Research Article

Preferential Mobilization and Egress of Type 1 and Type 3 Innate Lymphocytes in Response to Exercise and Hypoxia

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Abstract

The study examined the effect of exercise and hypoxia on the mobilization and egress of innate lymphocytes (ILCs) and adaptive T cell populations in the blood. The ILCs have emerged as a critical population of cells in immune regulation at mucosal surfaces in animals and humans. Eleven healthy male subjects performed (i) 45 min of exercise at 50% $\dot{V}O_2$ peak on a cycle ergometer under normoxia and (ii) hypoxia, or (iii) while resting in hypoxia. Blood samples were obtained pre-exercise, immediately post-exercise and 60 min post-exercise and were analyzed by flow cytometry to examine the type 1 and type 3 ILCs and CD4^{+} and CD8^{+} naive and memory cell populations. There was a significant increase in the number of type 1 (NK cells) and type 3 ILC22 cells in the blood in response to exercise under normal oxygen conditions followed by a significant egress of these cells following the cessation of exercise. Exercise performed under hypoxic conditions abrogated the mobilization response of NK cells and ILC22 cells. Type 3 LTi cells were mobilized into the blood only under hypoxic rest conditions. No significant changes were observed when we analysed total CD4^{+} and CD8^{+}T cell populations or the naive and memory subsets. This study highlights that distinct innate populations are mobilised under different environmental conditions and types of stress.

Keywords: Hypoxemia; Normoxia; Inflammation; Innate lymphocytes; Cycling

Introduction

The immune system comprises two inextricably linked innate and adaptive immune responses. The innate response provides the first line of protection involving physical barriers and a nonspecific cellular defence [1]. The adaptive immune system is composed of T and B lymphocytes that express clonally restricted antigen specific receptors that can differentiate into either short lived effector cells or memory cells to induce long lived immunological memory in response to antigen recognition [2]. T cells, B cells and natural killer (NK) cells are all lymphocytes and have previously been examined in the context of exercise. The adaptive immune response is mediated by CD4^{+} T cells which provide help to antigen-specific B cells in the form of cognate signals and soluble factors via the secretion of cytokines. In contrast, CD8^{+} T cells are required to kill virus infected cells. Several groups have identified preferential mobilisation of memory T cells into the blood in response to acute exercise, with a corresponding reduction in naive T cells [3-7]. However, these changes tend to be transient and cell populations return to baseline levels within a few hours after the cessation of exercise.

Innate lymphocytes cells (ILCs) are composed of three distinct subsets based on the expression of unique patterns of cell surface markers, lineage specific transcription factors and secretion of effector cytokines [8,9]. ILCs lack expression of antigen-specific receptors which are a hallmark of T cell populations. NK cells were the first innate lymphocyte to be characterised in the 1980s and has now been categorised as a Type 1 ILC that can be further subdivided into two distinct effector populations. The NK cell population is very responsive to an acute bout of exercise, with CD56dim subsets demonstrating greater mobilisation into the blood compared to the CD56 bright populations [10]. Type 2 ILCs (ILC2=nuocytes or natural helper cells) secrete interleukin (IL) IL-5 and IL-13 which is required for the secretion of amphiregulin which in turn promotes repair and regeneration of epithelial cells [11-13]. Type 3 ILCs (ILC22 and LTi cells) secrete IL-22 that plays an important role in epithelial cell repair, and can promote secretion of antimicrobial peptides such as β-defensin and Lipcalin-2 at mucosal epithelia [14-16]. A subset of type 3 ILCs secrete IL-17 a proinflammatory cytokine that promotes neutrophil recruitment, the secretion of antimicrobial peptides [17] and induce secretory IgA across intestinal epithelium [18,19]. Thus ILCs have emerged as crucial regulators of mucosal immunity with a specific role to regulate immune responses to the natural microbiota; which has now been identified as playing a key role in a number of disorders including insulin resistance [20]. The response of type 3 ILCs to exercise has not previously been investigated. Emerging evidence indicates that ILCs also influence the nature of the adaptive immune response in health and disease [9,21,22].

Immune cells and their progenitors are known to be exposed to variations in oxygen tensions as they develop, differentiate, proliferate and migrate throughout the blood, lymphoid organs, and various tissues [23]. A hypoxic extracellular environment is common at sites of

chronic inflammation and malignant diseases [24], and can occur transiently within normal tissues during strong immune responses [25]. Lymphocytes sense hypoxia through oxygen sensor relays by inducing transcription factors such as Hypoxia inducible factor-1α (HIF-1α) to regulate the cellular adaptation in response to low O_2 stress [26,27]. For decades, endurance athletes have undertaken a period of altitude training in an attempt to enhance their sporting performance during sea-level competitions by physiological conditioning. In addition a number of studies have identified hypoxic exercise can potentially improve the clinical outcomes of certain diseases which might or might not be related to changes to immune function [28-30].

The aim of the current study was to investigate the mobilization of specific lymphocyte populations in response to exercise as determined by their increased frequency in the blood in response to exercise. We examined the response of type 1 ($CD56^+$ NK cells) and type 3 ILCs (LTi and ILC22), as well as the naive and memory cell populations for $CD4^+$ and $CD8^+$ T cells. In this study we have focused on type 1 and type 3 ILCs as they appear to be the major innate cell populations recruited to microbial responses against bacteria and viruses, while Type2 ILCs seem to be restricted to parasite antigens [13]. Given the important role of ILCs in protection and repair of mucosal surfaces coupled with the previously reported effects of exercise on mucosal immunity [31], we hypothesized that moderate intensity exercise would mobilise both subsets of ILCs investigated into the blood; while T-cells would differentially respond to exercise, with an egress of naive cells from the blood observed as a decreased frequency of these cells with time and exercise. The second aim of the study was to investigate the effect of hypoxia on the mobilisation and egress of immune cells identified above. Given the important role of ILCs in mucosal immunity, we hypothesised that hypoxia would enhance the exercise-induced mobilization of ILCs into the blood.

Methods

Participants

Twelve young adult (18-40 years) lean (BMI<25), recreationally active (≥ 3 planned exercise training sessions per week) males were recruited from the local community through study flyers posted on public bulletin boards. Participants were excluded if they: previously experienced intolerance to oxygen insufficiency; were pregnant; taking medication for chronic illness or disease; smoked cigarettes; had a musculoskeletal injury or other health issues that significantly impaired their ability to perform exercise as assessed on stage one of the Exercise and Sports Science Australia (ESSA) pre-exercise screening questionnaire. Participants did not consume dietary supplements known to affect immune function and remained free from respiratory infection during the study period. All participants provided informed written consent to participate. The study received ethics approval from the Murdoch University Human Research Ethics Committee.

Experimental design

Each participant completed a familiarization and initial testing session followed by three experimental conditions (counterbalanced experimental design). The experimental conditions consisted of (i) resting hypoxia (HYPREST); (ii) cycling exercise under normoxia (NORMEX), and (iii) cycling exercise under hypoxia (HYPEX). The order of conditions was counterbalanced using a Latin-square approach. Participants were then randomly assigned to each sequence following a computer-generated random number list. The three experimental conditions were spaced at least 72 hours and participants were instructed to refrain from physical activity and alcohol for the 24 hours prior to each experimental condition. All participants were instructed to maintain consistent diets that closely mimicked their daily life on the day preceding each experimental condition and this was monitored by use of a 24 hour diet recall sheet. There was no caffeine intake in the 18 hours prior to the experimental conditions.

Familiarization and initial testing session

Anthropometric measurements (height and weight) were recorded for each participant prior to completion of the $\dot{V}O_2$ peak test. The $\dot{V}O_2$ peak test was performed on a cycle ergometer (Wattbike Pro) in normoxia (20.94% O_2) using a starting workload of 70 Watts which was increased 30 Watts every 3 minutes, followed by 30 Watt increments every minute when respiratory exchange ratio (RER) value exceeded 1.0. Heart rate (Polar A3 T31-coded HR monitor, Finland) and expired air (ParvoMedics TrueOne 2400 metabolic cart, UT, USA) were continuously monitored throughout the test. The $\dot{V}O_2$ peak ($ml \bullet kg^{-1} \bullet min^{-1}$) was determined as the highest oxygen uptake reading during a 15 sec epoch.

Experimental conditions

Participants arrived (0630-1030 h) at the laboratory following an overnight fast (> 8 hours). A blood sample (> 6 ml) was collected using an 8 ml Vacutainer cell preparation tube with sodium heparin (BD, Franklin Lakes, NJ, USA) via venupuncture from the antecubital vein while participants remained in a seated position. The exercise conditions (NORMEX and HYPEX) then consisted of cycling (Wattbike Pro) for 45 min at a workload corresponding with the power output at 50% $\dot{V}O_2$ peak. Heart rate, rating of perceived exertion (RPE; Borg scale 6-20) and SpO$_2$ were collected throughout each condition. Blood was sampled immediately after the 45 min exercise bout and then 60 min post-exercise via venupuncture as described above. During the hypoxia conditions (HYPREST and HYPEX), hypoxic air was delivered via tubing connected to a face mask using a hypoxicator (High performance hypoxicator, *GO$_2$ Altitude*, Biomedtech, Australia). The automatic biofeedback mode on the hypoxicator was used to maintain the SpO$_2$ at 85% using the in-built pulse oximeter. This setting enabled the systemic hypoxic-stress to be consistent between the HYPREST and HYPEX conditions. Participants wore a pulse oximeter (Onyx Model 9500, Nonin Medical, Plymouth, USA) on the index finger of the contralateral hand to confirm SpO$_2$ values. During the HYPREST condition, participants were asked to sit quietly on a chair for 45 minutes while breathing hypoxic air from the hypoxicator; all other procedures during the HYPREST condition were identical to those described for HYPEX and NORMEX. An indoor climate monitor (Davisi Perception II station, Hayward, CA) was used to record the temperature (22.3 ± 2.3^{0}C) and pressure (760.4 ± 3.8) in the laboratory.

Blood processing

Blood lactate (*Lactate Plus*, *Nova Biomedical*, Waltham, MA) and glucose (Accu-Chek Go, Roche, Australia, Perth) concentrations were analysed at the time that venous blood samples were collected. A 50 µL liquot of blood was tested immediately before exercise (Pre),

immediately post exercise (Post) and after subjects had recovered for 1 h post expersie (Rec).

Blood for immuno-staining and flow cytometry was processed within 2 h of collection. The PBMCs were collected by centrifugation for 30 minutes at 1500 Relative Centrifugal Force (rcf) at room temperature. The isolated PBMCs were then washed once by re-suspending with ice cold PBS containing 0.5% BSA.

The peripheral blood mononuclear cell (PBMC) layer was isolated on Histopaque followed by centrifugation. The PBMC layer was removed and washed with PBS+0.5% FCS (i.e., FACS wash) twice. PBMCs were stained using two different antibody cocktail mixtures containing 1.0×10^6 PBMCs per tube.

Panel 1 was used to analyze type 1 ILCs (Lin- CD56$^+$ NK Cells) (Figure 1a) and type 3 ILCs (LTi cells: Lin- CD56$^-$ CD117$^+$, CD127$^+$, NKp44$^-$ NKp46$^-$; ILC-22 cells: CD56$^+$ CD117$^+$, CD127$^+$, NKp44$^+$ NKp46$^+$) (Figure 1b and Table 1). Lineage positive markers (CD3, CD19, CD14, CD11c, TCRαβ, TCRγδ–all PE conjugated antibodies) were used to gate out T and B lymphocytes primarily, while type 1 were characterized by the following markers. Antibodies used to analyse ILC populations included CD56-PE-Cy7, CD127-FITC, CD117-Prcp Cy5.5, NKp44 (AlexaFluor647) and NKp46 (V450).

	Phenotype	Cell type	ILC Classification	Signature cytokines	Function	reference
Innate Lymphocytes	Lin-[a] CD56lo	NK cells	Type 1 ILCs (ILC1)	IFN-γ	Innate immunity against viral infections, tumour immunosurveillance	[8]
	Lin- CD117+/- CD127+ CD25+ ST2+, CRTH2+	ILC2	Type 2 ILCs (ILC2)	IL-5 and IL-13	Innate immunity against extracellular parasites and allergy	[11]
	Lin-CD56- CD117- CD127- NKp44+ NKp46+	Lymphoid tissue inducer (LTi) cells	LTi cells - a member of the type 3 ILC population	IL-17, IL-22	Mucosal immunity, ILF formation and T-independent IgA, tissue remodelling	[15,19]
	Lin - CD56+ NKp44+ NKp46+ CD117+, CD127+	ILC3 cells	ILC22 cells - a member of the type 3 ILC population	IL-17, IL-22	Epithelial homeostasis, intestinal immunity	[9,14,18,21]
Adaptive Lymphocytes	CD4+ CD45RA+ CD62L+	CD4+ naive T cells	NA		Antigen inexperienced Th cells	[36]
	CD4+ CD45RA- CD62L+/-	CD4+ memory T cells	NA		Antigen experienced Th cells	[36]
	CD8+ CD45RA+ CD62L+	CD8+ naive T cells	NA		Antigen inexperienced cytotoxic (Tc) cells	[36]
	CD8+ CD45RA- CD62L+/-	CD8+ memory T cells	NA		Antigen experienced cytotoxic (Tc) cells	[36]

Table 1: Properties of innate and adaptive lymphocyte cell populations ; [a]Lineage negative (Lin-) cells are selected for the absence of expression of lineage specific cell surface markers: e.g. CD3$^+$ CD4$^+$, CD8$^+$, CD14$^+$, TCRαβ$^+$ TCRγδ$^+$, NK T cells and CD19$^+$ B cells; NA=not applicable.

Panel 2 was used to analyze CD4$^+$ and CD8$^+$ naive (CD45RA$^+$, CD62L$^+$) and memory (CD62L$^{+/-}$ CD45RA$^-$) cells, where memory cells were further characterized as effector (CD62L- CCR7lo) or central (CD62L$^+$ CCR7hi) memory cell populations (Figure 1c). Panel 2 included the following markers: CD4APC-H7, CD8 PrcpCy5.5, CD45RA-PE, CD62L (V450), CCR 7-FITC. All antibodies were purchased from BD Pharmingen (San Jose, CA).

Cells were stained in the dark for 30 minutes, washed and transferred to polystyrene tubes and were stored in FACS buffer in the dark prior to flow cytometric analysis. Cells were analyzed on a FACS Canto II cell analyzer (Becton Dickinson Bioscience, San Jose, CA, USA). Data acquisition was performed with FACS DiVa v 6.1.2 software (BD, San Jose, CA, USA). For panel 1, approximately 300,000 to 1,000,000 events were recorded and at least 50,000 events were

recorded for panel. The post-acquisition analysis of the flow cytometry data was performed using the Flowjo analysis program v7.6.1 (Tree Star, Ashland, OR, USA) by transferring FCS files from FACS DiVa. Positive staining for cell surface markers was based on the use of FMO controls. The frequency and absolute cell numbers were calculated for individual samples for each cell population.

Data analysis

All data are expressed as mean ± Standard Deviation (SD), unless otherwise stated. All statistical calculations were performed using SPSS (statistical package for social sciences, version 21.0; IBM Corp, Armonk, NY). Individual mean values for physiological parameters (HR, RPE and SpO2) and performance (workload) were compared using a repeated measures (condition) one-way analysis of variance

(ANOVA). A Post hoc multiple comparisons test was used to contrast responses where significant interactions were obtained. All other data were compared using a 3 × 3 (Condition × Time) repeated measures ANOVA with post hoc pair-wise comparisons test being conducted to contrast changes between the main effects of condition (HYPREST, NORMEX and HYPEX) and time (pre, immediately-post and 60 min post exercise). A p value of less than 0.05 was considered as significant.
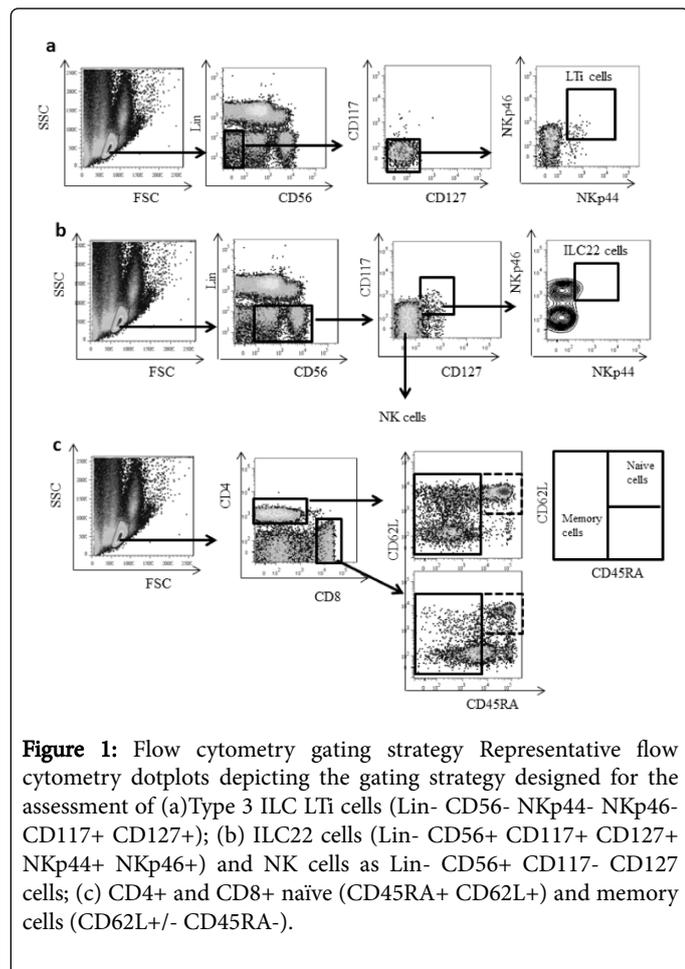

Figure 1: Flow cytometry gating strategy Representative flow cytometry dotplots depicting the gating strategy designed for the assessment of (a)Type 3 ILC LTi cells (Lin- CD56- NKp44- NKp46- CD117+ CD127+); (b) ILC22 cells (Lin- CD56+ CD117+ CD127+ NKp44+ NKp46+) and NK cells as Lin- CD56+ CD117- CD127 cells; (c) CD4+ and CD8+ naïve (CD45RA+ CD62L+) and memory cells (CD62L+/- CD45RA-).

Results

Twelve participants met the inclusion criteria, attended the familiarization session and enrolled into the study; however only eleven completed all trials and their data was used for all subsequent analysis. The participants were young (23.6 ± 4.9 y.o.), lean (BMI: 21.1 ± 2 .9 kg•m^{-2}) and recreationally active ($\dot{V}$O$_2$ peak: 46.4 ± 6.1 ml•min-1•kg^{-1}). Barometric pressure (HYPEX: 760.5 ± 4.5 mmHg; NORMEX: 760.6 ± 3.6 mmHg; HYPREST: 759.7 ± 3.3 mmHg; p=0.772), temperature (HYPEX: 22.7 ± 1.2°C; NORMEX: 22.0 ± 1.3°C; HYPREST: 22.2 ± 1.2°C; p=0.379) and humidity (HYPEX: 55 ± 6%; NORMEX: 59 ± 12%; HYPREST: 64 ± 11%; p=0.149) were not significantly different between conditions.

Workload (HYPEX *vs.* NORMEX: p=0.779 [95% CI for difference: -0.611–0.793]) and SpO2 (HYPEX *vs.* HYPREST: p=0.764 [95% CI for difference: -0.963–1.272]) were well matched between respective conditions (Table 2). Heart rate, RPE and lactate increased in response to exercise, with the greatest increase occurring with HYPEX (All

P<0.001; Figure 2a). There were no differences in blood glucose concentration (Figure 2b).

	HYPREST	NORMEX	HYPEX
Average Power (W)	-	104.3 ± 27.1	104.2 ± 27.3
Target Power (W)	-	103.5 ± 27.9	103.5 ± 27.9
SpO2 (%)	86.8 ± 1.3	97.6 ± 0.7a	86.9 ± 1.0b
O2 inspired1 (%)	13.3 ± 0.5	-	14.6 ± 0.8a
HR (bpm)	71 ± 9	133.3 ± 13.1a	148.3 ± 13.3 a,b
%HRmax	36.2 ± 4.7	67.9 ± 6.6a	75.6 ± 6.9 a,b
RPE	6.1 ± 0.3	10.9 ± 1.9a	12.2 ± 2.0a,b

Table 2: Physiological responses and workload measures recorded during respective conditions; 1O2 inspired data recorded from Hypoxicator; %HRmax calculated using formula from Tanaka et al (2001) [32]. The presence of a superscript 'a' is an indication of a significant difference in comparison to HYPREST, 'b' is an indication of a significant difference in comparison to NORMEX.
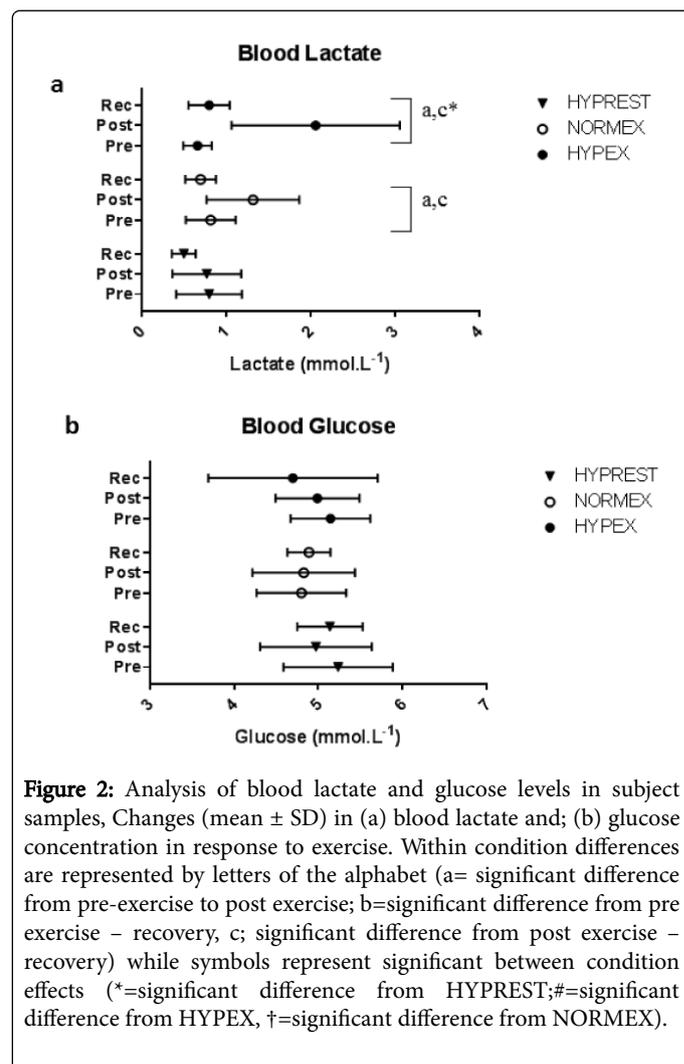

Figure 2: Analysis of blood lactate and glucose levels in subject samples, Changes (mean ± SD) in (a) blood lactate and; (b) glucose concentration in response to exercise. Within condition differences are represented by letters of the alphabet (a= significant difference from pre-exercise to post exercise; b=significant difference from pre exercise – recovery, c; significant difference from post exercise – recovery) while symbols represent significant between condition effects (*=significant difference from HYPREST;#=significant difference from HYPEX, †=significant difference from NORMEX).

Changes in NK cells and ILC populations in response to exercise

There was a significant increase in the proportion and absolute number of NK and ILC22 cells that were mobilized into the blood in response to NORMEX followed by a significant egress of both NK (Figures 3a and 3b) and ILC22 cells (Figures 3c and 3d) during the recovery period returning to baseline levels. The mobilization of ILC22 cells in response to NORMEX was significant compared to the HYPREST condition. There was also a significant increase in the percentage of NK cells mobilized into the blood in response to HYPEX followed by an egress of cells from the blood during the recovery period, but the absolute numbers of NK cells in the blood did not reach statistical significance in response to HYPEX (Figure 3a). There was a significant main effect of time (p=0.033) but not condition (p=0.171) on the number of circulating NK cells in the blood in response to exercise (Figure 3a). There was a significant main effect of condition (p=0.0471) and ILC22 cells were the only cell subset to show an effect of condition*time (p=0.026) (Figure 3b). The HYPREST condition did not affect the distribution of the NK or ILC22 cells in the blood.

There was a significant mobilization of Type 3 LTi cells into the blood in response to HYPREST, which was followed by an egress of these cells during the recovery period (Figure 3f). There was a significant main effect of condition with an increase in the absolute number of LTi cells during the HYPREST condition (p=0.005) (Figure 3f). There was also a significant difference in LTi cells during HYPREST compared to NORMEX and HYPEX conditions suggesting that exercise abrogated the mobilization of the LTi cells. There was no overall condition*time effect for the number of LTi cells in peripheral blood, but there was a significant main effect of time (p=0.039) observed in the percentage of LTi cells in the blood (Figure 3c).

Collectively these results indicate that type 1 and 3 ILCs display differential response to exercise with NK cells and ILC22 cells being mobilized into the blood in response to NORMEX but HYPEX conditions abrogated their recruitment. In contrast LTi cells were preferentially mobilized into the blood at rest under hypoxic conditions while exercise attenuated the movement.

Changes in the CD4+ and CD8+ lymphocyte subsets in response to different conditions

There was a significant decrease in the percentage of CD4+ T cells in the blood in response to NORMEX, followed by a significant increase of CD4+ T cells back into the blood during the recovery period (Figure 4a; Main effect for time: p<0.001). These changes were mirrored for CD4+ T cells in response to HYPEX (Figure 4a). There was a trend for a decrease in the number of CD4+ T cells in blood in response to NORMEX and HYPEX but these changes did not reach statistical significance (Figure 4b). In response to HYPREST there was a decrease in percentage of CD4+ cells in the blood and the percentage of cells remained the same during the recovery period. A similar trend was observed for the number of CD4+T cells in the blood but the observed changes did not reach statistical significance. For CD8+ T cells there was only a change in the percentage of CD8+ T cells during the recovery period following NORMEX. However the absolute number of CD8+ T cells did not change significantly in response to any of the conditions studied.

We also examined the naïve and memory cell subsets of both the CD4+ and CD8+ T cell populations but there were no significant changes in any of the subsets in response to any of the conditions tested for either the percentage or absolute cell numbers or at any time point (Data not shown).
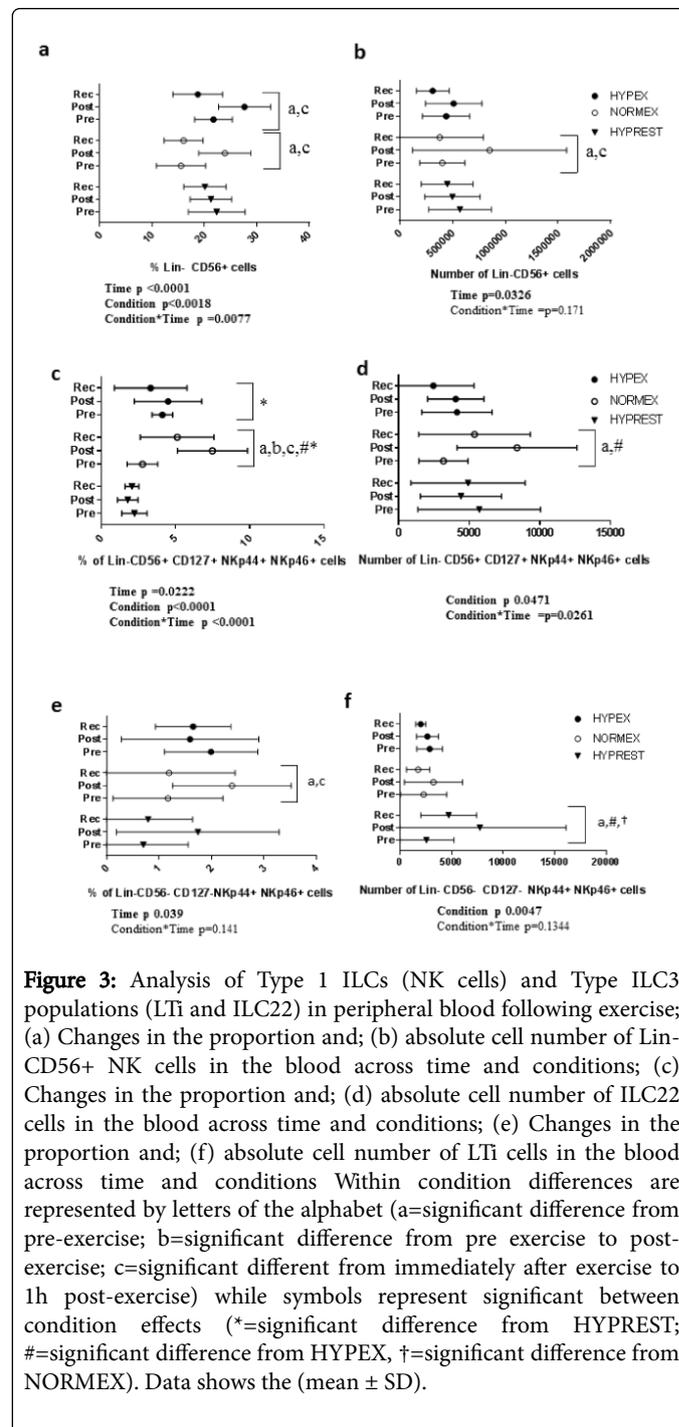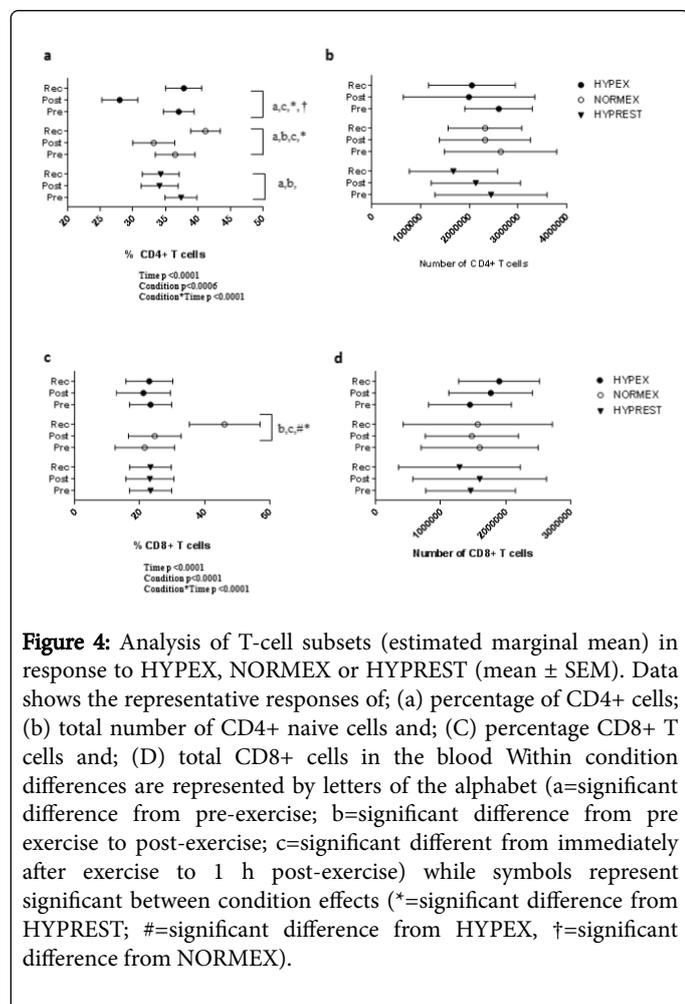

Figure 3: Analysis of Type 1 ILCs (NK cells) and Type ILC3 populations (LTi and ILC22) in peripheral blood following exercise; (a) Changes in the proportion and; (b) absolute cell number of Lin-CD56+ NK cells in the blood across time and conditions; (c) Changes in the proportion and; (d) absolute cell number of ILC22 cells in the blood across time and conditions; (e) Changes in the proportion and; (f) absolute cell number of LTi cells in the blood across time and conditions Within condition differences are represented by letters of the alphabet (a=significant difference from pre-exercise; b=significant difference from pre exercise to post-exercise; c=significant different from immediately after exercise to 1h post-exercise) while symbols represent significant between condition effects (*=significant difference from HYPREST; #=significant difference from HYPEX, †=significant difference from NORMEX). Data shows the (mean ± SD).

Figure 4: Analysis of T-cell subsets (estimated marginal mean) in response to HYPEX, NORMEX or HYPREST (mean ± SEM). Data shows the representative responses of; (a) percentage of CD4+ cells; (b) total number of CD4+ naive cells and; (C) percentage CD8+ T cells and; (D) total CD8+ cells in the blood Within condition differences are represented by letters of the alphabet (a=significant difference from pre-exercise; b=significant difference from pre exercise to post-exercise; c=significant different from immediately after exercise to 1 h post-exercise) while symbols represent significant between condition effects (*=significant difference from HYPREST; #=significant difference from HYPEX, †=significant difference from NORMEX).

Discussion

This study explored the effect of moderate-intensity-exercise (50% $\dot{V}O_2$ peak) performed under normoxic or hypoxic conditions on the mobilization of specific innate and adaptive lymphocyte subsets in the blood. Significant changes in the response to exercise were noted for the numbers of type 1 (NK cells) and type 3 (ILC22) ILCs that were mobilized into the blood in response to NORMEX but exercise under hypoxic conditions appeared to blunt the rise in both these cell subsets. There was a significant effect of condition*time observed for the ILC22 cell population in response to NORMEX. The LTi cells were mobilized into the blood in response to hypoxia alone, but this response was abrogated upon engagement with exercise under either normoxic or hypoxic conditions. In response to moderate intensity exercise there was only a modest effect on the mobilization of T cell subsets with a modest egress of CD4+ and CD8+T cells in response to NORMEX. This study represents the first description of the mobilization of the type 3 ILC22 cells in response to exercise in humans; and they support our original hypothesis that ILCs would be mobilized in response to exercise. However, hypoxia-mediated stress appeared to have a selective effect on type 3 LTi cells and only modest effect on the mobilization and egress of CD4+ and CD8+ T cell populations.

The directed migration of immune cells into and out of the blood is considered critical for the maintenance of immune homeostasis and

surveillance against infection [32]. The mechanical shear forces associated with muscle contraction during exercise was originally hypothesized to explain the mobilization of immune cells from peripheral tissues into the blood [33]. However this hypothesis is unlikely given the growing body of literature demonstrating both specific and predictable movement patterns in immune cells in response to exercise. There was an increase in NK cells which reached significance in the NORMEX condition only. This is in agreement with previous research demonstrating an exercise-mediated mobilization of type 1 ILCs (i.e., CD56 subsets of NK cells) in response to either high-intensity exercise, or moderate-intensity exercise supplemented with hypoxic (12% or 15% O_2) air [34]. In addition Campbell et al also demonstrated a rapid response back to baseline values within the NK subset populations at the completion of exercise similar to that observed here [6]

Within the type 3 ILC subset the proportion and the absolute number of ILC22 cells increased significantly in response to NORMEX, but mobilization of this subset was attenuated under hypoxic conditions. In contrast the proportion and the number of LTi cells in the blood increased in response to hypoxia only but the movement of LTi cells into the was abrogated by exercise. It was interesting to note that the absolute number of ILC3 cells as a population in the blood was ~10 fold lower compared to the NK cell population which supports the notion that that ILC3 cells would mainly reside within mucosal tissues such as the lung and the gut. The ILC22 cells secrete IL-22 which is an important cytokine for regulating epithelial repair and can induce secretion of antimicrobial peptides, mucins and chemokines [35] LTi cells can secrete IL-22 and IL-17; with IL-17 having a greater proinflammatory effect in the immune system. LTi cells also play an important role in the thymus to direct the development of AIRE+ medullary epithelial cells. Currently we do not understand why the different Type 3 ILC subsets (ILC22 and LTi cells) respond in opposing ways to exercise. Perhaps this may reflect differences in chemokine receptor expression that may restrict movement of cell subsets into the peripheral blood.

The redistribution of these lymphocyte subsets in response to exercise reflects the normal mechanisms associated with immune surveillance, and the tissue-specific migratory patterns of leukocytes are regulated by the coordinated expression of adhesion molecules and/or chemokine receptors [36]. At the site of infection innate cells such as macrophages, dendritic cells and ILCs become activated when they recognize pathogens with their germ line encoded pattern recognition receptors (e.g. Toll-like and NOD-like) and secrete cytokines and chemokines to attract effector lymphocytes into the infected site [1,37]. The movement of type 1 and type 3 ILCs, from the blood following exercise suggests a possible redistribution of these cells into peripheral tissues which are at greatest risk of harm (e.g. the lungs/intestines). To explore this possibility, future research may seek to determine the presence or modulation of other specific surface adhesion molecules/chemokine receptors on these cells in response to exercise. This information could provide critical insights on how exercise can beneficially influence migratory patterns of lymphocytes which to date, have not been explored.

On the whole we did not observe any major effect on the mobilization of CD4+ and CD8+ T cells in response to moderate intensity exercise which was surprising. In trying to explain these findings most studies have examined T cell subsets in response to moderate or high intensity exercise [6,38] where the largest changes in T cell populations were usually observed in response to high intensity

exercise. In the current study we observed a general egress of CD4+ cells from the blood in response to exercise but there were no significant changes when we examined naïve and memory T cell subsets.

This study was unique in the manner by which hypoxia was controlled. Where previous studies have provided a fixed concentration of oxygen in inspired air, this study provided a variable concentration of oxygen in the air with the goal of maintaining a consistent systemic hypoxia dose across HYPEX and HYPREST (i.e., ~86% SpO2 [5,6]. The acute exposure to hypoxia in the present study is unlikely to induce the same physiological changes as living or training at altitude, but nevertheless it does represent a significant acute stress on the immune system.

In conclusion, this study has demonstrated that innate lymphocytes including NK cells and ILC22 cells displayed increased mobilization into the blood in response to moderate intensity exercise and egress from the blood upon cessation of exercise, while hypoxia abrogated the movement of both NK and ILC22 cells. LTi cells were responsive to hypoxia and were mobilized into the blood but exercise attenuated their movement into the blood. There was no significant effect on naïve or memory T cells following either stress (moderate exercise or hypoxia). Future studies should investigate if similar changes in ILC subsets would be observed under conditions of high intensity exercise.

Perspectives

The recent discovery of a new subset of innate lymphocytes has underscored their important role in regulating mucosal responses in tissues such as the skin, lung and gut [9,21,22]. Here we have shown that type1 NK cells and type 3 ILC22 cells are both mobilized into the blood in response to moderate intensity exercise. However, exposure to hypoxic conditions during exercise abrogated this response. In contrast type 3 LTi cells were mobilised upon exposure to hypoxic conditions alone, whereas exercise prevented their movement into the blood. Therefore it appears that the mobilisation of distinct innate populations may be altered by changing the environmental conditions and types of stress. This may have important implications for athletes adopting hypoxia or altitude training, given that the innate lymphocytes are thought to control the migration of adaptive T cell subsets of CD4+ and CD8+ T cells which therefore directly influences the quality of the adaptive immune response [9]. Additionally, the use of hypoxic exercise was shown to improve insulin sensitivity and is therefore suggested for individuals with Type 2 Diabetes Mellitus [29]. Whether these improvements in insulin sensitivity may be explained by changes in the immune response, and specifically these ILC subsets, remains to be established.

Acknowledgement

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