The influence of exercise training status on antigen-stimulated IL-10 production in whole blood culture and numbers of circulating regulatory T cells.

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ABSTRACT

PURPOSE. Highly trained athletes are associated with high resting antigen-stimulated whole blood culture interleukin (IL)-10 production. The purpose of the present study was to examine the effects of training status on resting circulating T regulatory (Treg) cell counts and antigen-stimulated IL-10 production and the effect of acute bout of exercise on the Treg response. METHODS. Forty participants volunteered to participate and were assigned to one of the 4 groups: sedentary (SED), recreationally active (REC), sprint-trained athletes (SPR) and endurance-trained athletes (END). From the resting blood sample $CD4^+CD25^+CD127^{low/-}$ Treg cells and *in vitro* antigen-stimulated IL-10 production were assessed. Ten REC subjects performed 60 min cycling at 70% of maximal oxygen uptake and blood samples for Treg analysis were collected post- and 1 h post-exercise. RESULTS. IL-10 production was greater in END compared with the other groups ($P < 0.05$). END had a higher Treg percentage of total lymphocyte count compared with SED ($P < 0.05$). A smaller proportion of Treg $CD4^+$ cells was observed in SED compared with all other groups ($P < 0.05$). IL-10 production significantly correlated with the proportion of Tregs within the total lymphocyte population ($r_s = 0.51$, $P = 0.001$). No effect of acute exercise was evident for Treg cell counts in the REC subjects ($P > 0.05$). CONCLUSION. Our results demonstrate that high training loads in END are associated with greater resting IL-10 production and Treg cell count and suggest a possible mechanism for depression of immunity commonly reported in athletes engaged in high training loads.

Key words: Interleukin-10, T regulatory cells, immune system, exercise, training

INTRODUCTION

From its conception in the early 1990s, the 'J' shaped curve has commonly been used to express the association between habitual physical activity level and risk of upper respiratory tract infection (URTI) (Gleeson et al. 2012; Matthews et al. 2002; Nieman 1994). Defined within this relationship, regular moderate exercise is seen to enhance immunosurveillance and immune function (Nieman 2000), in turn reducing the risk of such infections. Conversely, very prolonged bouts of strenuous exercise and periods of heavy training are seen to result in a transient depression of immune function (Gleeson 2007), resulting in an 'open window' for infection and increased the susceptibility to URTI (Nieman 2000). Observations from the athletic population have given support to these findings, with high training loads (Gleeson et al. 2012; Nieman 1990) and intensified periods of training (Fahlman and Engels 2005) reported to be associated with a higher incidence of URTI. Although higher relative risk of infection in physically active individuals has been linked with hormonal and adrenergic effects impairing immune system function (Pedersen and Hoffman-Goetz 2000), more recent evidence suggests that an enhanced anti-inflammatory cytokine response to antigen challenges (e.g. interleukin (IL)-10 and IL-4 production) may be also involved in driving this relationship (Gleeson et al. 2012; Wang et al. 2012).

In the last decade, mounting evidence suggests that training status can have substantial influences on IL-10 secretion in response to antigen challenge *in vitro (*Walsh et al. 2011a,b). Regular moderate intensity exercise, in the form of a 12-week tai chi chuan intervention programme, was seen to result in significant elevations in IL-10 secretion responses in middle-aged individuals (Yeh et al. 2006). More recently, elevations in antigen-stimulated whole blood IL-10 production have been reported in individuals engaged in high levels (~14 h/week) of moderate-vigorous endurance exercise compared with those performing relatively

moderate (~5 h/week) training loads (Gleeson et al. 2012). In addition to this, the authors also highlighted a significantly higher incidence of URTI episodes in those individuals performing higher training loads. It is therefore plausible that habitual exercise could modulate alterations in immune status, via modification of cell counts and/or cell sensitivity, which result in an enhanced IL-10 response and a subsequent heightened immune-depressive effect. Alterations in one such immune parameter, the proportion and/or number of regulatory T cells (Tregs) could be an important factor in this altered cytokine response as they are proposed to be the primary source of IL-10 within the body (Gleeson et al. 2011b).

Treg cells, a T lymphocyte subset, express CD4 and high levels of CD25, a receptor for the highly potent T cell growth factor IL-2. Furthermore, these cells are known to express high levels of Fork-head box protein 3 (FoxP3), which is the most specific marker of this population to date (Liu et al. 2006). Due to its intra-cellular nature, identification of this structural marker is problematic. However, recent discovery of an inverse relationship between CD127 and FoxP3 expressed on $CD4^{+}CD25^{+}$ cells (Liu et al. 2006) allows more easily attainable estimations of this Treg cell population via flow cytometry. Evidence suggests that circulating levels of Treg cells increase following an acute bout of strenuous exercise in humans (Wilson et al. 2009) and in response to a period of high intensity exercise training in a murine model (Wang et al. 2012). These elevations in Treg cells have also been reported in individuals engaged in regular resistance exercise (i.e. weight lifting), with a 3-fold greater level seen when compared with control subjects (unpublished data, cited in Nandakumar et al. 2009). It is therefore possible that elevations in IL-10 secretion may be associated with increases in the numbers of circulating Treg cells in response to chronic exercise training; however, this relationship has yet to be explored.

Thus, the aims of the present study were to examine possible associations between antigen-stimulated IL-10 secretion, resting circulating Treg cell counts and habitual physical activity load, including possible differences between sprint-trained and endurance-trained athletes (i.e. the effect of training type). Specifically, we wished to determine whether the percentage or numbers of Treg cells correlated with IL-10 production within a population of varying levels of habitual activity. We also wished to examine the effect of an acute bout of exercise on Treg cell numbers before and after exercise. We hypothesized that individuals engaged in greater levels of physical activity would have higher IL-10 production in response to antigen challenge, and that this would be accompanied by larger percentage and/or numbers of circulating Treg cells. We also hypothesized that an acute bout of submaximal exercise would elevate the circulating Treg cell percentage and count.

METHODS

Two separate studies were performed: (1) To identify the effect of different physical activity levels on Treg cell counts and *in vitro* IL-10 production, a cross-sectional design study was carried out; (2) A second study examined the influence of an acute bout of exercise on the blood Treg cell count in recreationally active participants.

Participants

Between May and July forty healthy male university students ranging in levels of habitual activity volunteered to participate in the cross-sectional study. Subjects ranged from sedentary individuals to Olympic triathletes. All participants completed a comprehensive health-screening questionnaire before testing commenced, and had declared absence from cold symptoms (cough, sore throat, blocked sinuses) in the 2-week period prior to testing. Participants were only included if they had abstained from all exclusion criteria, including smoking, excessive alcohol consumption (>20 units/week) and blood donations in the previous 3 months; determined via a short questionnaire. Participants were informed fully about the rationale for the study and the procedures involved, before providing written informed consent to participate in the study. Approval for the study had earlier been received from the Loughborough University ethical advisory committee.

Study Design

Participants were assigned to one of four sub-groups for cross-sectional analysis, based on current exercise training load and sports performed at a competitive level. The groups identified were sprint-trained athletes (SPR), endurance-trained athletes (END), recreationally active (REC), and sedentary (SED) individuals (Table 1). Participants were classed as SED if they did not engage in sport or exercise training; REC if they performed

regular exercise (moderate-to-vigorous exercise 2 to 3 times per week) but did not train or compete within a sport at a competitive level; SPR if they trained for and competed in short-distance track running events (100 – 800 m) or END if they trained for and competed in events of a prolonged duration (≥ 1500 m events, triathlon). For the acute exercise study, 10 REC cycled for 60 min at 70% maximal oxygen uptake ($\dot{V}O_2max$).

Questionnaires

Participants were required to complete the short form of the International Physical Activity Questionnaire (IPAQ; https://www.ipaq.ki.se/downloads.htm); cataloguing the frequency and duration of walking, vigorous intensity and moderate intensity activity they had partaken in during the previous 7 days. This questionnaire allows subsequent quantification of physical activity in metabolic equivalents (MET) hours per week, and is seen to be a reliable estimate of physical activity levels in an adult population between the ages of 18 – 65 years (Craig et al. 2003). For additional information, participants were required to complete a secondary questionnaire detailing their primary sport, how often they trained for this sport per week and the total duration of training per week.

Laboratory Visit

Cross-sectional study

Participants arrived at the laboratory at 08:00-09:00 following an overnight fast of at least 10 h, during which time only water consumption was permitted. Participants had been instructed to abstain from physical activity, alcohol consumption and caffeine consumption in the 24 h prior to the visit. Information about the study was provided before each participant gave informed consent. Following this, participants sat quietly for 15 min while they completed a comprehensive health-screening questionnaire, questionnaires assessing habitual activity and

training habits, and the inclusion/exclusion criteria questionnaire. Venous blood was collected via venipuncture from an antecubital vein into two Vacutainer tubes (Becton Dickinson, Oxford, UK) containing either K_3EDTA (4 mL) or heparin (7 mL). Measures of height (to nearest cm) and body mass (to 0.1 kg) were then taken.

Acute study

The same 10 REC individuals participated in the acute exercise study. Their VO_2max was determined using an incremental cycling protocol performed on an electromagnetically braked cycle ergometer (Lode Excalibur Sport V4.67, Groningen, The Netherlands). After an overnight fast, participants began cycling at 70 W; power output was increased in 35 W steps every 3 min until volitional fatigue. Expired gas was collected via Douglas bags during the final minute of each 3-min stage and during the final minute of the test. Heart-rate, using short range telemetry (model A3, Polar, Kempele, Finland), and ratings of perceived exertion (RPE) (Borg 1973), were also recorded during these periods to confirm true maximal values had been achieved.

Expired gas samples were passed through a gas analyzer (series 1400, Servomex, Crowborough, Sussex, UK), which had been calibrated with gases of known concentrations, in order to determine oxygen consumption and carbon dioxide production. Volumes were determined using a dry gas meter (Harvard Apparatus, Edenbridge, Kent, UK). The oxygen uptake at each power output was plotted on a scatter graph and the linear relationship was used to estimate a power output which would elicit 70% maximum oxygen uptake; this power output was used in the main trial.

Main trial

Participants arrived at the laboratory at 09:00 after an overnight fast (10-12 h) having refrained from caffeine and alcohol and without partaking in vigorous exercise in the previous 24 h. Participants sat for 15 min before a 12 mL venous blood sample was acquired through venipuncture from an antecubital vein and collected into two Vacutainer tubes as described previously. Following a short (< 5 min), self-paced warm-up each participant at room temperature (~22°C) for 1 h at the power output estimated to elicit 70% VO_2max. Drinking water was provided ad libitum but no food was ingested during the exercise and the first hour of recovery. Expired gas samples were collected and analyzed every 15 min to ascertain exercise intensity, and power output was adjusted if necessary. Heart-rate and RPE were also measured every 15 min. Immediately upon completion of exercise a 12 mL blood sample was collected in the same manner as before. The participant remained seated for the following hour before a final 12 mL venous blood sample was collected.

Haematological analysis

Immediately after collection, blood cell counts [Red blood cells (RBC), total leukocytes, lymphocytes, monocytes, neutrophils], as well as haemoglobin concentrations and haematocrit levels, were determined from blood collected in the K_3EDTA vacutainer using an automated cell counter (AcT^{TM}5diff haematology analyzer, Beckman Coulter, High Wycombe, UK). Based on duplicate analysis of 80 samples the intra-assay coefficients of variation for all variables were <3%.

Lymphocyte subsets

Fluorescent-conjugated monoclonal antibodies were used to identify specific cell surface markers (CD4, CD25, CD127) using via four color flow-cytometry (FACS-Calibur, Becton

Dickinson, Oxford, UK) with CellQuest analysis software (Becton Dickinson Biosciences, Oxford, UK). Briefly, 10µL of human regulatory T cell cocktail (Becton Dickinson Biosciences, Oxford, UK) was added to 120 µL heparinized whole blood and incubated in the dark for 20 min on ice. Erythrocytes were then lysed by adding 1.5 mL of lysis solution (FACS lysis buffer, Becton Dickinson Biosciences); following which the sample was incubated for a further 10 min, before being spun at 3500 rpm for 6 min at 6°C. The supernatant was then aspirated and the cells being re-suspended in phosphate buffered saline solution (PBS) containing 0.1% bovine serum albumin and 2 mM EDTA. The mixture was then spun for a further 6 min at 3800rpm, the supernatant aspirated and the cells re-suspended in 400 µL PBS. Forward-scatter versus side-scatter plots were used to gate lymphocytes based on size and density, with 50,000 lymphocyte events acquired per analysis. $CD4^+$ lymphocytes were further gated to identify $CD25^+$ cells, and those that also were $CD127^{low/-}$ (Fig.1). Values obtained represented a percentage of total lymphocytes for $CD4^+CD25^+CD127^{low/-}$ (Treg cells), with absolute values for $CD4^+CD25^+CD127^{low/-}$ cells calculated using the total lymphocyte count obtained previously via the automated cell counter. This calculation process was repeated to determine $CD4^+$ and $CD4^+CD25^+$ cell counts.

Antigen-stimulated IL-10 production

Briefly, 0.25 mL of heparinized whole blood was added to 0.75 mL of RPMI 1640 medium (Sigma Chemicals, Poole, UK) with an added 40 µL of Pediacel vaccine cocktail (Sanofi Pasteur msd Limited, Maidenhead, UK), containing diphtheria, tetanus, acellular pertussis, poliomyelitis, and hemophilus influenzae Type b antigens, at a dilution of 1:100, before being incubated at 37°C and 5% CO_2 for 24 h. The stimulant dilution of 1:100 used in this study was based on a separate experiment (unpublished data), which established the dose–response

curve for the measured cytokines over the dilution range of 1:100–1:20 000. Samples were then centrifuged at 15000 rpm for 4 min at 4°C, following which the supernatant fluid was harvested and stored at -20°C. At a later date, samples were thawed at room temperature before high sensitivity enzyme- linked immunosorbent assay (ELISA) was used to assess IL-10 concentrations (Quantikine HS100C, R&D systems, Minneapolis, USA). The intra-assay coefficient of variation for IL-10 was 6.4%.

Statistical analyses

All statistical tests were performed using SPSS (SPSS for windows version 18.0, SPSS Inc., Chicago, IL). Age and anthropometric measures were compared between groups using one-way ANOVA. Blood cell counts, haematocrit levels, haemoglobin concentrations, CD4+ cell counts, CD4+CD25+ cell counts, Treg lymphocyte counts, Treg lymphocyte percentages and IL-10 production were compared between groups by one-way ANOVA for normally distributed data or via non-parametric Kruskal-Wallis tests for data that were not normally distributed or where variances were not homogenous between groups. *Post hoc* analysis using Sidak or Mann-Whitney tests were used for further analysis of significant differences between groups for data that were normally and not normally distributed, respectively. Correlations between IL-10 production, levels of physical activity and resting cell counts were quantified using Spearman's coefficient correlation. The effects of acute bout of exercise on haematological variables and Tregs were analyzed using within-measures design ANOVA or Friedman's ANOVA with *post hoc* Sidak and Wilcoxon test for normally and not normally distributed data, respectively. Effect sizes (ES), r and ω, for the differences between groups were also determined. Statistical significance was set at $P < 0.05$ for all analyses, with all results presented as means ± standard deviations unless specified otherwise.

RESULTS

Cross-sectional study

There was no significant difference in age and height between groups. A significant difference was found for participants' body mass between groups, $F_{(3,36)} = 7.11$, $P = 0.001$, ES = 0.56, with END being lighter than SED. Similarly, a significant difference for BMI among groups was observed, $F_{(3,36)} = 6.82$, $P = 0.001$, ES = 0.55, with SED having a greater BMI than REC and END.

According to the analysis of data obtained via the IPAQ, there were significant differences for training loads in MET-h during the week before the baseline data were collected ($F_{(3,39)} = 10.113$, $P < 0.05$, ES = 0.64) with SED having lower training loads than SPR and END. Additionally, REC had lower training loads than END (Fig. 2).

There were no significant differences for any haematological variables between groups (Table 2), although red blood cell counts tended to be lower in END than in SED ($P = 0.06$, ES = 0.31). Similarly, no significant differences were observed in circulating neutrophil, lymphocyte and monocyte counts.

Whole blood culture antigen-stimulated IL-10 production was significantly affected by the training status, $H_{(3)} = 11.01$, $P < 0.001$ (Fig. 3). Planned contrasts revealed that IL-10 production was significantly greater in END when compared with all other groups ($P < 0.05$, ES $\geq$ 0.49).

A significant difference was found for the $CD4^{+}CD25^{+}CD127^{low/-}$ cell percentage of total lymphocyte count, with values higher in END compared with SED ($F_{(3,36)} = 3.279$, $P < 0.05$,

ES = 0.38; Fig 4A). Similarly, a significant difference was observed in the $CD4^+CD25^+CD127^{low/-}$ cell percentage of $CD4^+$ cell count ($F_{(3,36)} = 10.492$, $P < 0.001$, ES = 0.55) with values lower in SED compared with all other groups (Fig. 4B). No significant difference was found between groups for the absolute numbers of $CD4^+CD25^+CD127^{low/-}$ cells ($F_{(3,39)} = 2.206$, $P > 0.05$, ES = 0.29; Fig. 4C), though values tended ($P = 0.104$) to be lowest in SED.

The *in vitro* production of IL-10 by antigen-stimulated whole blood culture significantly correlated with $CD4^+CD25^+CD127^{low/-}$ cell percentage of total lymphocyte population (r_s = 0.51, $P = 0.001$), $CD4^+CD25^+CD127^{low/-}$ cell percentage of $CD4^+$ cell count ($r_s = 0.41$, P = 0.01) and with the absolute concentration of $CD4^+CD25^+CD127^{low/-}$ cells ($r_s = 0.38$, $P <$ 0.05). Additionally, the $CD4^+CD25^+CD127^{low/-}$ cell percentage of $CD4^+$ cell count correlated positively with MET-h/week ($r_s = 0.47$, $P < 0.01$).

Acute study

The VO_2max of the REC participants was 49.2 ± 7.3 mL/kg/min. These ten participants completed the 1 h cycle at an average of 158 ± 28 W, relative exercise intensity of 72.9 ± 5.9% of VO_2max, heart rate of 160 ± 12 bpm and RPE of 15 ± 1, indicating that exercise was perceived as "Hard".

Table 3 shows the changes in haematological variables and the main effect of time for each parameter. Exercise induced a significant leukocytosis ($F_{(1.000, 9.000)} = 23.181$, $P = 0.001$, ES = 0.84); *post hoc* tests revealed that the total leukocyte count increased from baseline to post-exercise ($P < 0.001$) and continued to increase 1 h post-exercise ($P < 0.001$). This increase was also observed for neutrophils (χ (2) = 17.590, $P < 0.001$); neutrophils significantly

increased from pre-exercise to post-exercise ($P < 0.01$, ES = 0.63) and further increased 1 h later ($P < 0.01$, ES = 0.63). Similarly, lymphocytes exhibited a significant biphasic response to exercise ($F_{(1.000, 9.000)} = 21.785$, $P = 0.001$, ES = 0.84). Exercise induced a 30% increase in lymphocytes at post-exercise ($P = 0.001$) compared with baseline values; however, at 1 h post-exercise values were significantly lower than at pre-exercise (24%, $P < 0.01$) and post-exercise (42%, $P < 0.01$).

There was no significant effect of acute exercise on $CD4^+CD25^+CD127^{low/-}$ cell percentage of total lymphocyte count (main time effect: $F_{(2,18)} = 1.170$, $P > 0.05$; Fig 5A). Similarly, no significant influence of acute exercise was observed for the $CD4^+CD25^+CD127^{low/-}$ cell percentage of $CD4^+$ cells ($F_{(2,18)} = 0.011$, $P > 0.05$; Fig 5B) or for the absolute concentration of $CD4^+CD25^+CD127^{low/-}$ cells ($F_{(2,18)} = 1.788$, $P > 0.05$; Fig 5C).

DISCUSSION

The purpose of this study was to examine the effects of different training loads on resting circulating Treg cell counts and whole blood culture antigen-stimulated IL-10 production. A further aim was to investigate changes in the circulating Treg cell count in response to an acute bout of submaximal exercise. The main findings of this study demonstrate that physically active individuals had higher resting circulating Tregs as a percentage of total lymphocyte count and of $CD4^+$ cells. The other main finding of the present study was a higher *in vitro* IL-10 production in END compared with the other groups. These findings support our hypothesis that high training loads are associated with a higher resting Treg cell counts and greater IL-10 production following antigen stimulation. These findings suggest that endurance exercise training results in higher percentage of circulating Tregs at rest. Considering the finding that an acute bout of exercise did not affect the circulating Treg cell percentage or count (albeit shown only for the REC participants), it appears likely that the higher Treg percentage at rest in END represents a chronic adaptation to high training loads. Furthermore, the higher *in vitro* IL-10 production in END implies that those engaged in high training loads have a greater capacity of whole blood culture to secrete this anti-inflammatory cytokine, which may come from Tregs as both percentages and numbers of these cells were positively correlated with *in vitro* IL-10 production.

IL-10, an anti-inflammatory cytokine, exerts numerous inhibitory effects on the immune system (Saraiva and O'Garra 2010). The principal function of IL-10 appears to be to limit and ultimately terminate inflammatory responses (Moore et al. 2001). IL-10, originally termed cytokine synthesis inhibitory factor, induces the downregulation of major histocompatibility complex II (MHCII) molecules, the intercellular adhesion molecule-1, the costimulatory B7 molecules on antigen presenting cells (APCs), expression of several pro-

inflammatory cytokines (e.g. IL-12) and toll-like receptor 4 (Maynard and Weaver 2008; McCoy et al. 2010). Thus, it has the potential to depress immunity if produced in excess. Although, the number of URTI episodes was not directly assessed in the present study, we have previously demonstrated an association between *in vitro* IL-10 production and incidence of the common cold in highly physically active people (Gleeson et al. 2011a, 2012), which further supports the hypothesis that IL-10 plays a key role in the immune-suppressing effects of chronic strenuous physical activity. The precise source of the IL-10 remains unknown but the present study suggests that Tregs are a strong possibility.

Tregs have been shown in numerous animal- and human-based studies to be the key cells mediating dynamic balance of the immune system (LeGuern 2011; Shalev et al. 2011). Tregs exert their immune-modulating effects using cytokine-independent and cytokine-based signaling pathways including IL-10-mediated immune depression (Delves et al. 2011; Taylor et al. 2006). These suppressive actions of Treg cells include: apoptosis (programmed cell death) of APCs; binding to B7 molecules on APCs, rendering the latter incapable of activating naïve T cells; local inhibition of T cell effector functions mediated by changes in intracellular signaling molecules and inhibition of dendritic cell function (Delves et al. 2011; Shalev et al. 2011). The results of the present study provide evidence of an association between high training load, *in vitro* antigen-stimulated IL-10 production and the numbers of circulating Treg cells, supporting the hypothesis that Treg cell-mediated inhibitory effects on the immune system via greater IL-10 production in highly physically active individuals play an important role in modifying URTI risk (Gleeson et al. 2011a, 2012). This hypothesis is also partially supported by the recently reported finding that a greater resting Treg cell percentage coincided with an increase of URTI episodes in a group of elite swimmers (Teixeira et al. 2011).

Our data are in line with previous murine and human studies and further expand our knowledge of the effects of chronic, high volume physical activity on *in vitro* IL-10 production and resting circulating Treg cell counts. For example, in mice Wang et al. (2012) demonstrated higher *in vivo* IL-10 production and $CD4^+IL10^+/CD4^+FoxP3^+$ Treg percentage following 6 weeks of high-intensity training. Furthermore, higher absolute circulating $CD4^+CD25^+$ Treg cell counts and a 1.8-fold increase in *in vitro* IL-10 production were observed in response to a 12-week tai chi chuan exercise programme in 37 middle-aged participants (Yeh et al. 2006). Surprisingly, the same protocol did not induce an increase in Treg cell counts in the case-control study (Yeh et al. 2009). It is possible that methods could partially explain discrepancies in both studies, where in the first Tregs were assessed by labeling CD4 and CD25 cell surface receptors, while in the latter study qPCR was chosen to quantify FoxP3 mRNA, a Treg characteristic transcription factor. Since Yeh et al. (2006) examined middle-aged participants, it remained unknown whether these training-induced effects on resting Tregs and IL-10 would be evident in younger and more physically active individuals. Indeed, this was the key finding of the present study. Furthermore, compared with SED, although SPR exhibited a higher proportion of Treg $CD4^+$ cells, only END had greater resting numbers of circulating Tregs and IL-10 production, despite no significant difference in the IPAQ-based training volumes between the two athletic groups. Consequently, it may be the nature of the training (i.e. long-lasting, predominantly aerobic versus high-intensity, short bursts of relatively more anaerobic exercise) that accounts for these differences.

The published literature examining the relationship between exercise training and Tregs reports cell data as either absolute numbers, as a proportion of a cell population, or both. In

the present study only the proportion of Tregs within the total lymphocyte and CD4+ population differed between the groups, and not the absolute numbers of Treg cells. In the context of IL-10 release it could be argued that the absolute number of Tregs is perhaps the most relevant measurement as it reflects the absolute capacity for IL-10 release in a given volume of blood. On the other hand, it could be argued that the proportion of Tregs within the lymphocyte/CD4+ population gives a better indication of the lymphocyte response to training stress as it reflects the preferential mobilisation of a particular phenotype within the circulating lymphocyte pool. Alternatively, this finding may simply reflect the variance that exists within the absolute and relative data given the relatively small numbers of participants in each group.

The other main finding of this study was that an acute bout of exercise of moderate intensity does not result in an increase in the percentage or count of circulating Treg cells implying that acute bout of exercise does not affect Treg cell numbers. It is noted that this suggestion may be limited to REC group and the time frame over which Tregs were examined. Whether the same is true for the other training status groups or higher exercise intensities remains to be determined. This finding is in contrast to a study by Wilson et al. (2009), where numbers of $CD4^{+}CD25^{+}$ cells were shown to rise following 6 and 7 min bouts of race-pace swimming in a cohort of 22 elite adolescent swimmers. Differences in results may be accounted for the less specific identification of the Treg population in the study by Wilson et al. (2009) or by the higher relative intensity of exercise that was used with the swimmers. As illustrated in Figure 1B, gating on the $CD4^{+}CD25^{+}$ cell population does not give a clear delineation of a distinct (Treg) population and additional staining for CD127 is needed to achieve a sufficiently specific identification of Tregs as recently suggested by De Serres et al. (2011).

Collectively, our data imply that chronic strenuous physical activity results in higher numbers of Tregs and IL-10 and may provide a mechanistic explanation for higher IL-10 concentration being associated with a higher risk of common cold in physically active individuals. The actual mechanisms by which chronic exercise training induces Treg cell count upregulation remain, however, unknown. Based on our findings, an acute bout of moderate exercise does not appear to induce a rise in Tregs. There is some evidence for a stimulatory role of IL-2 on Treg cell proliferation, differentiation and activation (De Serres et al. 2011; Malek 2003) and plasma levels of IL-2 are increased for several hours after prolonged exercise (Castell et al. 1997). Thus, repeated elevations of IL-2 availability with regular prolonged exercise sessions could be responsible for the elevated Treg count with endurance training. . In addition, Treg cell activation state, and therefore potentially their ability to produce IL-10 may be also mediated by immunoregulatory effects of circulating hormones such as glucocorticosteroids, suggesting another loop which may explain the variability (Xiang and Marshall, 2011).

It is concluded that the present study confirms and further expands the existing evidence that chronic physical activity induces greater *in vitro* antigen-stimulated IL-10 production in comparison with less active individuals. Additionally, chronic but not acute exercise results in a higher Treg cell count which positively correlates with IL-10 production. Although this may impair the immune response in endurance athletes when a new pathogen is encountered, long term effects of the enhanced anti-inflammatory state could be regarded as beneficial in counteracting low-grade inflammation that is associated with risk of cardiovascular disease, type 2 diabetes mellitus, obesity and cancer (Gleeson et al. 2011b).

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Identification of T_{reg} cells via flow cytometry. **(A)** – Forward scatter (FSC-H) versus side scatter (SSC-H) gating the total lymphocyte population (R1). **(B)** - CD4 (FL1-H) versus CD25 (FL3-H), gating CD4+ cells (R4). **(C)** – CD25 (FL3-H) versus CD127 (FL4-H) identifying CD4+CD25+$CD127^{low/-}$ (Treg) cells (R5).

Fig. 2. Training loads in MET-h/week among groups. # different from SPR and END, $P < 0.01$; † different from END, $P < 0.05$. SED – sedentary; REC – recreationally active; SPR – sprint-trained; END – endurance-trained. Data are expressed as mean ± SD.

Fig. 3. Whole blood culture antigen-stimulated IL-10 production. # different from other groups, $P < 0.05$. SED – sedentary; REC – recreationally active; SPR – sprint-trained; END – endurance-trained. Data are expressed as mean ± SD.

Fig. 4. $CD4^{+}CD25^{+}CD127^{low/-}$ cells percentage of total lymphocyte count **(A)**, percentage of CD4+ cell **(B)**, and absolute concentration **(C)**. # different from SED, $P < 0.05$; † different from other groups, $P < 0.05$. SED – sedentary; REC – recreationally active; SPR – sprint-trained; END – endurance trained. Data are expressed as mean ± SD.

Fig. 5. Effect of acute exercise on $CD4^{+}CD25^{+}CD127^{low/-}$ cell percentage of total lymphocyte count **(A)**, $CD4^{+}CD25^{+}CD127^{low/-}$ cell percentage of $CD4^{+}$ cells **(B)** and absolute concentration of $CD4^{+}CD25^{+}CD127^{low/-}$ cells **(C)**. Pre – before exercise; Post – post exercise; 1h Post – 1h after exercise. Data are expressed as mean ± SD.

Table 1 Participant characteristics

	SED	REC	SPR	END
Age (years)	23 ± 1	22 ± 1	23 ± 3	24 ± 3
Height (cm)	177 ± 8	178 ± 6	182 ± 5	178 ± 5
Body mass (kg)	79.7 ± 6.6	72.4 ± 6.7	76.5 ± 6.9	67.3 ± 5.0*
BMI (kg/m^2)	25.7 ± 3.2	22.8 ± 1.4*	23.0 ± 1.9	21.2 ± 2.0*

Data are mean ± SD. n = 10 for all groups. * different from SED, $P \leq 0.05$. SED – sedentary; REC – recreationally active; SPR – sprint-trained; END – endurance-trained; BMI – Body Mass Index.

Table 2 Hematological variables and leukocyte counts

	SED	REC	SRP	END
RBC ($x10^{12}$/L)	4.80 ± 0.19	4.57 ± 0.40	4.60 ± 0.28	4.45 ± 0.22
Hb (g/L)	156 ± 8	156 ± 13	157 ± 7	147 ± 9
Hct (%)	45.2 ± 1.6	44.8 ± 4.0	44.3 ± 2.3	43.0 ± 2.5
WBC (x 10^9/L)	5.5 ± 1.6	5.7 ± 0.8	4.6 ± 1.1	5.6 ± 1.2
Ne (x 10^9/L)	2.7 ± 0.9	2.5 ± 0.6	2.2 ± 1.0	3.3 ± 1.6
Ly (x 10^9/L)	2.0 ± 0.6	2.4 ± 0.5	1.9 ± 0.3	2.0 ± 0.5
Mo (x 10^9/L)	0.50 ± 0.20	0.46 ± 0.09	0.41 ± 0.07	0.51 ± 0.15

Data are mean ± SD. n = 10 for all groups. RBC – red blood cells; Hct – hematocrit; Hb – hemoglobin; WBC – white blood cells; Ne – neutrophils; Ly – lymphocytes; Mo – monocytes; SED – sedentary; REC – recreationally active; SPR – sprint-trained; END – endurance-trained.

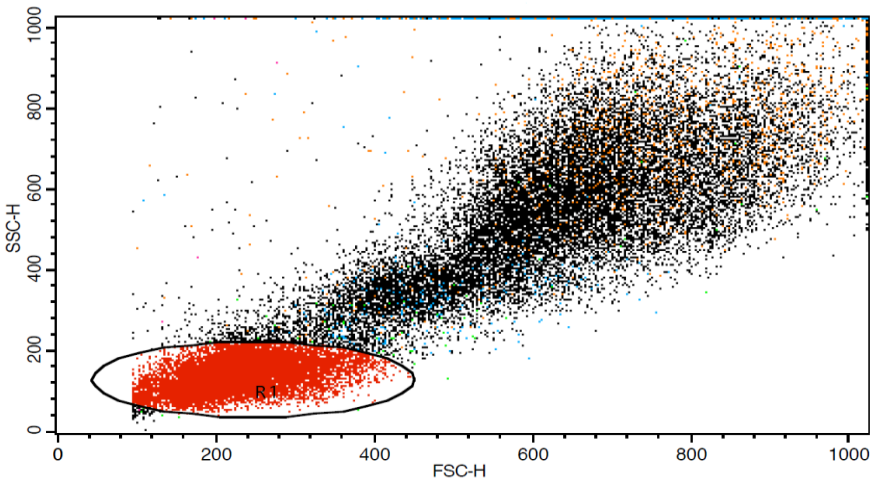
Table 3 Effect of acute exercise on hematological variables and leukocyte counts

	Pre-Exercise (n = 10)	Post-Exercise (n = 10)	1 h Post-Exercise (n = 10)
RBC (x 10^6/μL)	4.58 ± 0.40	4.85 ± 0.43*	4.64 ± 0.40†
Hct (%)	44.8 ± 4.0	47.8 ± 4.3*	45.7 ± 3.9†
Hb (g/L)	156 ± 13	165 ± 14*	158 ± 13†
WBC (x 10^9/L)	5.69 ± 0.84	7.76 ± 1.33*	11.40 ± 3.59*†
Ne (x 10^9/L)	2.50 ± 0.62	3.63 ± 1.07*	8.56 ± 3.15*†
Ly (x 10^9/L)	2.35 ± 0.53	3.07 ± 0.97*	1.79 ± 0.39*†

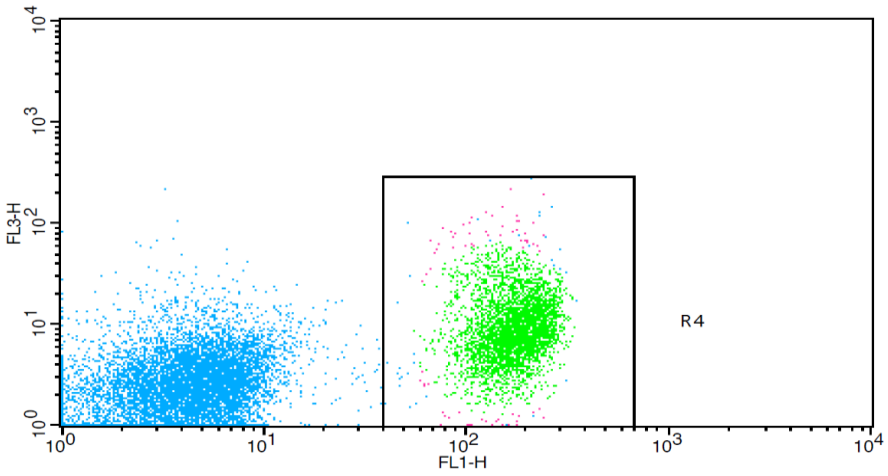
Data are mean ± SD. * different from pre-exercise, $P < 0.05$; † different from post-exercise, $P < 0.05$. RBC – red blood cells; Hct – hematocrit; Hb – hemoglobin; WBC – white blood cells; Ne – neutrophils; Ly – lymphocytes.

(Figure 1)

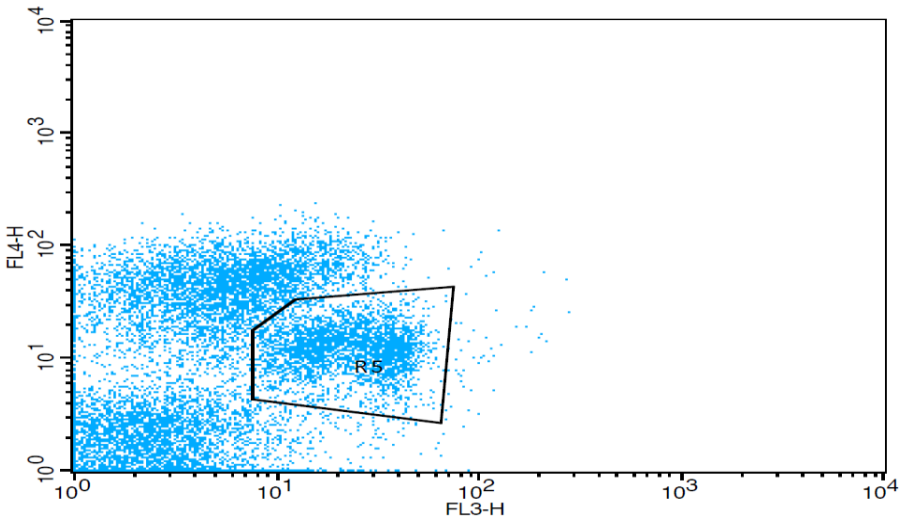
A



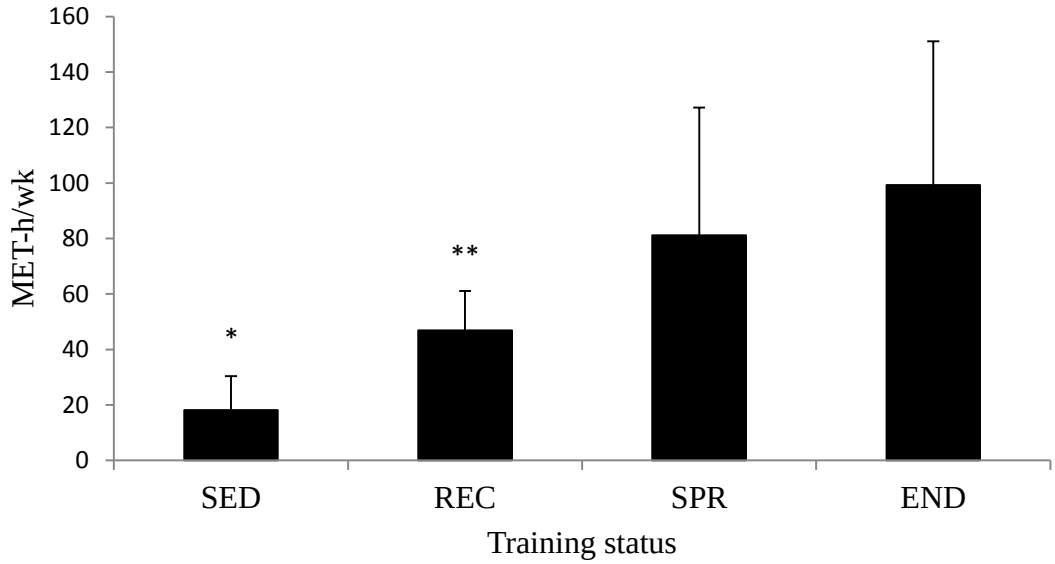
B



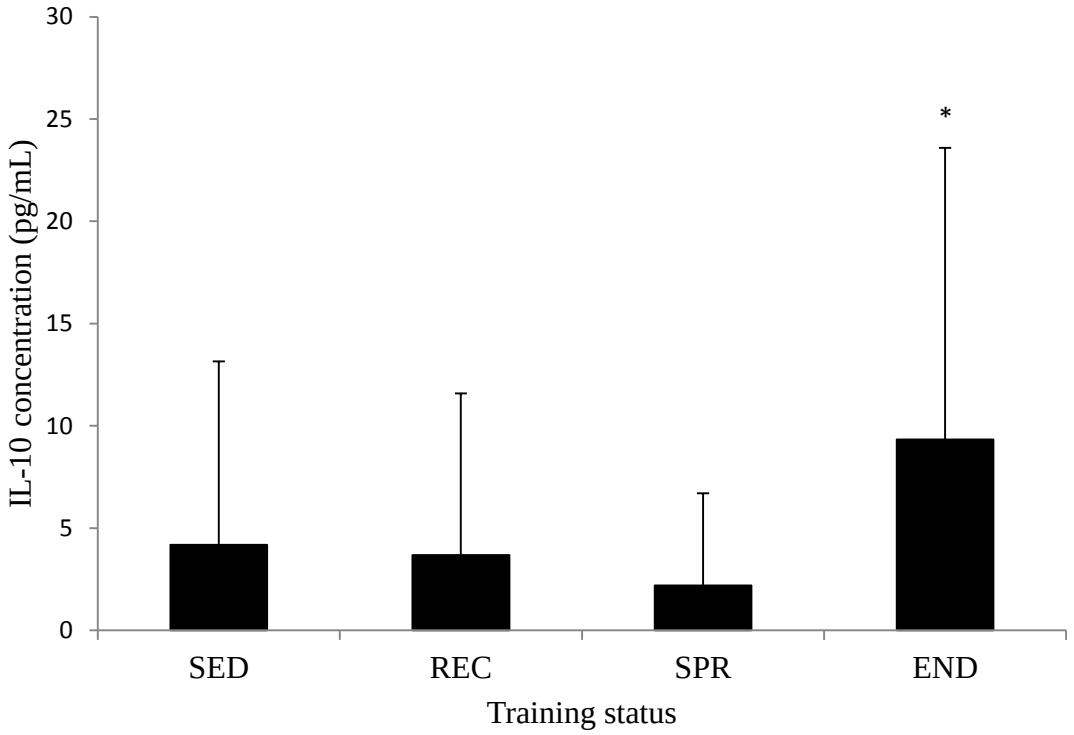
C



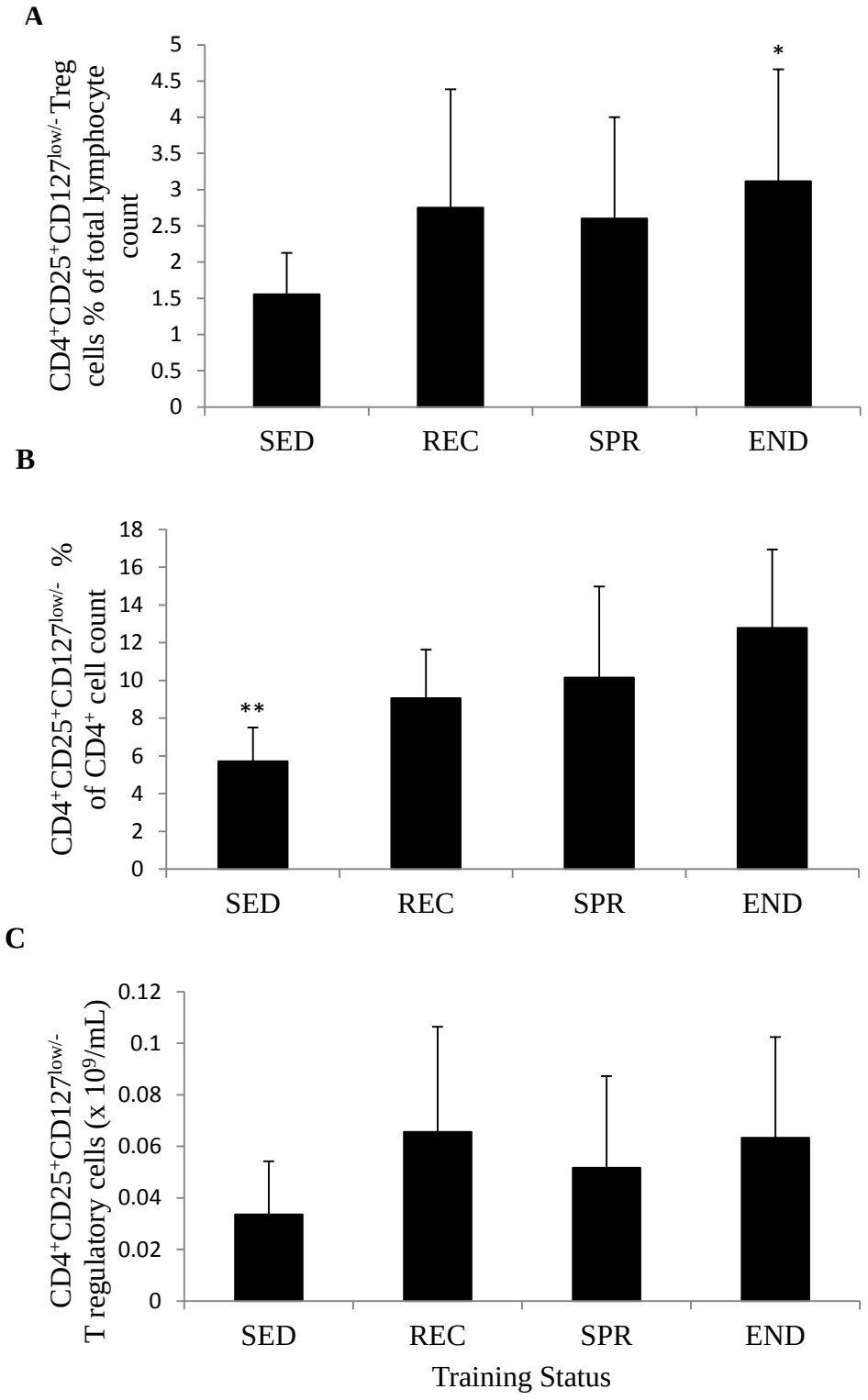
(Figure 2)


160
140
120
100
80
60
40
20
0
MET-h/wk
*
**
SED
REC
SPR
END
Training status

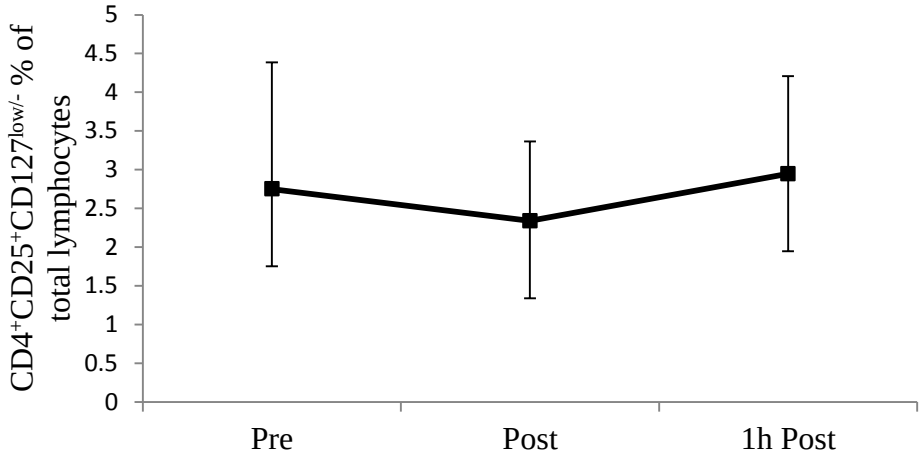
(Figure 3)


30
25
20
15
10
5
0
IL-10 concentration (pg/mL)
*
SED
REC
SPR
END
Training status

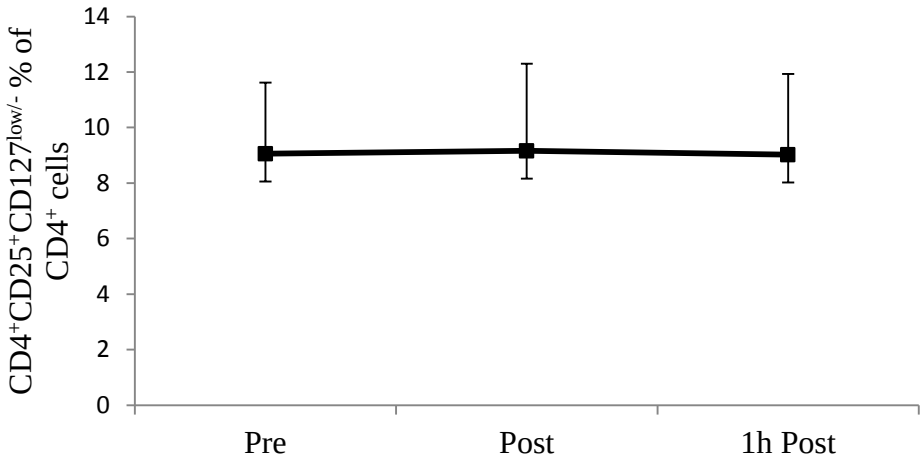
(Figure 4)


A
CD4+CD25+CD127low/- Treg cells % of total lymphocyte count
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
SED
REC
SPR
END
B
CD4+CD25+CD127low/- % of CD4+ cell count
18
16
14
12
10
8
6
4
2
0
**
SED
REC
SPR
END
C
CD4+CD25+CD127low/- T regulatory cells (x 10^9/mL)
0.12
0.1
0.08
0.06
0.04
0.02
0
SED
REC
SPR
END
Training Status

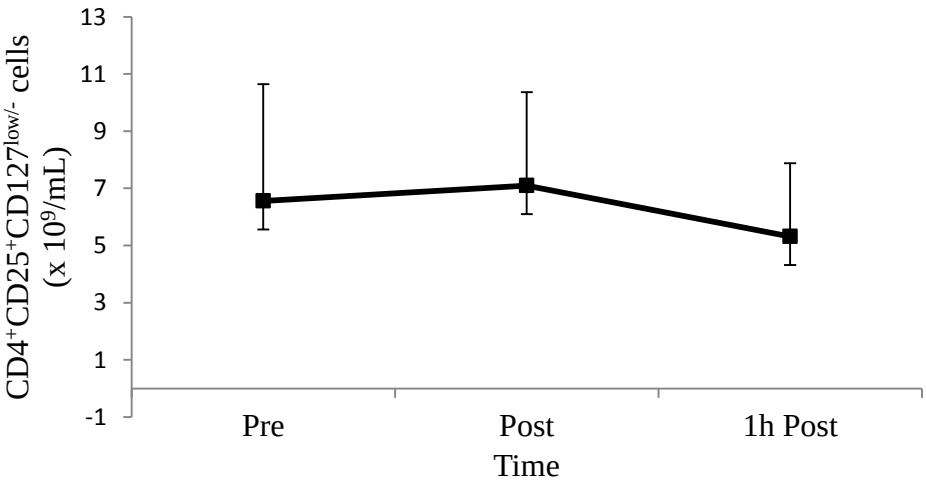
(Figure 5) **A**


CD4+CD25+CD127low/- % of total lymphocytes
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Pre
Post
1h Post

B


CD4+CD25+CD127low/- % of CD4+ cells
14
12
10
8
6
4
2
0
Pre
Post
1h Post

C


CD4+CD25+CD127low/- cells (x 10^9/mL)
13
11
9
7
5
3
1
-1
Pre
Post
1h Post
Time