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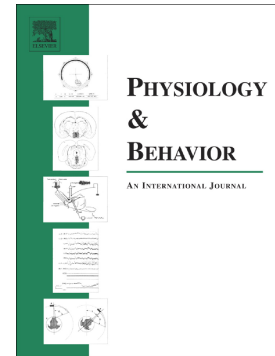
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# **Lymphocytes and monocytes egress peripheral blood within minutes after cessation of steady state exercise: a detailed temporal analysis of leukocyte extravasation**

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**Abstract:**

Acute exercise evokes an almost instantaneous lymphocytosis, followed by sustained lymphopenia that occurs within just 30-60 minutes after exercise cessation. The aim of this study was to characterize the immediate (order of minutes) post-exercise kinetics of lymphocyte and monocyte egress, and to determine whether this egress is associated with heart rate recovery following a single bout of steady state dynamic exercise. Eleven healthy subjects cycled for 30-minutes at ~70% of their estimated peak power. Blood samples were collected from an intravenous catheter before exercise, during exercise (E) at +15 and +30 minutes, and during passive recovery (R) at exactly +1, +2, +3, +4, +5 and +10 minutes after exercise cessation. Complete blood counts and flow cytometry were used to enumerate total monocytes, lymphocytes: CD3+ T-cells, CD4+ T-cells, CD8+ T-cells, NK-cells and  $\gamma\delta$  T-cells in whole blood. Both lymphocytes and monocytes displayed rapid egress kinetics, by R+3 the total numbers of all cell types examined were significantly lower than E+30. NK-cells egressed more rapidly than other lymphocyte subtypes, followed by CD8+,  $\gamma\delta$ , and then CD4+ T-cells. Further, the egress of NK-cells, CD4+, and CD8+ T-cells positively correlated with heart rate recovery after exercise cessation. In conclusion, lymphocyte and monocyte egress is rapid and occurs within minutes of exercise recovery, underscoring both the importance of collection time for post exercise blood samples, and the use of intravenous catheters to capture peak cell mobilization. The rate of egress may be dependent on how quickly hemodynamic equilibrium is restored on cessation of exercise and is, therefore, likely to be influenced by individual fitness levels.

**Key Words:** Exercise Immunology, Leukocytosis, T-Cell, NK-Cell, Neutrophilia, Trafficking, Lymphopenia

## 1.1 Introduction:

A single bout of dynamic exercise causes an almost instantaneous mobilization of leukocytes into the peripheral circulation [1,2]. Neutrophils account for the vast majority of this exercise-induced leukocytosis, although lymphocytes [3] and monocytes [4] also mobilize in relatively large numbers. During exercise recovery, the circulating neutrophil count may continue to rise [3], while lymphocyte numbers rapidly decline. Indeed, the circulating lymphocyte count may fall by 30-50% below resting levels within the first hour of exercise recovery, sometimes resulting in a transient clinical lymphopenia ( $<1.0 \times 10^6/\text{ml}$ ) that may persist for up to 6h [1,2,5]. The extent by which lymphocytes are redeployed between the blood and tissues with exercise is largely influenced by the intensity and duration of the bout. In the major lymphocyte subtypes, NK-cells exhibit the largest relative redeployment in response to exercise, followed by  $\gamma\delta$  T-cells, CD8+ T-cells, CD4+ T-cells and, lastly, B-cells [6,7]. Furthermore, the NK-cell and T-cell subtypes that are preferentially redeployed with exercise tend to have: greater cytotoxic and effector functions [8–11], heightened expression of adrenoceptors [7] and glucocorticoid receptors [12,13] and phenotypes associated with tissue migration [14], and antigen experience [15,16].

The mechanisms responsible for leukocyte redeployment in response to exercise are multifaceted, but are largely due to changes in hemodynamic shear stress and the actions of catecholamines and glucocorticoids [17]. Increases in cardiac output and heart rate amplify hemodynamic forces along the vascular endothelium and at target organ reservoirs, resulting in the demargination of adherent leukocytes from vessel walls [18] and from the pulmonary [19], hepatic, and splenic pools [20]. Additionally, either through the reduction in adhesion molecule expression or the obstruction of adhesion molecule receptor/ligand binding, catecholamines promote leukocytes to circulate freely [21]. Glucocorticoid secretion, resulting from the activation of the hypothalamic-pituitary-adrenal (HPA) axis, also influences leukocyte trafficking [13,22]. Where the mobilized cells traffic to following their egress from the peripheral blood compartment is not well understood. They may return to their pre-exercise location, or might migrate to areas/tissues that now require their sentinel and/or reparative action following activation of the biological stress response [17].

Although it is known that blood lymphocyte numbers can fall below pre-exercise levels within the first 30-60 minutes after exercise cessation [5], the kinetics of lymphocyte egress during the very early (order of minutes) stages of exercise recovery are not known. The very early egress kinetics could have important implications for studies that rely on standard phlebotomy to assess lymphocyte mobilization and egress in response to an acute stress task. For instance, it may take several minutes to situate the subject following the task, gain intravenous access, and draw the required volume of blood. The situation is further complicated by delays and inconsistencies in sample collection time (even in the order of minutes) that may occur between subjects and/or collection days. Individual and group variations in the rate of lymphocyte egress, could also compromise those studies designed to make between and within subject comparisons regarding leukocyte redeployment in response to an acute stress task. A potential factor in determining the rate of leukocyte egress during the early stage of exercise recovery is the time it takes to restore hemodynamic equilibrium. As heart rate returns to baseline after exercise, so too does cardiac output, vagal tone, and blood pressure, thus allowing circulating cells to adhere to their endothelial ligands and complete the process of diapedesis which facilitates their translocation to the tissues [23].

The aim of this study was to characterize the early (order of minutes) egress kinetics of blood lymphocytes, monocytes, and their subtypes during the immediate recovery phase following a single bout of steady state aerobic exercise. We hypothesized that lymphocytes and monocytes would egress the blood compartment immediately upon exercise cessation, resulting in circulating numbers falling below peak exercise values within just 5 minutes of passive recovery. We further hypothesized that the most rapid egress rate within the lymphocytes would be exhibited by NK-cells, followed by  $\gamma\delta$  T-cells, CD8+ T-cells, and then CD4+ T-cells. Finally, we hypothesized that the rate of lymphocyte egress from the peripheral circulation would be positively correlated with heart rate recovery following exercise cessation.

## 1.2 Methods:

### 1.2.1 Participants:

Eleven healthy and physically active individuals (7 males) between the ages of 26-38 participated in this study. All participants were considered 'low' risk for exercise testing as defined by being asymptomatic for cardiovascular disease with less than two risk factors in accordance with American College of Sports Medicine/American Heart Association (ACSM/AHA) criteria [24]. Physical activity status was determined using the Jackson PA-R questionnaire [25]. Participants reported regular engagement in vigorous and/or moderate intensity physical activity and did not report or exhibit any contraindications for participation in our study as determined by our inclusion/exclusion criteria. Exclusion criteria included excessive alcohol consumption, immunological impairments or diseases, and the taking of any medications/supplements known to affect the immune system or cardiovascular system. Additionally, all participants were required to be infection free for at least 6 weeks prior to their trial (confirmed by self report). Participants were asked to avoid strenuous activity 24 hours before each visit. We obtained written informed consent from each participant, and the Institutional Review Board (IRB) at the University of Houston granted approval for the study. Descriptive statistics for the participants are summarized in **(Table 1)**.

### 1.2.2 Experimental protocol:

Participants visited the lab on two separate occasions at the same time of day with no less than 24 hours and no more than 5 days between visits. On visit 1, participants completed an incremental submaximal cycling protocol on a stationary cycle ergometer (Velotron<sup>®</sup> LABs model Racermate Inc. Seattle, WA). The initial power output of 75W-100W was increased by 10W every three minutes until a steady state heart rate equivalent to 80% of age predicted maximum heart rate was attained, using the equation  $[191.5 - (.007 * \text{age}^2)] * 0.80$  [26]. Time to target heart rate ranged between 10-20 minutes. The relationships between heart rate and power output were plotted for each individual, and peak power was extrapolated from the curve to coincide with age-predicted maximum heart rate.  $\text{VO}_{2\text{max}}$  was estimated from the submaximal exercise test using the YMCA prediction equations [27]. On visit 2, an indwelling intravenous (IV) catheter was placed in a superficial forearm vein prior to exercise. Following 5 minutes of seated rest on the bike, a baseline blood sample was drawn into a 5ml vacuum tube spray coated with EDTA. Participants then completed a 30-minute bout of steady state cycling exercise. Each participant was asked to maintain the power output corresponding to 80% of their age-predicted maximum heart rate as determined from the submaximal cycling test

performed during Visit 1. Participants provided ratings of perceived exertion (RPE) every five minutes during exercise using the Borg scale [28], and power adjustments were made during the test only if the subject intimated that they would not be able to maintain the required power output for the entire 30-minute duration. Further blood samples were collected during exercise (E) at +15 and +30 minutes. Participants were asked to continue pedaling against the resistance of the cycle ergometer while the blood samples were being drawn. After collecting the blood sample at E+30, participants were asked to stop pedaling and remain seated on the bike for 10 minutes of passive recovery (R). Further blood samples were drawn at exactly +1, +2, +3, +4, +5 and +10 minutes following exercise cessation. The catheter was flushed with 3ml of non-heparinized isotonic saline to maintain patency as required. Whenever the catheter was flushed, a 3ml volume was collected from the IV catheter and discarded prior to collecting the blood sample. We recorded heart rate continuously using short-range telemetry (Polar RS300X Electro Oy, Kempele, Finland) during the exercise bout and the recovery period. The exercise performance measures collected during the test are shown in **Table 1**.

### *1.2.3 Complete Blood Counts, immunofluorescence and Flow Cytometry:*

Complete blood counts for leukocytes, lymphocytes, monocytes and granulocytes were determined in duplicate using a clinical grade Hematology Analyzer (Mindray BC 3200, Shenzhen, China). Direct immunofluorescence assays were performed to identify the numbers and proportions for the following: CD3+, CD3+/CD4+, CD3+/CD8+ T-Cells, CD3+/CD4-/CD8-  $\gamma\delta$  T-Cells, CD3-/CD56+ NK-Cells, and for CD14++/CD16- (classical monocytes), CD14++/CD16+ (intermediate monocytes), and CD14+/CD16++ (pro-inflammatory monocytes). Briefly, 50 $\mu$ l of whole blood was labeled with two separate antibody cocktails: (i) FITC-conjugated anti-CD56 (IgG1, TULY56), PE-conjugated anti-CD4 (IgG2b, OKT4), PerCy5-conjugated anti-CD8 (IgG1, RPA-T8) and APC-conjugated anti-CD3 (IgG2a, OKT3); (ii) FITC-conjugated anti-CD14 (IgG1, 61D3), PE-conjugated anti-CD16 (IgG1, CB16), PerCy5-conjugated anti-CD20 (IgG2b, 2H7) and APC-conjugated anti-CD66 (IgG2a, CD66a-B1.1). All antibodies were mouse anti-human monoclonal and purchased from eBioscience (San Diego, CA, USA). Single color control tubes were used to adjust for spectral overlap during analysis by 4 color flow cytometry, using either an Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI) with (CFlow<sup>®</sup> software V2) or MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) with (MACSQuantify<sup>™</sup> Version 2.8). The total number of lymphocyte and monocyte subtypes in peripheral blood was determined by multiplying the percentage of all gated cells (lymphocytes or monocytes) expressing the surface marker combinations of interest by the total blood lymphocyte or blood monocyte counts (as determined by the automated hematology analyzer).

### *1.2.4 Statistical Analysis*

Prior to analysis, the data were screened to ensure that all test assumptions had been met. Normality was confirmed using histograms and Fisher's skewness and kurtosis coefficient, as well as descriptive statistics and normal Q-Q plots. Mauchly's test indicated that the assumption of sphericity was violated for each of the cell types evaluated which led us to use the Greenhouse–Geisser correction test. Repeated-measures ANOVA was utilized to assess the change in leukocytes and lymphocytes over time as a main effect. Separate models were developed to evaluate all leukocyte and lymphocyte subsets present in the peripheral blood over time (resting, E+15, E+30, R+1, R+2, R+3, R+4, R+5 and R+10), as well as to assess the percentage difference from E+30 values at all recovery time points (R+1 to R+10). Bonferroni corrected, post hoc comparisons were used to determine which exercise recovery time points

were significantly different from peak exercise (E+30). Bivariate correlation was used to assess the relationship between the percent of E+30 heart rate and the percent of E+30 cell counts at all exercise recovery time points (R+1 to R+10) for all cell types analyzed. SPSS version 22 (IBM; Armonk, NY, USA) was used for statistical analyses with significance indicated as ( $p < .05$ ).

### 1.3 Results:

#### 1.3.1 *The egress of leukocytes and their subtypes is evident within just 1-3 minutes of exercise recovery*

There was a statistically significant main effect of time for leukocytes, lymphocytes, monocytes, and granulocytes, (**Table 2**). Total leukocytes, lymphocytes, monocytes and granulocytes were mobilized in response to exercise, increasing above resting values at E+15 and reaching peak concentrations in blood at E+30. Immediately upon cessation of exercise, cell numbers began to decline; compared to E+30, total leukocytes were significantly lower by R+1, while lymphocyte and granulocyte numbers declined to statistically significant values by R+2 ( $p < .05$ ). Monocyte numbers were significantly lower than E+30 values by R+3 ( $p < .05$ ) (**Table 2**). The R+3 and R+10 total monocyte numbers were not statistically different to rest ( $p > 0.99$ ). Individual data for these cell types expressed as a percent of the peak exercise cell counts at E+30 are shown in **Figure 1**.

Lymphocyte and lymphocyte subsets were mobilized in response to exercise and then decreased from their E+30 values upon completion of exercise. A statistically significant main effect of time was found for CD3+T-Cells, CD4+ T-Cells, CD8+ T-Cells,  $\gamma\delta$  T-Cells, and NK-Cells (**Table 2**). Circulating cell counts became significantly different from E+30 at R+2 for NK-Cells ( $p = .003$ ), CD8+ T-Cells ( $p = .012$ ), and CD3+ T-Cells ( $p = .025$ ). CD4+ cell counts became significantly different from E+30 at R+3, ( $p = .009$ ), (**Table 2**). There was no statistical difference between absolute cell counts when comparing rest to R+4, R+5 or R+10 for CD3+, CD4+ and CD8+ T-Cells, ( $p > 0.99$ ). Individual data for these cell types expressed as a percent of the peak exercise cell counts at E+30 in **Figure 2**, with the NK-cell data being presented in **Figure 3**.

Monocyte subpopulations increased during exercise and egressed immediately upon cessation of exercise, with a statistically significant main effect of time for classical monocytes, intermediate monocytes, and pro-inflammatory monocytes (**Table 2**). Classical monocytes showed a significant and consistent absolute cell number decline from E+30 ( $p < .009$ ), beginning at R+3 minutes and continuing through all measured post exercise time points. The egress for pro-inflammatory monocytes showed a transient statistical significance at R+3, (**Table 2**). Additionally, for all the subpopulations of monocytes, there was statistically no difference when comparing resting to recovery time points beyond R+3, ( $p > 0.99$ ). Individual data for these cell types expressed as a percent of the peak exercise cell counts at E+30 are shown in **Figure 3**.

#### 1.3.2 *Leukocyte subtypes egress peripheral blood at different rates during exercise recovery*

The relative rate of egress as a percentage of their respective peak values among the leukocyte and lymphocyte subtypes was compared. The relative rate of egress from E+30 was variable and contingent upon cell type, finding a significant main effect for time ( $F_{3,6, 143.26} = 129.61$ ,  $p < 0.001$ ) and a time x cell type interaction effect ( $F_{10,7, 143.26} = 3.99$ ,  $p < 0.001$ ). Though the relative egress from E+30 was statistically significant for all leukocyte and leukocyte subset counts at R+1, (all,  $p < .05$ ), monocytes showed the most rapid egress rate with a -14% change from E+30 at R+1. This was followed by -26% for R+2, a consistent -39-40% for R+3, R+4, and R+5, and



finally -46% at R+10. Furthermore, the percent decline for monocytes was significantly greater than leukocytes ( $p<.001$ ), lymphocytes ( $p<.002$ ), and granulocytes ( $p<.001$ ), starting at R+2, (**Figure 4, top**).

The rates of egress from E+30 values for lymphocytes and subsets again vary among the different cell types, with a significant main effect of both time ( $F_{3.5, 175} = 93.6, p<0.001$ ) and a time x cell type interaction effect ( $F_{14, 175} = 3.24, p<0.001$ ) being found. Though all lymphocyte subset counts were significantly different from E+30 as early as R+1 ( $p<.05$ ), NK-cells exhibited a more rapid egress compared to the other lymphocyte subsets. NK-cell counts decreased -10% from E+30 at R+1, -21% at R+2, -34% at R+3, -45% at R+4 and R+5 and -59% by R+10. Additionally, the relative egress of NK-Cells was significantly greater from CD3+ T-Cells ( $p<.001$ ), CD4+ T-Cells ( $p<.001$ ), CD8+ T-Cells ( $p<.004$ ), and  $\gamma\delta$  T-Cells ( $p<.05$ ), for all recovery time points starting at R+3, (**Figure 4, bottom**). NK-cells are followed, in order of egress rate, by CD8+,  $\gamma\delta$ , and CD4+ T-cells.

*1.3.3 Lymphocyte, but not monocyte, egress correlates with decreasing heart rate during passive recovery from exercise.*

To determine if the rate of cell egress correlates with recovery heart rate, cell numbers in blood during the recovery phase of exercise were expressed as a percentage of the E+30 cell count and correlated with recovery heart rate as a percentage of the E+30 heart rate. The rate of egress for CD3+, CD4+, and CD8+ T-Cells, as well as NK-Cells all correlated ( $r >.5$ ) significantly with the decrease in heart rate for the first 2 minutes into passive recovery. Recovery heart rate did not correlate with the egress of total lymphocytes, monocytes or monocyte subtypes (**Table 3**).

## 1.4 Discussion

We characterized for the first time the immediate (order of minutes) post-exercise kinetics of leukocyte, lymphocyte, and monocyte subsets as they egressed the peripheral circulation upon cessation of a single bout of steady state exercise. We determined that this egress occurs almost immediately, with the absolute numbers of all circulating leukocyte and leukocyte subtypes falling below peak exercise values within just two minutes of passive recovery. Further, total monocytes were not different to resting values after just 3 minutes of recovery, while CD3+, CD4+ and CD8+ T-cells were comparable to resting values after just 4, 5 and 10 minutes of passive recovery, respectively. These findings illustrate the rapid rate by which leukocytes are redeployed to and from the blood compartment in response to an acute stress task. This also underscores the importance of timing when collecting blood samples after exercise for the enumeration of circulating leukocytes and their subtypes, particularly when the purpose is to capture peak cell mobilization during the stress task. We also found that the rate of egress was associated with heart rate recovery for lymphocytes, indicating that the restoration of hemodynamic equilibrium could be a key factor governing the rate of lymphocyte egress from the blood compartment during the initial stages of exercise recovery.

Effective trafficking of immune cells through the lymphoid and peripheral tissues via the circulation is essential to both immunosurveillance and maintaining host immunity [29]. We contend that the speed of this exchange is also highly important because the lymphocyte and monocyte subtypes that are preferentially mobilized by exercise exhibit greater effector function (i.e. cytotoxicity), antigen experience and differentiation [7–11]. We further purport that the

capacity of the host to rapidly redeploy leukocytes between the blood and tissues in response to acute stress or exercise provides a global indication of immune system competence, as well as the likelihood that an individual will resolve challenges to the immune system during or immediately after a short-term stress task, [30]. Thus, for a given biological stress response under controlled conditions (i.e. dose-controlled epinephrine infusion, individualized intensity controlled exercise), a larger and swifter exchange of leukocytes between the blood and tissues would be indicative of enhanced immunosurveillance and better global immunity, whereas a smaller and/or slower rate of exchange might be indicative of compromised immunosurveillance and an impaired ability to resolve immunological challenges. Although this remains to be determined empirically, establishing the magnitude and rate of lymphocyte and monocyte egress during exercise recovery may help stratify individuals with robust and compromised immune responses to stress. This could be useful, for example, to identify athletes at an increased risk of infection or 'burnout' in response to overreaching or high volume/intensity training. Indeed, it has been shown that lymphocyte redeployment is reduced in highly trained athletes at a given intensity of exercise following a period of functional overreaching [31]. Witard et al. showed that just one week of intensified cycling training impaired the redistribution (mobilization and egress) of CD8+ T-cells in response to a single bout of exercise, implying that high intensity exercise training may impair immune surveillance in athletes [31].

Pro-inflammatory (non-classical) CD16+ monocytes and NK-cells egress the peripheral circulation more rapidly than other leukocytes. This is likely due to the importance of their specific and immediate roles in host defense as part of the innate immune response. Additionally, NK-cell egress may be related to the direct influence of macrophage derived cytokines [32]. Both NK-cells and monocytes have higher relative densities of  $\beta_2$ -adrenergic receptors on their cell surface in comparison to the other leukocytes and lymphocytes [33,34]. This perhaps governs their rapid mobilization kinetics, [35], however their egress is more likely influenced by exercise induced increases in both glucocorticoids [22,36,37,42] and a myriad of inflammatory chemokines/cytokines [38–41]. For instance, in a detailed temporal analysis of lymphocyte trafficking using blood and saliva samples collected every 15-minutes in a 8h period, Trifonova et al. showed that diurnal changes in lymphocyte trafficking, including NK-cells, was strongly linked to salivary cortisol, [42]. Monocytes may egress the blood compartment under the influence of IL-6, which is known to be secreted at relatively high levels during exercise by contracting skeletal muscle even in the absence of muscle damage [43,44]. Monocytes are highly responsive to IL-6 as it stimulates their differentiation into macrophages [45], where they phagocytize foreign substances at the tissues and aid in tissue repair. Though this differentiation is not likely to occur in the 2 minutes of passive recovery, the monocytes might still home to areas where IL-6 is being produced. IL-6 also drives post-exercise NK-cell homing [46], and due to NK-cell tissue homing receptor densities they predominantly home to lung, spleen and muscle [47], where they can respond rapidly to opportunistic invading pathogens or reactivating viruses. It is likely that a combination of catecholamines, glucocorticoids and cytokines facilitate the rapid egress of discrete leukocyte subpopulations from the peripheral blood compartment after exercise allowing their homing to areas where reparative and sentinel action is required.

We also found a positive correlation between heart rate recovery and lymphocyte egress during the first two minutes of exercise recovery. At this point, the heart rate had dropped ~28% from peak values and, because heart rate recovery from exercise is more rapid among trained individuals compared to those who are untrained [48], variability in egress rate is likely due to

the broad range of aerobic fitness levels in this cohort of participants, (estimated  $VO_{2max}$  29 - 65.5 ml·kg<sup>-1</sup>·min<sup>-1</sup>). Lymphocyte egress from peripheral blood involves the physical process of diapedesis. This requires the migrating leukocytes to first adhere to the vascular endothelium, initially through tethering and rolling adhesion that is mediated by selectins and endothelial ligands such as CD34 [23]. This is followed by activation and firm adhesion, which requires ligation of integrins with stronger adhesion molecules on the endothelium such as ICAM-1, allowing the leukocyte to attach firmly against the forces of blood flow for subsequent migration through adjacent endothelial cells toward the tissues [23]. We purported, therefore, that a swifter restoration towards hemodynamic equilibrium (i.e. lowering of cardiac output, blood flow and blood pressure) during exercise recovery would allow for diapedesis and a more rapid extravasation of the leukocytes previously mobilized by exercise to take place. As such, our finding that lymphocyte egress correlated positively with heart rate recovery (used here as a global indicator of hemodynamic restoration) during the first few minutes of exercise cessation was expected. This does indicate, however, that the rate of lymphocyte egress during the early stages of exercise recovery is likely to be influenced by aerobic fitness, and a future study comparing lymphocyte and monocyte egress rates between trained and untrained individuals after intensity controlled exercise would be illuminating. It will also be important for future studies to consider the many other factors that could also play a role in the rate of leukocyte egress after exercise such as age, sex, catecholamines, glucocorticoids, cytokines, infection history, leukocyte composition, adrenergic/glucocorticoid receptor density/sensitivity and cardiovascular responses, and also to characterize, in great detail, leukocyte trafficking beyond 10-minutes of recovery.

These findings also have practical implications for research studies in exercise and stress immunology. Many studies in this field are concerned with the effects of acute bouts of physical (i.e. exercise) or psychological stress on leukocyte redistribution to and from the blood compartment. Oftentimes, the post-stress blood draw is collected 'immediately' after the stress task under the assumption that this will represent peak cell numbers mobilized during the stress task. While there is a clear lack of consistency and specificity when detailing the time-course of when blood samples are collected relative to the onset and cessation of acute stress [49], some exercise studies are specific in detailing the time elapsed after exercise when the 'post-exercise' blood sample was actually drawn, which can sometimes be as long as 5-minutes [50–52]. As standard venipuncture is most often used for the collection of 'post-exercise' blood samples, it can be assumed that these blood draws actually occur during the first few minutes of exercise recovery. Our findings would indicate that blood samples collected, even just a few minutes after exercise cessation, are suboptimal for identifying peak cell mobilization during exercise. Moreover, even if the amount of time elapsed following exercise cessation is defined (i.e. 5-minutes) and consistent prior to blood collection, it is still likely that differences in lymphocyte and monocyte egress rates among subjects, possibly due to differences in fitness levels, would remain an important confounder. It is clear from our findings that, in order to accurately capture peak cell mobilization during exercise, future studies should use indwelling intravenous catheters over standard phlebotomy. This would allow samples to be drawn while the subject is still performing the exercise bout at the prescribed intensity, ensuring that the window to capture peak cell mobilization is not missed.

## 1.5 Conclusion

Lymphocytes and monocytes begin to egress the peripheral blood compartment immediately upon cessation of a single bout of steady state exercise. Significant numbers of lymphocytes and monocytes extravasate the bloodstream within as little as 3 minutes of passive recovery, with those monocyte and lymphocyte subtypes known to have phenotypes associated with increased effector function and tissue migration (i.e. pro-inflammatory monocytes, NK-cells, CD8+ T-cells) exhibiting the most rapid egress kinetics. The rate of monocyte and lymphocyte egress may be dependent on how quickly hemodynamic perturbations are restored following an acute bout of exercise, indicating that individual fitness levels are likely to play a role in determining how quickly certain leukocyte subtypes can exit the blood compartment and migrate toward the tissue during exercise recovery.

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### **Conflicts of Interest**

None

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Figure 1. Leukocytes and leukocyte subtypes rapidly egress the peripheral circulation immediately upon cessation of steady state exercise. Individual data shown as a percentage of E+30 cell counts during exercise recovery (n=11). The mean of all participants is shown as a bold solid line.

Figure 2. T-Cells rapidly egress the peripheral circulation immediately upon cessation of steady state exercise. Individual data shown as a percentage of E+30 cell counts during exercise recovery (n=11). The mean of all participants is shown as a bold solid line.

Figure 3. NK-cells and monocyte subtypes rapidly egress the peripheral circulation immediately upon cessation of steady state exercise. Individual data shown as a percentage of E+30 cell counts during exercise recovery (n=11). The mean of all participants is shown as a bold solid line.

Figure 4. The relative egress of leukocytes/leukocyte subtypes (*top*) and lymphocyte subtypes (*bottom*) from peripheral blood immediately upon cessation of steady state exercise. Values are mean  $\pm$  SE. \* indicates statistically significant difference from E+30 for all cell types,  $p < .005$ , # indicates statistically significant difference between monocytes (*top*) and all other cell types (*top*), and between NK Cells all other cell types (*bottom*),  $p < 0.05$ .

Table 1. Physical characteristics and exercise performance measures of the participants ( $n=11$ ). All exercise performance measures were averaged across the 30-minute bout.

Physical Characteristics	Mean	SD	Range
Age (Years)	31.0	4.4	25-38
Height (cm)	173.2	10.1	156-185.4
Mass (kg)	72.1	7.8	56.3-82
Body Mass Index ( $\text{kg}\cdot\text{m}^{-2}$ )	24.0	1.9	20-27
Maximum Heart Rate (bpm) <sup>1</sup>	184.6	1.9	181-187.1
Physical activity rating (0-7) <sup>2</sup>	5.1	1.8	2.0-7
Estimated Peak Cycling Power (W)	225.6	59.6	100.3-288.3
$\text{VO}_{2\text{max}}$ ( $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) <sup>3</sup>	46.2	10.5	29-65.5
Exercise Performance Measures			
Cycling Power (W)	150.6	36.3	75-200
Cycling Power (% of peak cycling power)	67.7	6.9	60-77
Exercising Heart Rate (bpm)	152.9	11.3	126-167
Exercising Heart Rate (% of predicted max)	82.9	6.6	67-91
Perceived Exertion (Borg's 6-20 Scale) <sup>4</sup>	14.1	1.5	11.7-17

<sup>1</sup> Maximum Heart Rate was estimated using the equation  $191.5 - (.007 \cdot \text{age}^2)$  [26].

<sup>2</sup> Physical activity rating was determined on a 0-7 point scale: None-0, Minimal-1, Moderate-2-3, Vigorous-4-7 [25].

<sup>3</sup> Maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) was estimated from the submaximal exercise test [27].

<sup>4</sup> Rating of perceived exertion (RPE) was determined using the Borg 6-20 scale [28].

Table 2. Absolute number of circulating cells (cells/ $\mu$ L) for each experimental time point, with results from the repeated measure ANOVA, ( $n=11$ ). Values are mean  $\pm$  SD.

	Rest	E+15	E+30	R+1	R+2	R+3	R+4	R+5	R+10	F (df)	p
Leukocytes	5429 $\pm$ 816*	8067 $\pm$ 1277	8533 $\pm$ 1506	8058 $\pm$ 1380*	7550 $\pm$ 1294*	7129 $\pm$ 1247*	6858 $\pm$ 1116*	6683 $\pm$ 1192*	6225 $\pm$ 1092*	58.2 (3,30)	<0.001
Lymphocytes	1679 $\pm$ 205*	2746 $\pm$ 519	2946 $\pm$ 663	2745 $\pm$ 566	2554 $\pm$ 484*	2395 $\pm$ 519*	2316 $\pm$ 502*	2225 $\pm$ 452*	2129 $\pm$ 513*	39.96 (3.1,31)	<0.001
Monocytes	383 $\pm$ 153*	783 $\pm$ 272	887 $\pm$ 360	766 $\pm$ 323	654 $\pm$ 278	545 $\pm$ 249*	550 $\pm$ 194*	533 $\pm$ 217*	483 $\pm$ 186*	18 (3,30)	<0.001
Granulocytes	3367 $\pm$ 825*	4546 $\pm$ 1115	4704 $\pm$ 1135	4546 $\pm$ 1228	4350 $\pm$ 1156*	4195 $\pm$ 1099*	3991 $\pm$ 1021*	3916 $\pm$ 1086*	3600 $\pm$ 853*	44.16 (2.4, 24)	<0.001
CD3+ T-cells	884 $\pm$ 354	1291 $\pm$ 679	1427 $\pm$ 745	1351 $\pm$ 676	1259 $\pm$ 679*	1209 $\pm$ 670*	1115 $\pm$ 637*	1140 $\pm$ 627*	982 $\pm$ 518*	11.2 (2.2, 22)	<0.001
CD4/CD3+ T-cells	517 $\pm$ 233*	668 $\pm$ 330	709 $\pm$ 341	680 $\pm$ 330	636 $\pm$ 328	627 $\pm$ 322*	579 $\pm$ 325*	591 $\pm$ 323*	515 $\pm$ 271*	9.29 (3.2,32)	<0.001
CD8+/CD3+ T-cells	295 $\pm$ 123	467 $\pm$ 275	531 $\pm$ 304	515 $\pm$ 292	454 $\pm$ 267*	432 $\pm$ 269*	395 $\pm$ 251*	410 $\pm$ 250*	350 $\pm$ 209*	11.61 (2.2,22)	<0.001
$\gamma\delta$ T Cells	194 $\pm$ 161	394 $\pm$ 307	425 $\pm$ 268	407 $\pm$ 273	391 $\pm$ 235	339 $\pm$ 230	323 $\pm$ 200	313 $\pm$ 200	303 $\pm$ 224	13.71 (1.7, 17)	<0.001
NK-cells	139 $\pm$ 86*	442 $\pm$ 226*	523 $\pm$ 269	469 $\pm$ 245	413 $\pm$ 245*	345 $\pm$ 230*	290 $\pm$ 196*	289 $\pm$ 169*	215 $\pm$ 138*	32.41 (2.21)	<0.001
Classical Monocytes	295 $\pm$ 119*	598 $\pm$ 228	652 $\pm$ 288	563 $\pm$ 260	489 $\pm$ 226	407 $\pm$ 202*	386 $\pm$ 203*	390 $\pm$ 189*	348 $\pm$ 150*	16.62 (3.4,34)	<0.001
Intermediate Monocytes	35 $\pm$ 27	58 $\pm$ 31	80 $\pm$ 67	71 $\pm$ 54	56 $\pm$ 38	48 $\pm$ 43	44 $\pm$ 35	46 $\pm$ 39	40 $\pm$ 30	6.71 (2.7,27)	<0.002
Pro-Inflammatory Monocytes	27 $\pm$ 17	52 $\pm$ 46	74 $\pm$ 53	57 $\pm$ 35	49 $\pm$ 33	38 $\pm$ 31*	41 $\pm$ 25	43 $\pm$ 32	31 $\pm$ 20	5.61 (3,30)	<0.003

\* indicates statistically significant difference from Exercise Phase +30 minutes (E+30) in accordance with post-hoc Bonferroni correction,  $p < .05$ .

Table 3. Bivariate correlations of heart rate recovery and cell egress when expressed as a percentage of E+30 values during the early stages of recovery following cessation of steady state exercise ( $n=11$ )

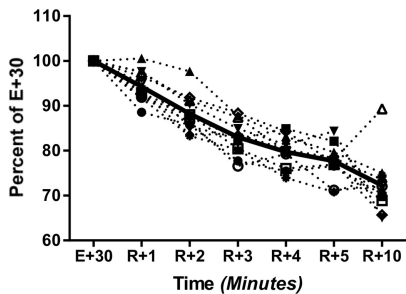
	Recovery Time Point					
	R+1	R+2	R+3	R+4	R+5	R+10
<b>Lymphocytes</b>	0.599	0.454	0.187	0.124	0.434	-0.071
<b>CD3+ T-Cells</b>	0.623 <sup>*</sup>	0.609 <sup>*</sup>	0.572	0.504	0.538	0.521
<b>CD4+ T-Cells</b>	0.622 <sup>*</sup>	0.589 <sup>*</sup>	0.559	0.454	0.492	0.458
<b>CD8+ T-Cells</b>	0.614 <sup>*</sup>	0.615 <sup>*</sup>	0.538	0.479	0.535	0.494
<b>NK Cells</b>	0.585 <sup>*</sup>	0.570	0.434	0.426	0.476	0.397
<b><math>\gamma\delta</math> T-Cells</b>	0.470	0.043	0.272	0.392	.674 <sup>†</sup>	0.204
<b>Total Monocytes</b>	0.121	0.207	-0.130	0.414	0.201	0.032
<b>Granulocytes</b>	-0.028	0.133	0.195	0.432	0.208	0.137
<b>Classical Monocytes</b>	-0.414	-0.555	0.276	-0.008	-0.134	-0.147
<b>Intermediate Monocytes</b>	-0.469	-0.27	0.057	-0.076	0.082	-0.560
<b>Pro-Inflammatory Monocytes</b>	-0.367	-0.336	0.232	0.105	0.498	-0.420

Numbers presented in the table are Pearson correlation coefficients ( $r$ ). \* indicates statistically significant correlation between % of E+30 heartrate and % of E+30 cell counts at the specified time point during exercise recovery,  $p < .05$ .

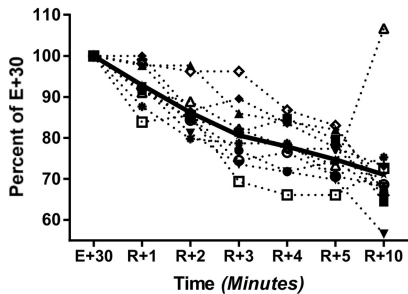
**“Core findings”****Highlights:**

- Lymphocytes and monocytes egress blood immediately upon cessation of acute exercise
- This cellular egress is rapid and evident within just 3 minutes of passive recovery
- Monocytes egress the peripheral circulation more rapidly than other leukocytes
- NK-Cells egress the peripheral circulation more rapidly than other lymphocytes
- The rate of lymphocyte egress correlates with recovering heart rate after exercise

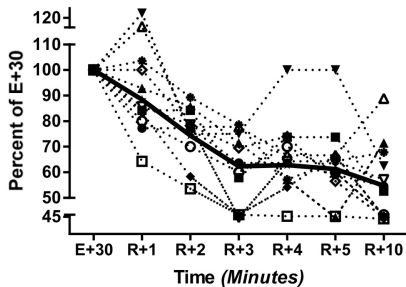
### Leukocytes



### Lymphocytes



### Monocytes



### Granulocytes

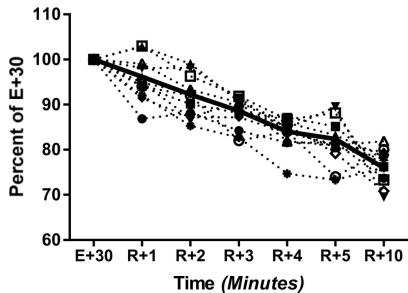
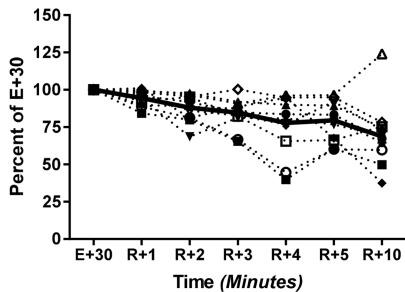
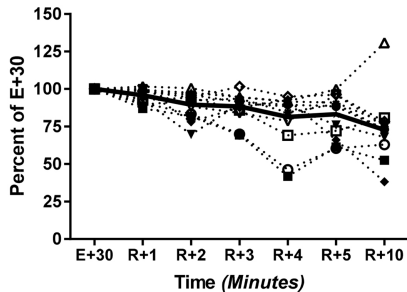


Figure 1

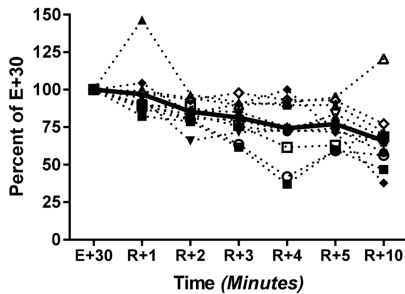
### CD3+ T-Cells



### CD4+ T-Cells



### CD8+ T-Cells



### $\gamma\delta$ T-Cells

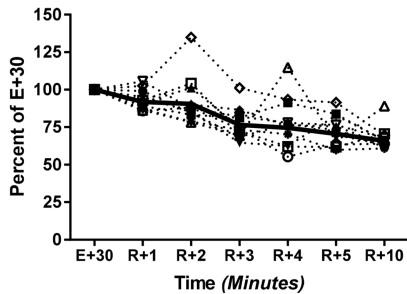
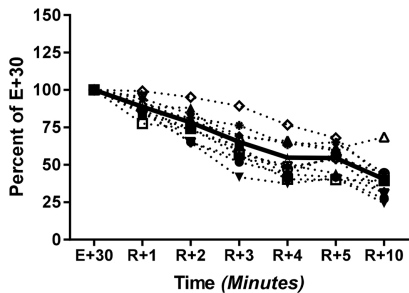
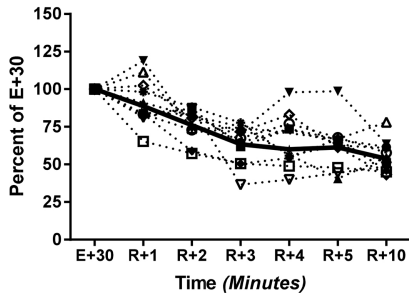


Figure 2

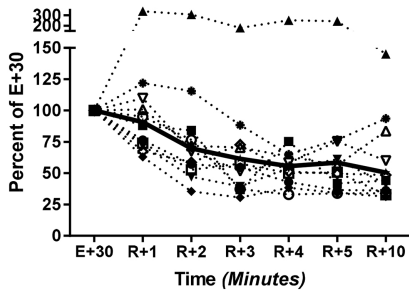
### NK-Cells



### Classical Monocytes



### Intermediate Monocytes



### Pro-Inflammatory Monocytes

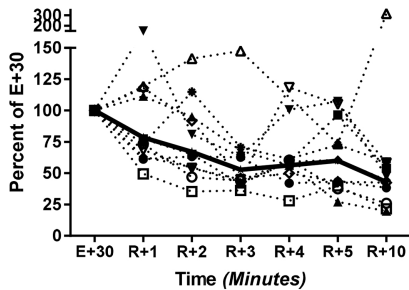


Figure 3



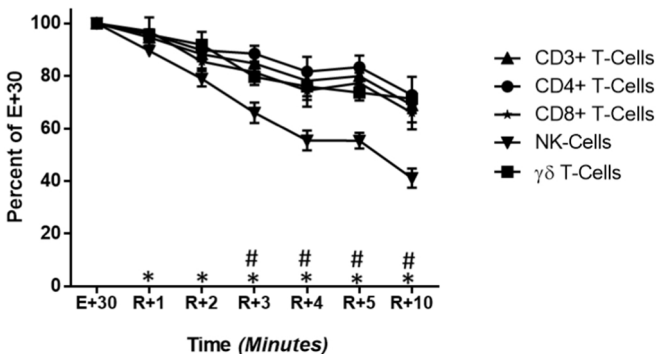
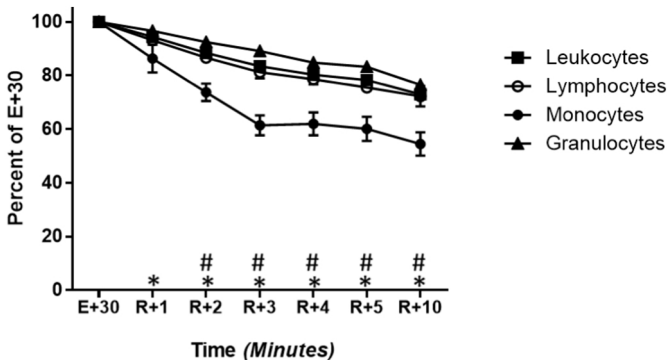


Figure 4