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Influence of training load on upper respiratory tract infection incidence and antigen-stimulated cytokine production

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Running head: Training load and infection risk in athletes

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Word count (text): 3550
Word count (Abstract): 200

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Abstract

This study examined the effect of training load on upper respiratory tract infection (URTI) incidence in men and women engaged in endurance-based physical activity during winter and sought to establish if there are training associated differences in immune function related to patterns of illness. Seventy five individuals provided resting blood and saliva samples for determination of markers of systemic immunity. Weekly training and illness logs were kept for the following 4 months. Comparisons were made between subjects (n=25) who reported that they exercised 3-6 h/week (LOW), 7-10 h/week (MED) or ≥11 h/week (HIGH). The HIGH and MED groups had more URTI episodes than the LOW group (2.4 ± 2.8 and 2.6 ± 2.2 *vs* 1.0 ± 1.6, respectively: $P < 0.05$). The HIGH group had ~3-fold higher IL-2, IL-4 and IL-10 production (all $P < 0.05$) by antigen-stimulated whole blood culture than the LOW group and the MED group had 2-fold higher IL-10 production than the LOW group ($P < 0.05$). Other immune variables were not influenced by training load. It is concluded that high levels of physical activity are associated with increased risk of URTI and this may be related to an elevated anti-inflammatory cytokine response to antigen challenge.

Keywords: exercise, immunity, leukocytes, illness, interleukins

Introduction

Exercise, depending on its intensity, can have either positive or negative effects on immune function and general health (Pedersen & Hoffman-Goetz, 2000). Regular moderate-intensity exercise enhances immune functions above those typically found in sedentary individuals. These functions include the potentiation of T cell-mediated immunity, natural killer (NK) cell cytotoxicity, pro-inflammatory cytokine production, and the Th1 reaction in human or animal models (Sugiura et al., 2001; Davis et al., 2004; Murphy et al., 2004; Okutsu et al., 2008; Wang et al., 2011). These effects may explain why regular moderate exercise reduces upper respiratory tract infection (URTI) incidence by ~20-45% compared with a sedentary lifestyle (Matthews et al., 2002; Nieman et al., 2010). In contrast, very prolonged strenuous bouts of exercise and periods of intensive training and competition may impair immune function, increasing susceptibility to URTI by decreasing saliva secretory immunoglobulin A (S-IgA) secretion, NK cell activity, and pro-inflammatory cytokine production (Peters & Bateman, 1983; Nieman et al., 1990; Heath et al., 1992; Gleeson et al., 1999; Nieman, 2000; Steensberg et al., 2001; Suzuki et al., 2002; Bishop, 2005; Fahlman & Engels, 2005). However, not all studies on high-level athletes have found significant associations between training load and URTI incidence (e.g. Fricker et al., 2005).

The aims of the present study were to examine URTI incidence and its possible associations with resting immune variables including salivary and plasma immunoglobulin concentrations, numbers of circulating leukocyte subsets and cytokine production by antigen-stimulated

whole blood culture in an athletic population. In particular, we wished to determine if whether URTI incidence and any immune variables differed between subjects who practiced regular moderate amounts of exercise (defined as 3-6 hours of exercise per week) and those who engaged in substantially more hours of endurance-based training. Differences in saliva and blood immune parameters were examined, and one measure of immune function was antigen-stimulated cytokine production by whole blood culture in order to simulate exposure to a pathogen challenge. We hypothesised that high volume training would be associated with a higher incidence of URTI and an impaired pro-inflammatory cytokine response and/or an elevated anti-inflammatory cytokine response to the multi-antigen challenge compared with subjects engaged in lower levels of physical activity. Our study population was a group of university students on a single campus site so that environment and pathogen exposure were likely to be similar for all subjects.

Materials and methods

Subjects

Ninety healthy university students who were engaged in regular sports training (predominantly endurance-based activities such as running, cycling, swimming, triathlon, team games and racquet sports) volunteered to participate in the study. Subjects ranged from recreationally active to Olympic triathletes. Of these 90 subjects 40 were female and 50 were male with baseline characteristics (mean ± SD) as follows: age 22.5 ± 4.0 years, body mass 71.5 ± 11.6 kg, height 175 ± 9 cm, body mass index 23.2 ± 2.6 kg/m^2 and self-reported weekly training load 9.3 ± 4.3 h/week. Subjects were required to complete a comprehensive health-screening questionnaire prior to starting the study; they were free of URTI symptoms for at least 2 weeks and had not taken any medication in the 4 weeks prior to the study. All

subjects were fully informed about the rationale for the study and of all experimental procedures to be undertaken. Subjects provided written consent to participate in the study, which had earlier received the approval of Loughborough University ethical advisory committee. Subjects were enrolled after having fulfilled all inclusion criteria, and presenting none of the exclusion criteria (determined by both questionnaire and interview).

Subjects could be included if they were currently healthy, aged 18-35 years, had been involved in endurance training for at least 2 years, and engaged in at least 3 sessions and at least 3 h of total moderate/high intensity training time per week. For data analysis subjects were allotted to one of three groups according to their self-reported hours of weekly training: 3-6 h/week, 7-10 h/week and 11 or more h/week, designated as low (LOW), medium (MED) and high (HIGH) volume training groups, respectively. Subjects representing one or more of the following criteria were excluded from participation: Smoking or use of any regular medication, abnormal haematology (e.g. erythrocyte or leukocyte counts outside the normal range), suffered from or had a history of cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal, haematological or psychiatric illness.

Seventy-five subjects (44 males and 31 females) completed the 4-month study period. Reasons for dropout were given as foreign travel, injury or persistent non-respiratory illness (preventing subjects from performing training) or due to undisclosed reasons. There were 25 subjects in the LOW group (10 females, 15 males), 25 in the MED group (14 females, 11 males) and 25 in the HIGH group (7 females, 18 males). The anthropometric characteristics of the groups were similar, although subjects in the LOW group were slightly older than in the other two groups (Table 1).

Laboratory visit

The study began in the month of November. Subjects arrived at the laboratory in the morning at 08.30-10:30 following an overnight fast of approximately 12 h. Each subject was asked to empty their bladder before body mass and height were recorded. Subjects then sat quietly for 10 min and completed health and training questionnaires before providing a saliva sample. With an initial swallow to empty the mouth, unstimulated whole saliva was collected by expectoration into a vial for 2 min with eyes open, head tilted slightly forward and making minimal orofacial movement. All saliva samples were stored at –20^{o}C until analysis. Subsequently, a venous blood sample (11 ml) was obtained by venepuncture from an antecubital vein and blood was collected into two Vacutainer tubes (Becton Dickinson, Oxford, UK) containing either K_3EDTA or heparin. Haematological analysis was immediately carried out on the EDTA sample as detailed below.

Questionnaires

During the 4-month subsequent study period subjects were requested to continue with their normal training programmes and they completed a health (URTI symptoms) questionnaire on a weekly basis. Supplements (vitamins and minerals, etc.) were not permitted during this period. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required, on a weekly basis, to report any unprescribed medications taken, visits to the doctor and any prescribed medications.

The illness symptoms listed on the questionnaire were: sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and

pains, weakness, headache and loss of sleep. The non-numerical ratings of light, moderate or severe (L, M or S, respectively) of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis (Fricker et al., 2005; Gleeson et al., 2011a,b) and the total symptom score for every subject each week was calculated by multiplying the total number of days each symptom was experienced by the numerical symptom severity rating. In any given week a total symptom score ≥12 was taken to indicate that a URTI was present. This score was chosen as to achieve it a subject would have to record at least 3 moderate symptoms lasting for 2 days or 2 moderate symptoms lasting for at least 3 days in a given week. A single URTI episode was defined as a period during which the weekly total symptom score was ≥12 and separated by at least one week from another week with a total symptom score ≥12. Subjects were also asked to rate the impact of illness symptoms on their ability to train (normal training maintained, training reduced or training discontinued; L, M or S, respectively). The same questionnaire was used in two previous studies that examined sex differences in URTI incidence in athletes (Gleeson et al., 2011a) and the influence of probiotic supplementation on URTI incidence in an endurance athlete cohort Gleeson et al., 2011b). Subjects were also asked to fill in a standard short form International Physical Activity Questionnaire (IPAQ; http://www.ipaq.ki.se/downloads.htm) at weekly intervals, thus providing quantitative information on training loads in metabolic equivalent (MET)-h/week (Craig et al., 2003).

Blood cell counts

Blood samples in the K_3EDTA vacutainer (4 ml) were used for haematological analysis (including haemoglobin, haematocrit and total and differential leukocyte counts) using an automated cell-counter ($A^c.T^{TM}$5diff haematology analyser, Beckman Coulter, High

Wycombe, UK). The intra-assay coefficient of variation for all measured variables was less than 3.0%.

Lymphocyte subsets

Lymphocyte subsets (CD3, CD4, CD8, CD19, CD56) to enumerate total T cells, T-helper cells, T-cytotoxic cells, B cells and NK cells, respectively were determined by three-colour flow cytometry (Becton Dickinson FACS-Calibur) with CellQuest analysis software (Becton Dickinson Biosciences, Oxford, UK) as described previously (Lancaster et al., 2004). Forward scatter versus side scatter plots were used to gate on the lymphocyte population by morphology and 10,000 lymphocyte events were acquired per analysis. Estimations of the absolute $CD3^+$, $CD3+CD4^+$, $CD3+CD8^+$, NK cell (CD3-CD56+) and B cell (CD3-CD19+) numbers were derived from the total lymphocyte count.

Monocyte Toll-like receptor 4 expression

The cell surface expression of toll-like receptor 4 (TLR4) on CD14+ monocytes (geometric mean fluorescence intensity) corrected for non-specific binding using an isotype control) was determined according to Oliveira & Gleeson (2010).

Antigen-stimulated cytokine production

Stimulated whole blood culture production of cytokines (IFN-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8 and IL-10) was determined as described previously (Gleeson et al., 2011a,b). The stimulant was a commercially available multi-antigen vaccine (Pediacel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. Briefly, heparinised whole blood was cultured with vaccine at 37°C and 5% CO_2 for 24 h. After centrifugation at 1500 *g* for 10 min at 4ºC, supernatants were collected and stored frozen at -80°C prior to analysis of cytokine concentrations using an Evidence Investigator System using the cytokine biochip array EV3513 (Randox, County Antrim, UK). The intra-assay coefficient of variation for all measured cytokines was less than 5.0%.

Plasma immunoglobulins

The remaining blood in the K_3EDTA tube was centrifuged at 1500 *g* for 10 min at 4 ºC within 10 min of sampling. The plasma obtained was immediately stored at –80 ºC prior to analysis of immunoglobulins A, G and M (immunoturbidometric assay on Pentra 400 autoanalyser, Horiba, France using the manufacturer's calibrators and controls). The intra-assay coefficient of variation for immunoglobulins A, G and M was 3.2%, 1.9% and 2.3%, respectively.

Saliva IgA

Duplicate saliva samples were analysed for secretory IgA using an ELISA kit (Salimetrics, Philadelphia, USA). The intra-assay coefficient of variation for IgA was 3.6%.

Statistical Analysis

The number of URTI episodes, blood leukocyte, neutrophil, monocyte, eosinophil and lymphocyte counts, lymphocyte subset counts, concentrations of secreted cytokines, plasma and saliva immunoglobulin concentrations were compared between the groups using one way ANOVA for normally distributed data. Where significant F values were found, Newman-Keuls tests were used for comparisons between groups. The cytokine and saliva IgA data were not normally distributed and these data were analysed using the Kruskal-Wallis test (nonparametric equivalent of one way ANOVA) with post hoc Dunns test. Relationships between variables were examined using Pearson's product moment correlation coefficient. Statistical significance was accepted at $P < 0.05$. Data are expressed as mean ± SD or median and 25% and 75% percentiles as appropriate.

Results

Training loads and URTI incidence

The LOW, MED and HIGH groups reported participating in moderate-vigorous exercise for 5.1 ± 0.9, 8.5 ± 1.0 and 14.5 ± 3.4 h/week, respectively ($P < 0.001$). Analysis of the IPAQ questionnaires confirmed that the weekly training loads were substantially higher in the HIGH group compared with the MED and LOW groups (Table 1). The IPAQ scores in MET-h/week significantly correlated with subjects' self-reported training loads at the start of the study ($r = 0.583$, $P < 0.001$, $n=75$).

Analysis of the URTI symptom questionnaires indicated that the HIGH and MED groups experienced 2.4 ± 2.6 and 2.6 ± 2.2 episodes, respectively during the 4-month period which were both significantly more than the LOW group (1.0 ± 1.7 episodes, $P < 0.05$). The

proportion of subjects who suffered one or more episodes of URTI was also higher in the HIGH (0.72) and MED (0.84) groups compared with the LOW (0.36) group. The proportion of all subjects who stated that training was negatively affected when suffering URTI symptoms (N=48) was 0.81. When an URTI episode was present, the proportion of subjects who took medication was 0.65 and the proportion of subjects who visited their doctor was 0.22.

Influence of training load on blood and saliva immune variables

There were no differences in haematological variables (Table 1), circulating numbers of leukocytes, neutrophils, monocytes, eosinophils, lymphocytes or lymphocyte subsets (CD3+, CD3+CD4+, CD3+CD8+, CD3-CD19+, CD3-CD56+) or monocyte TLR4 expression (Table 2) between the groups. The HIGH subjects had significantly higher IL-2, IL-4 and IL-10 production by antigen-stimulated whole blood culture than the LOW subjects (all $P < 0.05$; Table 3). The MED subjects had significantly higher IL-10 production by antigen-stimulated whole blood culture than the LOW subjects ($P < 0.05$). The production of other cytokines (IL-1α, IL-1β, IL-6, IL-8, IFN-γ and TNF-α) was not significantly influenced by training load although IFN-γ tended to be higher in the HIGH group ($P = 0.054$). None of the measured cytokines correlated with monocyte TLR4 expression (all r values <0.15). There were no significant differences in plasma immunoglobulin or saliva IgA concentrations between the groups (Table 4).

DISCUSSION

The main findings of the present study were that athletes engaged in high training loads had higher URTI incidence and higher IL-2, IL-4 and IL-10 production in response to antigen

252 challenge than subjects who were only moderately active. Thus, our hypothesis was only
253 partially correct in that high volume training was associated with a higher incidence of URTI
254 and an elevated anti-inflammatory cytokine response to antigen compared with subjects
255 engaged in lower levels of physical activity but there was no statistically significant effect of
256 training load on the pro-inflammatory cytokine response to antigen (other than a tendency for
257 IFN-γ production to be higher in the HIGH group). Other studies have reported increased
258 URTI incidence following marathon and ultramarathon events (Peters & Bateman, 1983;
259 Nieman et al., 2001) or during periods of intensive training and competition (Fahlman &
260 Engels, 2005) and some (Heath et al., 1992; Nieman et al., 1990; Nieman, 2000) but not all
261 (Matthews et al., 2002; Fricker et al., 2005) studies have reported a positive association
262 between URTI incidence and training load. Our data, in combination with the findings of
263 studies by Matthews et al. (2002) and Nieman et al. (2010) support the notion of a "J-shaped"
264 relationship between URTI incidence and exercise training load (Nieman 2000) although a
265 limitation of the present study was that a sedentary control group was not included. These
266 previous studies indicated that URTI risk is reduced in adults performing regular moderate
267 exercise compared with those having a sedentary lifestyle and our findings indicate that
268 exercise training loads above that which could be considered to be moderate levels of
269 physical activity result in increased URTI risk.
270
271 Associations between URTI risk and blood immune parameters have not been extensively
272 examined, though an impaired IFN-γ production in unstimulated whole blood culture has
273 been reported in fatigued and illness-prone endurance athletes (Clancy et al., 2006).
274 However, the relevance of this measure of immune function to infection risk is unclear as
275 cytokine production in the unstimulated state is very low compared with the response to an

infectious agent or antigen challenge. In the present study we examined cytokine production in response to an *in vitro* multi-antigen challenge in order to simulate the response to an infection. We observed significant differences in the production of IL-2, IL-4 and IL-10 between the LOW and HIGH training volume groups. Similarly, Sugiura et al. (2002) observed a 2-fold higher mitogen-stimulated IL-2 production by splenic lymphocytes in exercise trained mice compared with sedentary counterparts. None of the other immune variables measured in the present study were significantly influenced by training load.

A difference in TLR expression on monocytes was considered as one possible reason for a difference in cytokine production in response to an antigen challenge (Lancaster et al., 2005) and others have reported lower TLR4 expression in trained compared with sedentary subjects in both cross sectional (McFarlin et al., 2006) and longitudinal training studies (Stewart et al., 2005). However, in the present study we found no influence of training load on monocyte TLR4 expression and production of none of the measured cytokines correlated with monocyte TLR4 expression. Specific differences in the expression of cytokine genes could also account for differences in susceptibility to URTI (and other infectious diseases) by influencing the function of immune cells or the cytokine response to pathogens (Ollier, 2004). In a recent study IL-2 and IL-10 polymorphisms were associated with respiratory tract infection incidence in elderly humans (Besisle et al., 2010). However, the present study suggests that training volume may have a direct influence on the pattern and magnitude of cytokine production in response to antigen challenge and that a high anti-inflammatory cytokine (IL-4 and IL-10) response may make individuals more susceptible to URTI.

IL-10 is known to be produced by both innate and adaptive immune cells and its principal role appears to be containment and suppression of inflammatory responses so as to downregulate effector adaptive immune responses and minimize tissue damage in response to microbial challenges (Moore et al., 2001; Fujio et al., 2010). Accordingly, IL-10 induces downregulation of major histocompatibility complex (MHC) antigens, the intercellular adhesion molecule-1, as well as the costimulatory molecules CD80 and CD86 on antigen presenting cells, and it has been shown to promote differentiation of dendritic cells expressing low levels of MHC class II, CD80, and CD86 (Maynard & Weaver, 2008). Thus, IL-10 is able to limit the ability of antigen presenting cells to promote the differentiation and/or proliferation of CD4+ T cells, thereby regulating both initiation and perpetuation of adaptive T-cell responses.

There is now extensive evidence from both murine and human studies that IL-10 production usually imposes some limits on the effectiveness of antipathogen immune responses, especially innate immunity and adaptive Th1 responses (van der Sluijs et al., 2004; Blackburn & Wherry, 2007). The Th2 cytokine IL-4 which was also secreted in higher amounts in the HIGH subjects also exerts inhibitory effects on Th1 differentiation. Th1 cells drive cell-mediated immunity which is important in the defence against viral infections. Therefore, we suggest that decreased Th1 responses, mediated by high IL-10 responsiveness in particular, may be partly responsible for the higher incidence of URTI symptoms in the HIGH and MED athletes. In the present study, the higher URTI incidence in the HIGH and MED subjects could not be attributed to decreased production of pro-inflammatory cytokines but rather to an increased production of IL-10 and/or IL-4.

T-regulatory (Treg) cells (Fujio et al., 2010) and B-regulatory (Breg) cells (Blair et al., 2010) are thought to be the main secretors of IL-10 and one possibility is that heavy exercise training induces an increase in the number of Treg and/or Breg cells. Indeed, several recent human (Yeh et al., 2006; Teixeira et al., 2011) and animal (Wang et al., 2011) studies have reported higher circulating Treg cell numbers following increases in training load. The study by Yeh et al. (2006) reported an increase in the numbers of circulating CD4+CD25+ Treg cells following a 12-week exercise training programme which was associated with a 1.8 fold increase in antigen-stimulated IL-10 production by cultured mononuclear leukocytes. Interestingly, Treg cells respond to the presence of IL-2 by rapid proliferation (Malek, 2003) and plasma levels of IL-2 are increased for several hours after prolonged exercise (Castell et al., 2007).

Limitations of the present study were that an unvalidated questionnaire was used to assess URTI incidence and the immune measures were made on only a single baseline sample. However, we had established from an earlier unpublished study of 48 healthy active subjects that differential leukocyte counts and antigen-stimulated cytokine production were reasonably consistent in resting blood samples collected 2 months apart. The same immune variables were measured and we found that coefficients of variation for were <20% for total leukocytes, neutrophils, monocytes, lymphocytes, T cells and antigen-stimulated IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ production by whole blood culture. Furthermore, rank correlations among the 48 subjects for the relationship between 0-month and +2-month samples for these same variables had r values of 0.70-0.98.

346 It is concluded that high training loads are associated with increased risk of URTI and that
347 alterations in the cytokine response to antigens – in particular elevated production of the anti-
348 inflammatory cytokines IL-4 and IL-10 – with heavy training may, at least in part, account
349 for the greater susceptibility to URTI in highly physically active individuals.
350
351 **Perspectives**
352 The present confirms that high training loads are associated with increased incidence of
353 URTI during the winter months. This study is the first, to our knowledge, to report that IL-10
354 responsiveness to antigen challenge is influenced by training load and presumably this is due
355 to higher numbers of IL-10 producing cells in the circulation. Although this may impair
356 immune response effectiveness against the common cold it could also be a contributing factor
357 to the anti-inflammatory effects of regular exercise which are seen to be beneficial to long-
358 term health (Gleeson et al., 2011c).
359
360 **Acknowledgements**
361 This study was sponsored by Yakult Honsha Co., Ltd., Japan and GlaxoSmithKline, UK**.**
362 Pedro Tauler received a “José Castillejo” grant from the Spanish Ministry of Science and
363 Education.
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Table 1. Anthropometric and haematological characteristics of the low (LOW), medium (MED) and high (HIGH) volume training groups

	LOW (n=25)	MED (n=25)	HIGH (n=25)
Females (F), Males (M)	10F, 15M	14F, 11M	7F, 18M
Age (years)	24.6 ± 5.1	21.2 ± 2.4**	22.0 ± 3.3*
Height (cm)	1.74 ± 0.10	1.75 ± 0.08	1.77 ± 0.08
Body mass (kg)	71.7 ± 12.1	70.9 ± 11.2	72.2 ± 12.5
BMI (kg/m^2)	23.5 ± 2.8	23.1 ± 2.2	23.0 ± 2.7
Training load (h/week)	5.1 ± 0.9	8.5 ± 1.0**	14.5 ± 3.4**##
IPAQ (MET-h/week)	45.5 ± 22.1	58.8 ± 23.0*	86.0 ± 33.4**##
RBC count ($x10^{12}/L$)	4.76± 0.49	4.61 ± 0.43	4.79 ± 0.44
Haematocrit (%)	41.2 ± 3.6	41.3 ± 4.0	41.4 ± 2.8
Haemoglobin (g/L)	140 ± 13	139 ± 14	141 ± 10

Values are expressed as mean (±SD).
Significantly different from LOW: *P<0.05; **P<0.01
Significant difference between HIGH and MED: #P<0.05; ##P<0.01

Table 2. Blood leukocytes, lymphocyte subsets and monocyte TLR4 expression in the low (LOW), medium (MED) and high (HIGH) volume training groups

	LOW (n=25)	MED (n=25)	HIGH (n=25)	P
Leukocyte count (x10^9/L)	6.09 ± 1.60	5.52 ± 1.39	5.75 ± 1.37	0.382
Neutrophil count (x10^9/L)	3.12 ± 1.38	2.85 ± 1.03	2.87 ± 1.11	0.669
Monocyte count (x10^9/L)	0.52 ± 0.18	0.49 ± 0.17	0.48 ± 0.14	0.685
Eosinophil count (x10^9/L)	0.21 ± 0.14	0.19 ± 0.14	0.17 ± 0.11	0.480
Lymphocyte count (x10^9/L)	2.18 ± 0.56	1.86 ± 0.46	2.14 ± 0.62	0.189
CD3+ cell count (x10^9/L)	1.31 ± 0.44	1.10 ± 0.38	1.31 ± 0.45	0.148
CD3+CD4+ cell count (x10^9/L)	0.71 ± 0.25	0.63 ± 0.19	0.70 ± 0.25	0.429
CD3+CD8+ cell count (x10^9/L)	0.54 ± 0.23	0.42 ± 0.20	0.52 ± 0.25	0.175
CD3-CD19+ cell count (x10^9/L)	0.24 ± 0.15	0.18 ± 0.09	0.20 ± 0.10	0.155
CD3-CD56+ cell count (x10^9/L)	0.28 ± 0.15	0.23 ± 0.19	0.24 ± 0.12	0.459
Monocyte TLR4 expression (GMFI)	24.1 ± 10.3	25.4 ± 16.3	20.9 ± 12.0	0.473

Values are expressed as mean (±SD). There were no significant effects of training load on any of these variables.

P values are outcome of one-way ANOVA.

Table 3. Antigen stimulated cytokine production by whole blood culture in the low (LOW), medium (MED) and high (HIGH) volume training groups

	LOW (n=25)	MED (n=25)	HIGH (n=25)	P
IL-1α production (pg/ml)	0.5 (0.2-1.5)	0.5 (0.2-1.2)	0.4 (0.2-1.3)	0.840
IL-1β production (pg/ml)	4.8 (3.6-10.2)	4.6 (3.4-8.9)	4.8 (3.4-7.9)	0.765
IL-2 production (pg/ml)	19.6 (5.6-94.4)	44.8 (7.6-186.6)	152.8 (42.6-282.6)**	0.006
IL-4 production (pg/ml)	1.8 (1.3-2.6)	2.5 (1.8-3.8)	3.1 (2.2-6.3)**	0.003
IL-6 production (pg/ml)	103 (28-207)	74 (16-340)	128 (43-285)	0.724
IL-8 production (pg/ml)	938 (373-1855)	839 (239-1855)	1343 (553-1855)	0.431
IL-10 production (pg/ml)	1.1 (0.8-2.4)	2.8 (1.1-5.6)*	4.4 (1.7-6.2)**	0.008
IFN-γ production (pg/ml)	9.3 (2.9-30.4)	8.1 (2.5-34.4)	23.8 (9.9-44.4)	0.054
TNF-α production (pg/ml)	8.6 (4.0-16.3)	7.0 (3.8-19.6)	22.6 (6.5-32.8)	0.153

Values are expressed as median (with 25% and 75% percentiles).
Significantly different from LOW: *P<0.05; **P<0.01
Significant difference between HIGH and MED: #P<0.05; ##P<0.01
P values are outcome of Kruskal-Wallis test (non-parametric equivalent of one-way ANOVA).

Table 4. Plasma and saliva immunoglobulins in the low (LOW), medium (MED) and high (HIGH) volume training groups

	LOW (n=25)	MED (n=25)	HIGH (n=25)	P
Plasma IgA (g/L)	1.55 ± 0.56	1.47 ± 0.80	1.62 ± 0.66	0.744
Plasma IgG (g/L)	11.4 ± 2.6	10.8 ± 2.1	10.8 ± 1.8	0.521
Plasma IgM (g/L)	1.26 ± 0.63	1.46 ± 0.89	1.54 ± 0.72	0.396
Saliva IgA (mg/L)	114 (89-174)	125 (88-183)	126 (88-199)	0.932

Plasma values are expressed as mean (±SD). Saliva values are expressed as median (with 25% and 75% percentiles).
There were no significant effects of training load on any of these variables. P values are outcome of one-way ANOVA for plasma immunoglobulins and Kruskal-Wallis test (non-parametric equivalent of one-way ANOVA) for saliva IgA.