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Changes in naïve and memory T-cells in elite swimmers during a winter training season

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Abstract

High intensity training regimens appear to put athletes at a higher risk of illness. As these have been linked to alterations in the proportions of differentiated T cells, how training load affects these populations could have important implications for athlete susceptibility to disease. This study examined the effect of a winter training season on the proportions of circulating naïve and memory T cells subsets of high competitive level swimmers.

Blood samples were taken at rest at 4 time-points during the season: before the start of the season (t0-September), after 7 weeks of an initial period of gradually increasing training load (t1-November), after 6 weeks of an intense

training cycle (t2-February) and 48 hours after the main competition (t3-April) and from eleven non-athlete controls at 2 similar time-points (t2 and t3). CD4, CD8 and gamma-delta ($\gamma\delta$) T cells expressing the naïve (CCR7⁺CD45RA⁺), central-memory (CM-CCR7⁺CD45RA⁻), effector-memory (EM-CCR7⁺CD45RA⁻) and terminal effector (TEMRA-CCR7⁺CD45RA⁺) were quantified by flow cytometry. Statistical analyses were performed using multilevel modeling regression.

Both T CD4⁺ naïve and CM presented a linear increase in response to the first moment of training exposure, and had an exponential decrease until the end of the training exposure. As for TCD4⁺ EM, changes were observed from t2 until the end of the training season with an exponential trend, while TCD4⁺ TEMRA increased linearly throughout the season. TCD8⁺ naïve increased at t1 and decreased exponentially thereafter. TCD8⁺ TEMRA values decreased at t1 and increased exponentially until t3. $\gamma\delta$ T-EM had an increase at t1 and an exponential decrease afterwards. In contrast, $\gamma\delta$ T-TEMRA decreased at t1 and exponentially increased during the remaining 20 weeks of training.

An increase in TEMRA and EM T cells alongside a decrease in naïve T cells could leave athletes more susceptible to illness in response to variation in training stimulus during the season.

Keywords: training load; CCR7; CD45RA; $\gamma\delta$ T cells; exercise

Introduction

Periods of heavy training appear to increase the incidence of upper respiratory tract symptoms (URS) in elite athletes (Bermon 2007) with infectious episodes often impairing training and performance and reducing the likelihood of success in high-level competition (Pyne et al, 2006). The mechanisms that could explain this increased susceptibility to infection are likely to be multi-factorial and could include alterations in the natural killer (NK) cell and T cell compartments. Such changes could be caused by repeated elevations of stress hormones (e.g. cortisol) and anti-inflammatory cytokines (e.g. interleukin-10) that occur in response to prolonged strenuous exercise during periods of heavy training.

There is only limited information on the effect of training load during a training season on the recirculation of lymphocytes between the tissues and the blood, their maturation status and how this may affect susceptibility to disease. Recently we reported that the numbers of CD56dim NK cells in the peripheral blood (PB) decrease during a swimming training season (Rama et al. 2013). This study suggests that periods of highly demanding training have a negative impact on innate immunity mediated by NK cell subsets, which could partially explain the higher frequency of URS observed during these training phases.

Another lymphocyte population with cytotoxic functions and important in host defense is the gamma-delta ($\gamma\delta$) T cell subset which are cells that possess a distinct T cell receptor (TCR) on their surface. Most T cells have a TCR composed of two glycoprotein chains called α and β TCR chains (so called $\alpha\beta$ T cells). In contrast, $\gamma\delta$ T cells, have a TCR that is made up of one γ chain and one δ chain. $\gamma\delta$ T cells represent approximately 5% of total T cells in PB, but comprise up to

1 50% of T cells in epithelial tissues, such as the lining of the gastro-intestinal tract
2 where they act as sentinel cytotoxic cells involved in the elimination of bacterial
3 infection (Nakasone et al. 2007) and wound repair (Girardi 2006). They are also
4 have immunomodulatory and antigen presenting capabilities (Moser and
5 Brandes 2006). They are known to be mobilized during acute psychological
6 stress, exercise and β -adrenoreceptor agonist infusion (Anane et al. 2009). To
7 date, no chronic studies on the effects of varying exercise training loads on
8 circulating numbers of $\gamma\delta$ T cells have been reported.
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10 It is possible to distinguish between naïve and memory/effector T cells subsets
11 by the reciprocal expression of the CD45RA or CD45RO isoforms and the
12 expression of CCR7 (Sallusto et al. 1999), a chemokine receptor that that controls
13 homing to secondary lymphoid organs distinguishes human memory T cells into
14 two functionally distinct subsets. CCR7⁻ memory cells express receptors for
15 migration to inflamed tissues and display immediate effector function. On the
16 other hand, CCR7⁺ memory cells express lymph-node homing receptors and lack
17 immediate effector function, but stimulate dendritic cells and differentiate into
18 CCR7⁻ effector cells upon secondary stimulation (Sallusto et al. 1999). Four
19 different T cell subsets can be identified: one naïve CD45RA⁺CCR7⁺ and three
20 memory subsets CD45RA⁻CCR7⁺ termed central memory (CM), CD45RA⁻CCR7⁻
21 termed effector memory (EM) and CD45RA⁺CCR7⁻ termed terminally
22 differentiated effector memory cells (TEMRA) (Sallusto et al, 1999). The CCR7⁺
23 subsets also express high levels of L-selectin (CD62L), the receptor for homing to
24 peripheral and mesenteric lymph nodes, while the CCR7⁻ subsets lack CD62L but
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express high levels of $\beta 1$ and $\beta 2$ integrins which are required for homing to inflamed tissues (Lanzavecchia and Sallusto 2005).

The aim of this study was to longitudinally examine the effect of chronic training on the levels of circulating naïve and memory T cells subsets in PB of high competitive level swimmers during a winter training season and to relate them to training load variation and the occurrence of Upper Respiratory Symptoms (URS). To this end we examined the changes in the proportion of $\alpha\beta$ and $\gamma\delta$ T cells that expressed the CD45RA and CCR7 antigens within the CD4+ and CD8+ T-cell subsets, at different times of the training season, including moments of increased training volume and intensity.

2. Methods

2.1. Participants

A group of 13 high level Portuguese swimmers, 10 males (17.2 ± 1.1 years, 175 ± 3 cm height and 64.6 ± 4.0 kg weight) and 3 females (16.1 ± 0.6 years, 168 ± 10 cm height and 57.6 ± 2.5 kg weight) and a group of non-athletes, 7 males (18.6 ± 1.1 years; 172 ± 5 cm height and 79.9 ± 14.6 kg weight) and 4 females (19.8 ± 0.5 years; 162 ± 3 cm height and 58.5 ± 6.9 kg weight) were recruited for this study which was approved by the Ethics and Human Subjects Review Board at the Faculty of Sports Science and Physical Education, University of Coimbra. All the participants and their tutors were informed of the objectives and procedures of the study and gave their written informed consent.

2.2. Experimental design

1 Training load, volume, intensity and participation in competition events, were
2 monitored during 29 weeks of a winter swimming season from September to
3 April. According to the usual methodologies used to quantify the training
4 workload in swimming, the activities were categorized in different training
5 zones. Dry land training activities were quantified by time and converted to
6 swimming distance as proposed by Mujika and colleagues (Mujika et al., 1995).
7 The distance values were multiplied by the intensity factor and totaled. The
8 magnitude of the load was expressed in arbitrary units of load (AUL), quantified
9 from the calculated sum of the volumes swam in each of the zones, multiplied by
10 the respective intensity factor, and the total volume effectively completed
11 (Mujika et al., 1995).
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28 Blood samples were taken, at rest, at four specific times during the swimming
29 winter season: before the start of the season and after an off season period of 5
30 to 6 weeks (t0), after the first 7 weeks of gradual increasing the training load
31 (t1), in the 24th week of training after an intense training cycle (t2), and in the
32 29th week of training, 48 hours after a major competition (t3).
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41 During a similar winter period blood samples were obtained from the non-
42 athlete group at two time points (February and April) matching the time points
43 of the swimmers' group. These volunteers were not regularly involved in sport
44 and did not spend more than 1 hour/week in recreational physical activities.
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52 At the times of blood collection both swimmers and controls were in good health
53 and were not using medication that could affect immune function and
54 inflammation. All subjects were non smokers and non alcohol consumers
55 (Spence et al. 2007).
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1 URS episodes in athletes and controls were monitored using daily log books, (1
2 episode was defined as repetition of more than two symptoms on at least two
3 consecutive days). A new episode was considered after a minimum interval of 10
4 days following the previous one (Bishop, 2006). The list of symptoms was in
5 accordance with the influenza-like-illness survey - European Influenza
6 Surveillance Scheme (EISS) - and included cold, cough, nasal secretion,
7 headache, sore throat, muscle pain, diarrhoea, abdominal pain, cold shivers, itchy
8 eyes, sneezing and fever. Symptoms related to allergic episodes (itchy eyes,
9 sneezing), gastrointestinal or muscular pain related to injuries were careful
10 analysed and discarded (Spence et al., 2007).
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27 2.3 Blood collection

28 20 mL of peripheral blood (PB) from an antecubital vein were collected by
29 venepuncture into EDTA containing tubes, always at the same time of day, before
30 the first afternoon workout of the week (16:00), after 30 minutes of resting in
31 sitting position. A time lapse of 36 to 48 hours of rest after the last training
32 session or competition was always maintained.
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45 2.4 Immunofluorescent staining

46 Specific lymphocyte populations were identified by immunofluorescent staining
47 of whole blood performing a four-colour immunophenotyping strategy. The
48 following monoclonal antibodies (mAbs) combinations were used: CD45RA-FITC
49 (clone HI 100; BD Pharmingen, San Diego, C.A. USA), CD3-PerCP-CY 5.5 (Clone
50 SK7, BD Biosciences, San Jose, CA, USA), CD195 (CCR7) PE (clone 3D12; BD
51 Pharmingen) and CD8-APC (clone: SK1;BD Bioscience). Another experiment was
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performed to $\gamma\delta$ T cells characterization: CD45RA-FITC (clone HI 100; BD Pharmingen), CD3-PerCP-CY 5.5 (Clone SK7, BD Biosciences, San Jose, CA, USA), CD195 (CCR7) PE (clone 3D12; BD Pharmingen) $\alpha\gamma\delta$ TCR-APC (clone. B1; BD Pharmingen).

Briefly, PB aliquots were labelled and incubated for 10 minutes at room temperature in the dark. After this incubation period, 2 ml of FACS Lysing Solution (BD Biosciences) diluted 1:10 (vol/vol) in distilled water was added and after 10 minutes of incubation the cells were washed with 2 ml of PBS and resuspended in 0.5 ml of PBS, and stored in the dark at 4°C, until acquisition in the flow cytometer.

2.5 Flow cytometry data acquisition and analysis

The data acquisition was performed in two consecutive steps in a FACSCalibur flow cytometer (BD Biosciences) equipped with an argon ion laser and a red diode laser. In the first step, 20.000 events, corresponding to all nucleated cells present in the sample, were collected and information stored. In the second step, information was stored exclusively for those cells included in a live gate in the lymphocyte region containing 20.000 events. The FACSCalibur machine was routinely calibrated using Calibrite beads (BD Biosciences) and single labelled antibody tubes were used for further compensation adjustments. Total Lymphocytes were gated using forward versus side scatter.

Data analysis was performed in the Infinicyt version 1.4 software (Cytognos, Salamanca, Spain). Absolute counts were calculated using a dual platform methodology (flow cytometry and hematological cell analyser – Beckman Coulter LH 750, Miami, USA).

1 T lymphocytes were identified according to their positivity for CD3 and typical
2 light scatter. Among positive CD3 cells, CD4 T cells were analyzed by the
3
4 expression of CD3 and absence of CD8; instead, CD8 T cells were identified by
5
6 the co-expression of both markers after exclusion of the $\gamma\delta$ T cell subset
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8 according to their higher reactivity with anti-CD3 monoclonal antibody and
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10 typical light scatter. $\gamma\delta$ T cells were identified by the positive expression for both
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12 CD3 and $\gamma\delta$ TCR. They were subsequently further differentiated into naïve, CM,
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14 EM and TEMRA subsets by CD45RA expression in combination with CCR7.
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23 **Statistical Analysis**

24 2.6. Statistical analysis

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27 Initially visual inspection of stem-and-leaf plots was made to inspect the
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29 distribution of immunological responses with training. Descriptive statistics for
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31 all dependent variables at all measurement points are presented as mean $\pm$
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33 standard deviation. Changes in immunological responses as a consequence of
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35 training were examined based on multilevel modeling regression using a
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37 polynomial model. At Level 1, each participant's successive measurements over
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39 time were defined by an individual change trajectory, considering both the
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41 possibility of linear (linear term) and non-linear (linear term squared) changes,
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43 and random error. At level 2, differences in trajectories between groups of
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45 individuals were examined. All parameters were fixed with the exception of the
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47 constant (intercept term) linear change trajectory and squared change trajectory
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49 parameters, which were allowed to vary randomly at level 2 (between
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51 individuals). Inspection of autocorrelation between correlation between
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successive measurements over time and dependent variables variance at each measurement moment were considered in the adoption of covariance matrixes at both Level 1 and Level 2. Akaike information criterion (AIC), that take into account the different number of fitted parameters in the two model structures to be compared, as well as visual inspection of residual plots were used to determine the model's validity to fit immunological responses as a consequence of training. Significance was set at $p < 0.05$. Statistical analyses were performed using mixed linear procedures available on SPSS version 20.0 (SPSS, Chicago, IL).

3. Results

3.1. Training workload

The training program followed the usual approach in swimming sport (Maglischo 2003), showing a gradual increment in training load in the first phase (first 7 weeks), followed by periods of increased intensity (from week 8 to week 24) while maintaining high volume during the central period of the season and a decrease of volume towards the end of the winter training season (week 30) in preparation for the main competition. Training data representing the weekly volume swum in each intensity zone is shown in table 1. Intensity was calculated as proposed by Mujika and colleagues (Mujika et al. 1996; Mujika et al. 1995). Training volume was significantly higher at t1 and lower at t3. Intensity increased significantly at t2 and remained elevated at t3 as was expected in the taper period just before the main winter competition ($p < 0.05$).

3.2. URS occurrence

A total of 23 episodes that lasted 10.5 (SD = 9.5) days corresponding to a mean of 1.8 (SD = 1.7) URS episodes per athlete were reported during the study period. When looking at the 4 weeks before and the 2 weeks after the selected time points we found that 71% of the episodes occurred between the 7th and 24th weeks of training, i.e., the hardest training phases. Data analysis confirmed that the higher frequency of URS occurred during and soon after times of hard training, with some URS concurrent with a seasonal flu peak in December. The control group reported no URS episodes in the two weeks previous to the analyzed time-points. Other reported episodes out of this period of time were not considered as they occurred too much apart from the time of blood collection. No associations between changes in cell populations and experience of URS were found.

3.3. Lymphocytes

Table 2 displays the summary data and statistical analysis results for the lymphocyte and lymphocyte subset numbers and percentage at each time point analysed during the training season. Multilevel regression analysis for leukocytes, lymphocytes, T lymphocytes, CD3⁺CD4⁺, CD3⁺CD8⁺ and $\gamma\delta$ T cell changes with training in the athletes are summarized in table 3. All the variables showed no significant change with training at all measurement points. No changes were observed for any of the lymphocyte populations in the control group. However, when the athletes' baseline values were compared to those of the controls, differences were found for the number of CD3⁺T cells ($p < 0.001$), number and percentage of TCD8⁺ ($p = 0.012$; $p = 0.035$) and number of $\gamma\delta$ T cells ($p < 0.001$), these cell populations being higher for the control group.

3.4. CD8 and CD4 $\alpha\beta$ and $\gamma\delta$ T lymphocyte subsets

The summary data and statistical results for the CD8⁺ and CD4⁺ $\alpha\beta$ and $\gamma\delta$ T lymphocyte subsets, as characterized by CD45RA and CCR7 expression are shown in tables 4 and 5.

Multilevel regression analysis for TCD4⁺ subpopulation changes to training in the athletes are summarized in Table 4 and Figure 1. With the exception of TCD4⁺ TEMRA, all variables show a curvilinear trend in response to training. Both TCD4⁺ naïve and TCD4⁺ CM presented a significant linear increase in response to the first moment of training exposure ($p < 0.05$), and had a significant exponential decrease until the end of the training exposure ($p < 0.05$). As for TCD4⁺ EM, no significant change was observed after the first 7 weeks of training ($p < 0.05$), but an exponential increase was present at mean rate of 1.1% thereafter until the end of the training season ($p < 0.05$). A significant positive linear trend was observed for TCD4⁺ TEMRA in response to training ($p < 0.05$).

Multilevel regression analysis for TCD8⁺ and $\gamma\delta$ T subpopulation changes to training in the athletes are summarized in Table 5 and Figures 2 and 3. Both TCD8⁺ CM and TDC8⁺ EM subsets showed no significant response to training at all measurement points. TCD8⁺ naïve cells had a significant positive response to the first period of training, and a significant exponential decrease thereafter at a mean rate of 4.7% ($p < 0.01$). Also TCD8⁺ TEMRA cells had a non-linear response to training; however, initially their values significantly decreased and after the first 7 weeks of training exposure the responses increased exponentially at a rate of 3.4% ($p < 0.01$). Interestingly, non-linear trends of response with similar

1 magnitudes, but opposite directions were observed for $\gamma\delta$ T EM and $\gamma\delta$ T TEMRA
2 subsets in response to training. $\gamma\delta$ T EM cells had an increase with initial
3 exposure to training and a significant exponential decrease afterwards until the
4 end of the training season. In contrast, $\gamma\delta$ T TEMRA cells had a decrease after the
5 first 7 weeks of training exponentially increased during the remaining 20 weeks
6 of training.
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10 When comparing the control to the athlete group at baseline, differences were
11 found for the number of both CD4⁺ and CD8⁺ naïve (both $p < 0.001$), EM (both
12 $p < 0.001$) and TEMRA (both $p < 0.001$) $\alpha\beta$ T cell subsets. Naïve cell values were
13 higher in the swimmers while the EM and TEMRA cell values were lower. The
14 percentage of EM $\gamma\delta$ T cells was also lower in the swimmers ($p < 0.002$) while the
15 percentage for the TEMRA $\delta\gamma$ T cell subset was higher.
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31 Overall, the results from the multilevel models suggest there was significant
32 residual variance within individual responses (level 1) to be explained for all
33 dependent variables. With the exception of TCD8⁺ EM, $\gamma\delta$ T EM, $\gamma\delta$ T TEMRA and
34 CD3⁺CD8⁺, there was no significant residual variance to be explained across
35 individuals, i.e., at level 2, indicating that the mean response is consistent
36 between the athletes.
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48 **4. Discussion**

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50 This study examined the impact of training load during a 29-week swimming
51 winter season on the percentages of naïve, central memory, effector memory and
52 terminal effector memory within CD4⁺ and CD8⁺ T-cell subsets, and $\gamma\delta$ T cells, in a
53 cohort of national level swimmers. It was found that the response to the first 7
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1 weeks of training, with a gradual increase in training volume, induced an
2 increase in the percentages of both CD4⁺ and CD8⁺ naïve T cells, followed by a
3 decrease until the end of the season. This was accompanied by an increase in the
4 percentages of EM and TEMRA CD4⁺ cells throughout the season, and a decrease
5 in the percentages of TEMRA CD8⁺ and $\gamma\delta$ T cells in the first 7 weeks of training
6 followed by an increase of these subpopulations until the end of the season. A
7 study on the impact of a 6-month training preparation for and Ironman triathlon
8 race on naïve and memory T cells (Cosgrove et al, 2012) also reported an
9 increase with training in CD8⁺ effector memory (CD45RA⁻/CD45RO⁺) T-cells and
10 CD4⁺ and CD8⁺ terminal effector memory (CD45RA⁺/CD45RO⁺) T cell-subsets. A
11 recent study by Witard and colleagues (Witard et al. 2012) indicated that the
12 mobilization and egress of CD8⁺ TEMRA cells and naïve CD8⁺ T cells were
13 impaired by one week of high intensity training. It is possible that repeated bouts
14 of high intensity training may lead to an increase of these populations in the
15 athletes' PB and could also explain why only after the increase in training
16 intensity (at week 24) the number of EM and TEMRA CD4⁺ and CD8⁺ cells
17 increased in the PB, whereas the training intensity done until week 7 was
18 probably not high enough to induce a reduction in the egress of these cells from
19 the PB during recovery periods (Witard et al. 2012). Several studies (Campbell et
20 al. 2009; Simpson et al. 2007) have shown that high intensity acute exercise
21 preferentially mobilizes TEMRA and EM CD8⁺ T cells into the PB, including
22 senescent T cells that express KLRG1 and CD57 on their surface. The fact that
23 this highly cytotoxic cells were shown by Witard and colleagues (Witard et al,
24 2012) to have impaired egress capacity from the PB after one week of high
25 intensity training, could explain why the maintenance for large periods of a high

1 intensity training load could compromise the capacity of these cells to migrate to
2 the peripheral tissues, which in turn, might be associated with compromised
3 immune surveillance. Another explanation for the increase of memory and
4 terminal effector memory T cells with training in our study could be the
5 accumulation of these cells due to recent encounters of naïve or with pre-
6 existing CM T cells with antigens eliciting their differentiation into effector
7 memory T cells. In fact both the absolute numbers of TCD4⁺ and TCD8⁺ naïve
8 (148 to 112,4 and 89,1 to 66,6 cells/ μ l respectively) and of CD4⁺ and CD8⁺ CM T
9 cells (36.7 to 19.3 and 14.23 to 9.8 cells/ μ l respectively) decrease from baseline
10 to t1. In fact our results show that 71% of the URS episodes reported were
11 clustered around the more demanding training phases.

12 Maintaining a high magnitude of training load until the end of the training season
13 could explain the may impaired naïve cell output and the increased percentage in
14 terminally differentiated cells. Moreover it is possible that the stress induced by
15 a major competition could also help increase the mobilization of terminally
16 differentiated cells into the PB (Anane et al, 2009).

17 The higher percentage of naïve and the lower percentages of EM and TEMRA
18 CD4⁺, EM CD8⁺ and $\gamma\delta$ T cell-subsets in these athletes after 5 to 6 weeks of off-
19 season training (summer vacations) when compared to the control group are
20 consistent with the model proposed by Simpson (Simpson, 2010), that suggests
21 that exercise may act as a mechanism for the depletion of senescent T cells,
22 which accumulate with aging and take up "immune space" causing the naïve T-
23 cell repertoire to shrink and increasing infection risk. Exercise would help
24 remove these senescent cells by preferentially mobilizing them to the blood

1 compartment, most probably under the influence of catecholamines, where the
2 increased production of reactive oxygen species, glucocorticoids and
3 proinflammatory cytokines by exercise, would expose them to proapoptotic
4 stimuli. Several studies have documented blood lymphocytes DNA damage,
5 increased oxidative stress-induced apoptosis and changes in cell surface death
6 receptors after a single bout of acute exercise (Hoffman-Goetz (Hoffman-Goetz
7 and Quadrilatero 2003; Kruger et al. 2009). These cells, together with
8 undamaged naïve and memory T cells, would egress the blood compartment
9 during the recovery phase of exercise and migrate to peripheral tissues. Some of
10 these “marked” cells would then undergo apoptosis (Takahashi et al. 2005), thus
11 creating vacant space. This creates a positive feedback loop that increases the
12 naïve T-cell output from the thymus or sites of extrathymic T-cell development
13 such as the liver and intestines. These new cells fill the vacant space and increase
14 the naïve T-cell repertoire. This model is supported by the finding that aerobic
15 fitness was associated with a lower age-related accumulation of senescent blood
16 T-cells in men (Spielman et al, 2011). The higher values of TEMRA CD8⁺ T and
17 $\gamma\delta$ T cells in the athletes, found in our study, may be explained by the cumulative
18 effect of exercise during several years of practice. When looking at the $\gamma\delta$ T-cell
19 subset populations in the athletes we see an increase in the EM subset in the
20 periphery while the TEMRA subset increases in the PB. Although in this study we
21 did not stain for senescent markers like KLRG1, this could mean a better capacity
22 for an immune response in the periphery while some of the less able to replicate
23 senescent cells within the TEMRA subset are being targeted for apoptosis in the
24 PB.
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Taken together and interpreted in the context of the model proposed by Simpson (Simpson 2011), our results suggest that fewer EM and TEMRA cells may be removed from the T-cell repertoire during long high-intensity training periods leaving the athletes more susceptible to illness. This however, maybe a small price to pay when considering the long term benefits of an increased naïve T-cell repertoire due to exercise practice.

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Table 1

Table 1 – Weekly training loads leading up to me points 1, 2 and 3.

Training zones	Time points		
	T1 (week 7)	T2 (week 24)	T3 (week 29)
Warm-up & recovery (m)	14820	14816	9633
Aerobic (m)	26925	29223	8673
Anaerobic (m)	1805	2513	1200
Sprint (m)	310	739	658
Total Volume (m)	42783	41228	20508
Intensity (aul)	12.3	14.1	13.8
Dry land training (hours/week)	3	4,5	3

m – meters; aul - arbitrary units of load

Table2

Table 2. Mean (SD) cell number (cells/ μ L) and % changes on leucocyte subpopulation and on naïve and memory T-cell subsets during a 29-week winter season training in swimmers (n=13) and control individuals (n = 11). * indicates statistical differences ($p < 0.05$) between athletes and controls at baseline based on student T-test analysis.

	Athletes				Controls	
	Pre-season	7 weeks	24 weeks	29 weeks	24 weeks	29 weeks
Leukocytes (Cells/ μ L)	7611 (1489)	9300 (2326)	8223 (1519)	8508 (1728)	6864 (1306)	7840 (1497)
Lymphocytes (%)	32.2 (8.2)	24.1 (7.3)	35.5 (6.3)	31.1 (9.3)	31.6 (5.32)	27.5 (12.3)
Lymphocytes (Cells/ μ L)	2449 (877)	2156 (537)	2911 (689)	2528 (518)	2166 (365)	2157 (961)
T Lymphocytes (%)	20.4 (14.9)	15.4 (3.8)	21.7 (5.1)	21.9 (7.4)	22.5 (3.2)	21.7 (4.2)
T Lymphocytes (Cells/ μ L)	500 (367)*	332 (124)	632 (257)	555 (273)	1520 (251)*	1550 (718)*
CD3 ⁺ CD4 ⁺ (%)	60.6 (5.3)	62.2 (7.1)	58.8 (6.8)	56.2 (9.7)	58.9 (6.8)	59.9 (5.6)
CD3 ⁺ CD4 ⁺ (Cells/ μ L)	297 (201)	203 (74)	367 (145)	297 (125)	902 (212)	939 (479)
CD3 ⁺ CD8 ⁺ (%)	32.5 (6.0)*	33.1 (7.1)	34.4 (7.4)	36.1 (7.8)	41.1 (6.8)*	40.0 (5.63)*
CD3 ⁺ CD8 ⁺ (Cells/ μ L)	174 (155)*	113 (55)	221 (108)	206 (132)	617 (108)*	611 (273)*
γ δT (%)	4.9 (6.0)	3.7 (2.3)	5.8 (5.3)	7.0 (4.7)	9.8 (4.5)	9.4 (1.8)
γ δT (Cells/ μ L)	19.6 (18.0)*	12.6 (10.0)	38.3 (46.1)	46.9 (55.5)	138.9 (49.5)*	141.8 (59.2)*
TCD4 ⁺ naïve (CD45RA ⁺ CCR7 ⁺) (%)	49.2 (6.8)*	52.9 (6.6)	50.6 (6.1)	48.4 (7.6)	21.7 (7.0)*	33.2 (9.5)*
TCD4 ⁺ naïve (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	146.1 (20.2)*	107.4 (13.4)	185.7 (22.4)	143.8 (22.6)	195.7 (63.1)*	311.7 (89.2)*
TCD4 ⁺ CM (CD45RA ⁺ CCR7 ⁺) (%)	12.2 (2.5)*	9.1 (2.0)	12.3 (3.9)	14.9 (4.6)	27.6 (6.5)*	43.1 (9.8)*
TCD4 ⁺ CM (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	36.2 (7.4)*	18.5 (4.1)	45.1 (14.3)	44.3 (13.7)	249.0 (58.6)*	404.7 (92.0)*
TCD4 ⁺ EM (CD45RA ⁺ CCR7 ⁺) (%)	35.6 (5.5)*	36.5 (6.4)	34.3 (5.4)	30.8 (5.4)	41.9 (14.8)*	25.8 (16.0)*
TCD4 ⁺ EM (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	105.7 (16.3)*	74.1 (13.0)	125.9 (19.8)	91.5 (16.0)	377.9 (133.5)*	242.3 (150.2)*
TCD4 ⁺ TEMRA CD45RA ⁺ CCR7 ⁺ (%)	2.9 (2.0)*	1.5 (0.8)	4.5 (8.1)	5.9 (3.9)	11.7 (4.5)*	5.7 (4.3)*
TCD4 ⁺ TEMRA CD45RA ⁺ CCR7 ⁺ (Cells/ μ L)	8.6 (5.9)*	3.1 (1.6)	16.5 (29.4)	17.5 (11.6)	105.5 (40.6)*	53.5 (40.4)*
TCD8 ⁺ naïve (CD45RA ⁺ CCR7 ⁺) (%)	48.8 (12.2)*	57.3 (11.4)	50.2 (11.0)	39.8 (14.4)	25.8 (9.1)*	31.5 (10.9)
TCD8 ⁺ naïve (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	84.9 (11.8)*	64.7 (12.9)	110.9 (24.3)	82.0 (29.7)	159.5 (40.6)*	193.0 (66)*
TCD8 ⁺ CM (CD45RA ⁺ CCR7 ⁺) (%)	7.8 (2.6)	8.4 (2.4)	7.8 (3.2)	7.5 (3.4)	7.1 (2.1)	9.6 (4.0)
TCD8 ⁺ CM (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	13.5 (4.5)*	9.5 (2.7)	17.2 (7.1)	15.5 (7.0)	43.8 (13.0)*	58.7 (24.4)*
TCD8 ⁺ EM (CD45RA ⁺ CCR7 ⁺) (%)	16.2 (5.3)*	16.8 (5.2)	16.8 (5.5)	21.0 (13.6)	53.4 (6.9)*	39.5 (8.6)*
TCD8 ⁺ EM (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	28.2 (9.2)*	19.0 (5.9)	37.1 (12.2)	43.3 (28.0)	329.5 (42.5)*	241.3 (52.5)*
TCD8 ⁺ TEMRA CD45RA ⁺ CCR7 ⁺ (%)	24.8 (12.6)*	17.4 (9.8)	25.5 (11.3)	31.7 (14.9)	17.6 (8.0)	17.2 (10.5)
TCD8 ⁺ TEMRA CD45RA ⁺ CCR7 ⁺ (Cells/ μ L)	43.2 (21.9)*	19.7 (11.0)	56.4 (24.0)	65.3 (30.7)	108.6 (49.4)*	105.1 (64.2)*
γ δT EM (CD45RA ⁺ CCR7 ⁺) (%)	48.5 (15.4)*	65.0 (15.4)	53.8 (11.3)	57.0 (15.2)	74.8 (21.0)*	76.1 (13.7)*
γ δT EM (CD45RA ⁺ CCR7 ⁺) (Cells/ μ L)	9.5 (3.0)*	8.2 (1.9)	20.6 (4.3)	26.7 (7.0)	103.9 (29.0)*	108.9 (19.4)*
γ δT TEMRA CD45RA ⁺ CCR7 ⁺ (%)	51.5 (15.4)*	35.0 (15.4)	46.2 (11.3)	43.0 (15.2)	25.1 (18.8)*	24.0 (13.6)*
γ δT TEMRA CD45RA ⁺ CCR7 ⁺ (Cells/ μ L)	10.1 (3.0)*	4.4 (1.9)	17.7 (4.3)	20.2 (7.0)	34.8 (26.0)*	34.0 (19.3)*

Table 3. Multilevel regression analysis of Leukocytes, Lymphocytes, T Lymphocytes, CD3⁺CD4⁺, CD3⁺CD8⁺ and γδT cell changes with training.

	Leukocytes (Cells/μL)	Lymphocytes (%)	T Lymphocytes (%)	CD3 ⁺ CD4 ⁺ (%)	CD3 ⁺ CD8 ⁺ (%)	γδT (%)
Variance-Covariance Matrix of Random Variables						
<i>Level 1 (within individuals)</i>						
Intercept	8169 (328)**	29.6 (1.8)**	17.2 (1.2)**	61.9 (1.5)**	32.2 (1.3)**	4.1 (1.0)**
Linear growth	161 (237)	0.8 (1.1)	1.2 (0.7)	-1.7 (0.9)	1.9 (1.2)	0.8 (0.6)
Variance-Covariance Matrix of Random Variables						
<i>Level 1 (within individuals)</i>						
Repeated measures variance	2324782 (580650)**	53.9 (13.0)**	22.0 (5.4)**	33.3 (8.4)**	10.9 (3.4)**	14.6 (3.9)**
<i>Level 2 (between individuals)</i>						
Variance	269895 (182766)	3.5 (3.5)	3.0 (1.8)	5.0 (3.1)	13.9 (6.3)*	2.1 (1.5)
-2 Restricted Log Likelihood	897	361	320	342	318	301
Akaike's Information Criterion	901	366	324	346	322	305

** p ≤ 0.01; * p ≤ 0.05

Table 4. Multilevel regression analysis for CD4+ T cell subpopulations changes to training in swimming athletes.

	TCD4+ naïve (CD45RA ⁺ CCR7 ⁺) (%)	TCD4+ CM (CD45RA ⁺ CCR7 ⁺) (%)	TCD4+ EM (CD45RA ⁺ CCR7 ⁺) (%)	TCD4+ TEMRA (CD45RA ⁺ CCR7 ⁺) (%)
Fixed Explanatory Variables				
<i>Exponent value (standard error)</i>				
Intercept	49.5 (1.4)**	11.9 (0.8)**	35.7 (1.2)**	1.9 (0.9)*
Linear growth	4.0 (2.0) *	-3.2 (1.3)*	1.6 (1.7)	1.2 (0.6)*
Quadratic growth	-1.5 (0.6) *	1.4 (0.4) **	-1.1 (0.5)*	-
Variance-Covariance Matrix of Random Variables				
<i>Level 1 (within individuals)</i>				
Repeated measures variance	18.1 (5.1) **	8.8 (2.1)**	19.9 (11.1)**	14.3 (3.2)**
<i>Level 2 (between individuals)</i>				
Variance	3.6 (1.9)*	0.7 (0.5)	5.6 (0.0)	1.5 (0.9)
-2 Restricted Log Likelihood	322	270	309	297
Akaike's Information Criterion	326	274	313	301

** p ≤ 0.01; * p ≤ 0.05

Table 5. Multilevel regression analysis for CD8+ and Gamma-delta T cell subpopulations changes to training in swimming athletes.

	TCD8+ naïve (CD45RA ⁺ CCR7 ⁺) (%)	TCD8+ CM (CD45RA ⁺ CCR7 ⁺) (%)	TCD8+ EM (CD45RA ⁺ CCR7 ⁺) (%)	TCD8+ TEMRA (CD45RA ⁺ CCR7 ⁺) (%)	Tyδ EM (CD45RA ⁺ CCR7 ⁺) (%)	Tyδ TEMRA (CD45RA ⁺ CCR7 ⁺) (%)
Fixed Explanatory Variables						
<i>Exponent value (standard error)</i>						
Intercept	48.9 (3.2)**	15.5 (1.5)**	8.1 (0.5)**	23.9 (2.3)**	50.9 (3.5)**	49.1 (3.5)**
Linear growth	11.1 (3.7)**	1.4 (1.5)	-0.1 (0.8)	-7.4 (3.3)*	11.1 (5.2)*	-11.1 (5.2)*
Quadratic growth	-4.7 (1.1)**	-	-	3.4 (0.9)**	-3.2 (1.6)*	3.2 (1.6)*
Variance-Covariance Matrix of Random Variables						
<i>Level 1 (within individuals)</i>						
Repeated measures variance	134.3 (55.1)*	30.8 (7.8)**	3.4 (0.9)**	44.9 (13.0)**	129.8 (35.3)**	129.8 (35.3)**
<i>Level 2 (between individuals)</i>						
Variance	3.0 (5.9)	8.2 (4.1)*	1.1 (0.5)*	28.2 (13.1)*	23.9 (15.1)	23.9 (15.1)
-2 Restricted Log Likelihood	381	345	238	374	401	401
Akaike's Information Criterion	389	349	242	378	405	405

** p ≤ 0.01; * p ≤ 0.05

Figure(s)

Figure 1. Mean changes in naïve and memory CD4⁺ T-cell subsets during a 29-week winter training season in swimmers. Panel A: significant increase in TCD4⁺ naïve cells until the first 7 weeks, followed by significant exponential decrease in cell expression throughout the remaining training season ($p \leq 0.05$); Panel B: significant decrease in TCD4⁺ CM cells until the first 7 weeks ($p \leq 0.05$), followed by significant exponential increase in cell expression throughout the remaining training season ($p \leq 0.01$); Panel C: significant exponential decrease in TCD4⁺ EM cells expression only after the first 7 weeks of training and until the end of the training season ($p \leq 0.01$); Panel D: significant linear increase in TCD4⁺ TEMRA cells expression throughout training season ($p \leq 0.05$).

Figure 2. Mean changes in naïve and memory CD8⁺ T-cell subsets during a 29-week winter training season in swimmers. Panel A: significant increase in TCD8⁺ naïve cells until the first 7 weeks, followed by significant exponential decrease in cell expression throughout the remaining training season ($p \leq 0.01$); Panel B: no significant changes in TCD8⁺ CM cells throughout the training season ($p > 0.05$); Panel C: no significant changes in TCD8⁺ EM cells throughout the training season ($p > 0.05$); Panel D: significant decrease in TCD8⁺ TEMRA cells until the first 7 weeks ($p \leq 0.05$), followed by significant exponential increase in cell expression throughout the remaining training season ($p \leq 0.01$).

Figure 3. Mean changes in memory $\gamma\delta$ T-cell subsets in during a 29-week winter training season in swimmers. Panel A: significant increase in T $\gamma\delta$ EM cells until the first 7 weeks, with the significant exponential exponent indicating attainment of a plateau between 7th and 24th weeks of training and a decrease thereafter ($p \leq 0.05$); Panel B: significant decrease in T $\gamma\delta$ TEMRA cells until the first 7 weeks, with the significant exponential exponent indicating a deceleration of the decreases in these cells' expression between the 7th and 24th week and an increase after the 24th week until the end of the training season ($p \leq 0.05$).