table 1

Total cell number of peripheral blood T-cells and subsets in response to 10km TT bout (mean ± SEM)

Cell subset	Pre (cells·μl^{-1})	Post (cells·μl^{-1})	1hr Post (cells·μl^{-1})	Main effects of exercise
CD3^{+} T-cells	1377 ± 78	3235 ± 342	1121 ± 91	$F_{(2,22)} = 46.299$; $p = 0.000$, $\eta^2 = 0.81$
CD4^{+} T-cells	560 ± 51	987 ± 116	553 ± 051	$F_{(2,22)} = 23.764$; $p = 0.000$, $\eta^2 = 0.68$
CD8^{+} T-cells	307 ± 34	717 ± 90	265 ± 032	$F_{(2,22)} = 36.976$; $p = 0.000$, $\eta^2 = 0.77$

Table 2

Circulating $CD31^+$ and $CD31^-$ T-cell response to 10km TT bout (mean ± SEM)

Cell subset	Pre ($cells \cdot \mu l^{-1}$)	Post ($cells \cdot \mu l^{-1}$)	1hr Post ($cells \cdot \mu l^{-1}$)	Main Effect of Time	Time x Phenotype Interaction
$CD3^+CD31^+$	514 ± 45	1040 ± 128*γ	464 ± 39	$F_{(2,22)} = 29.232$; $p = 0.000$, $\eta^2 = 0.74$	$F_{(2,22)} = 0.451$; $p = 0.520$, $\eta^2 = 0.001$
$CD3^+CD31^-$	477 ± 51	1040 ± 17*γ	441 ± 44γ	$F_{(2,22)} = 19.515$; $p = 0.001$, $\eta^2 = 0.66$	
$CD3^+CD4^+CD31^+$	224 ± 23	375 ± 47*γ	217 ± 21	$F_{(2,22)} = 21.580$; $p = 0.000$, $\eta^2 = 0.67$	$F_{(2,22)} = 9.059$; $p = 0.010$, $\eta^2 = 0.03$
$CD3^+CD4^+CD31^-$	336 ± 34	612 ± 79*γ	337 ± 36	$F_{(2,22)} = 21.126$; $p = 0.001$, $\eta^2 = 0.68$	
$CD3^+CD8^+CD31^+$	236 ± 27	505 ± 64*γ	218 ± 26	$F_{(2,22)} = 27.632$; $p = 0.000$, $\eta^2 = 0.74$	$F_{(2,22)} = 38.584$; $p = 0.000$, $\eta^2 = 0.11$
$CD3^+CD8^+CD31^-$	71 ± 8	213 ± 32*γ	47 ± 7*	$F_{(2,22)} = 32.461$; $p = 0.000$, $\eta^2 = 0.79$	

**p<0.05 vs. pre-exercise, γ p<0.05 vs. 1hr post-exercise.*

Table 3

CXCR4 cell surface expression (mean fluorescence intensity) on $CD31^+$ T-cells and subsets in response to 10km TT bout (mean ± SEM)

	Pre (AU)	Post (AU)	1 hr Post (AU)	Main Effects of Exercise
$CD3^+CD31^+$ (T_{ANG})	11.78 ± 0.44	12.25 ± 0.51	11.97 ± 0.60	$F_{(2,22)} = 2.724$; $p = 0.088$, $\eta^2 = 0.20$
$CD4^+$ T_{ANG}	12.80 ± 0.50	13.08 ± 0.46	12.70 ± 0.51	$F_{(2,22)} = 1.860$: $p = 0.179$, $\eta^2 = 0.15$
$CD8^+$ T_{ANG}	10.21 ± 0.83	10.99 ± 0.93	10.18 ± 0.86	$F_{(2,22)} = 14.127$; $p = 0.000$, $\eta^2 = 0.59$