Cytokine kinetics in nasal mucosa and sera: new insights in understanding upper-airway disease of marathon runners

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Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa de São Paulo (FAPESP), São Paulo, Brazil (ref. no. 2009/51800-1).

Authors' Disclosure Statement: The authors declare that there is no conflict of interest. All authors participated in the study design, data interpretation and writing of the manuscript.

ABSTRACT

Recently, many authors have proposed that mechanisms such as inflammation and/or allergies could be partly responsible for cases of upper respiratory tract illnesses that affect athletes after exhaustive exercise. Here we studied the kinetics of cytokines in the serum and nasal mucosa of athletes after a marathon. We were able to demonstrate an increase in serum levels of all interleukins studied immediately after the marathon in athletes that present or not with upper airways symptoms followed by a return to basal levels 72 hours after the race, as described in the literature. Interleukin (IL)-10 behaviour differed in the group of asymptomatic athletes. Measurement of this cytokine in protein extract of nasal mucosal cells showed increase 72 hours after the marathon. Levels of this cytokine in sera were increased at rest in athletes that did not present symptoms. These fin-

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dings suggest that the maintenance of a non-inflammatory environment in the mucosal airways is an active process that requires participation of the systemic and mucosal immune systems. We propose that the understanding of the upper airway disease of the athlete involves the study of mucosal and systemic immune systems.

Keywords: upper respiratory tract infections, cytokines, secretory immunoglobulin A, exhaustive exercise, mucosal immune system.

INTRODUCTION

Peters and Bateman (17) were the first authors to report an increased prevalence of respiratory tract infection in athletes after competitions or periods of intensive training. It has been suggested that a relationship exists between training load and susceptibility to infection (9, 15). Among the hypotheses put forward to explain these findings, it is worth mentioning the "open window" and "J curve" theories, which suggest that infection may be caused by systemic immunosuppression. However, these theories are still under considerable debate (16) and fail to explain why systemic immunosuppression in these athletes usually causes only upper respiratory tract infections (URTIs) without any other manifestations.

Spence and others (19), examining athletes with symptoms of URTIs, reported that inflammatory allergic reactions associated with vasomotor phenomena and the inhalation of cold and dry air could be observed in most cases, and that confirmed infection by pathogen identification could be detected in a smaller number of cases. Bermon (3) also suggested that the mechanism that causes manifestations of symptoms of upper respiratory tract illness symptoms (e.g. sore throat, runny nose) in the respiratory tract can be associated with either infections or inflammation/allergies. Cox (6) reported similar results, showing that allergy and asthma were responsible for at least 30% of non-infectious cases. Thus, the question of whether sore throats are actually caused by infections or are a reflection of other inflammatory stimuli continues to be the subject of debate (20).

The study of local factors involved in upper respiratory tract illnesses essentially focuses on determining the concentration of secretory immunoglobulin A (SIgA) in athletes' saliva, which has been reported to decrease after prolonged endurance events. Exercise has an important effect on salivary SIgA levels, which vary according to the level of the athlete's training. The absolute concentration of SIgA has been reported to fall after prolonged strenuous exercise. Thus, there appears to be a relationship between repeated high-intensity training or competitions and altered production of mucosal immunoglobulins and, therefore, the local immune response (20).

However, the role played by the immune system in this regulatory response is not yet fully understood (8). For this reason, the study of immune system cells involved in mechanisms that protect the upper respiratory tract, as well as their products, such as immunoglobulins and cytokines, is of fundamental importance

in understanding the mechanisms responsible for breaking the homeostasis, which manifest as infection, clinical inflammation or allergy.

In spite of the importance of cytokines in the regulation of the immune response, their role in respiratory tract infection in athletes has yet to be clarified. As shown by Cox (7), athletes prone to respiratory illness had low absolute plasma concentrations of IL-10, IL-1ra and IL-8 when at rest, and high absolute plasma concentrations of IL-6 after exercise, suggesting a possible association between impaired inflammatory regulation and respiratory illness. However, the studies cited above only investigated levels of cytokines in peripheral blood samples. Despite the differences between systemic and mucosal immune responses, local factors in the upper airway, after exhaustive exercise, have not been studied to date, except for the SIgA response.

The aim of this study was to evaluate the immune response elicited by exhaustive exercise in different compartments, namely, the local (upper airway mucosa) and systemic (serum) compartments, by comparing athletes that presented or not with symptoms of upper airway disease after completing a marathon.

MATERIAL AND METHODS

Subjects and study design

The study population consisted of 22 recreational male marathon runners with a mean age of 41.4±9.4 years living in the city of São Paulo. All the athletes were aware of the possible risks involved in the study, having given their agreement to the study protocol and signed a consent form, both of which were approved by the UNIFESP-EPM Ethics Committee. This study meets the ethical standards defined by Harris and Atkinson (10).

The volunteers were evaluated before the race by clinical examination and those with upper respiratory symptoms were excluded. Athletes were instructed to report any manifestations of respiratory illness symptoms in the two weeks following their participation in the race. The individuals recruited to the study kept to their usual training/physical exercise schedules throughout the study (mean of 100 km/week and 12 hours/week) to prepare for the race. The last training session was performed 24 hours before samples were collected. We recommended that all marathon runners ingested 500 mL of water before the marathon. The same drink was offered by the marathon organisation team during the race and consumed according to each runner's needs. One week after the race, the volunteers were divided into two groups based on the athletes' reports: asymptomatic individuals (n=15) and symptomatic individuals (n=7). Symptoms began within five to seven days after the marathon and lasted for three to five days. The main complaints reported by the athletes were coryza (nasal congestion) and an itchy and runny nose. All athletes who reported upper-airway symptoms were examined by a doctor.

Collection of the samples
Samples of serum, saliva and cells from the nasal mucosa, which were chosen as being representative of the upper airways, were collected at three different times: at rest, immediately after and 72 hours after the marathon.

One millilitre of saliva sample from each volunteer was collected directly into sterile 15-mL Falcon® tubes without prior stimulation. A total of 250 µL of each sample was transferred to 1.5-mL Eppendorf tubes and kept frozen at -80°C until SIgA concentration was measured. No buffers or preservatives were added. Each sample was centrifuged at 3000 rpm for 5 minutes, and the supernatant was used to measure SIgA concentration. Samples containing blood were discarded and replaced with newly collected samples.

Samples of nasal cells (representative of the upper airways) were collected from the nasal cavities with a cytobrush (Kolplast LTDA, São Paulo, Brazil), and the total protein extract was obtained as previously described (11). A total of 200 µL of supernatant was collected and frozen at -80ºC for subsequent analysis of intracellular cytokines.

Cell collection for cytogram
At rest and after 72 hours, nasal cells were collected to determine the percentage of neutrophils in the sample. These data were used together with the report of the athletes and blind clinical evaluation performed by an otolaryngologist to determine the inflammatory status of their upper airway.

Determination of salivary immunoglobulin A (SIgA) concentration
Salivary IgA concentration was determined by ELISA using the Salivary Secretory IgA Indirect Enzyme Immunoassay kit (Salimetrics™) according to the manufacturer's instructions. Standards, positive and negative controls were prepared as described by the manufacturer.

Determination of cytokine concentrations in serum and nasal cell extract
The concentration of the cytokines IL-6 and IL-10 in serum and in the nasal cell extract previously stored at -80°C was measured using the Luminex bead-based system for human cytokines with the LINCOplex kit for simultaneous multi-analyte detection (Linco Research, Inc. St Charles Missouri, IL) following the manufacturer's instructions. The cytokine concentrations obtained in the nasal cell extract were normalised by the Bradford method (5), using the ratio of total protein (in milligrams) to cytokine concentrations in each sample.

Statistical analysis
Secretory immunoglobulin A (SIgA) and cytokine concentrations are shown as means and standard deviation (SD). Data analysis was performed using a Two-Way ANOVA test followed by a Fisher's LSD post hoc test to compare if the cytokine or SIgA concentrations and three different times of sample collection were significant between each group of marathon runners. The Mann-Whitney test was used to determine whether the relation between neutrophils infiltration

percentage PRE and 72 hours after (POST) marathon [(POST/PRE)*100] were significant. The significance level was set at 5% ($p < 0.05$).

RESULTS

Performance and maintenance of hydration during the marathon race

The athletes performed the marathon in a mean time of 4 hours (h) and 31 minutes (min) (SD ± 29 min). No statistically significant differences in times were observed between the groups [asymptomatic group = 4h 36min (SD ± 31min) and symptomatic group = 4h 25min (SD ± 29min)]. Immediately after the marathon, the haematocrit percentage did not alter [asymptomatic group = 44.7±4.1 (baseline) and 43.4±3.9 (immediately after); symptomatic group = 44.9±5.2 (baseline) and 45.1±5.8 (immediately after)], indicating that the athletes were adequately hydrated during the race.

Differences in salivary SIgA levels observed before and after the marathon race

Analysis of salivary SIgA levels in all the athletes immediately after the marathon showed a significant reduction compared with baseline levels ($p<0.005$), returning to the levels at rest, 72 hours after the marathon (Table 1). When we evaluated the concentration of salivary SIgA levels at rest, comparing asymptomatic with symptomatic athletes, there was no significant difference between the groups (symptomatic = 215±35 mg/L and asymptomatic = 243±47 mg/L, Figure 1). In both groups, SIgA levels were reduced in relation to levels at rest immediately after the marathon (symptomatic = 168±31 mg/L and asymptomatic = 153±18 mg/L, $p=0.001$). No difference was observed between the groups (Figure 1). Seventy-two hours after the race, salivary SIgA levels in both groups had returned to their respective baseline values (symptomatic = 223±47 mg/L and asymptomatic = 237±12 mg/L), with no statistical difference between the groups (Figure 1).

Cellular analysis of nasal mucosa

The cytological study of cells obtained from the nasal cavities at rest and 72 hours after the marathon showed that upper-airway complaints were compatible with alteration in the nasal cellularity. While in the symptomatic group there was an increase from 1.1±1.6 to 21.7±14.6% in the percentage of neutrophils infiltration, in the asymptomatic group the percentage of neutrophils after the marathon (0.7±1.2) was similar to that

Table 1. Salivary secretory immunoglobulin A (SIgA) levels (mg/L) of all marathon runners on three different occasions: at rest (baseline), immediately after and 72 hours after running a marathon. The results are shown as means and standard deviation.

Salivary SIgA (mg/L)	Marathon runners (n=22)
Baseline (at rest)	274±24
Immediately after the marathon	168±19*
72 hours after the marathon	266±28

* $p<0.005$ (immediately after X baseline and 72 hours after the run)

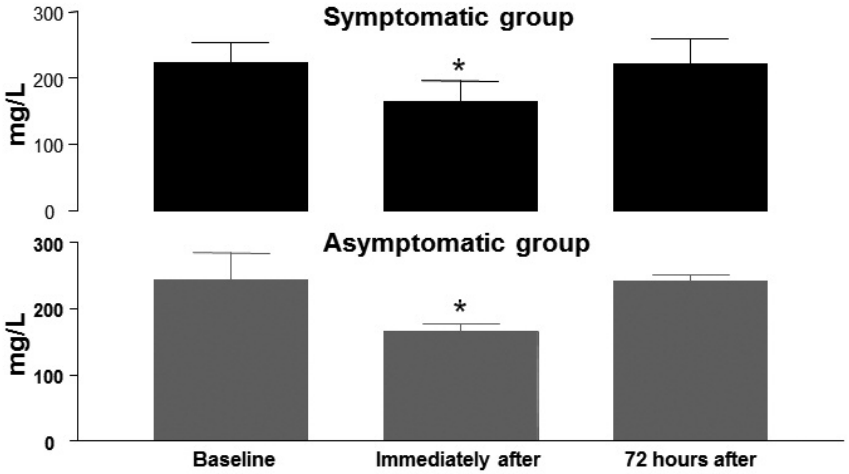

Figure 1. Secretory immunoglobulin A levels (mg/L) in saliva of marathon runners in the asymptomatic group and symptomatic group were evaluated on three different occasions: at rest (baseline), immediately after and 72 h after running a marathon. The values ware expressed as mean and standard deviation with a significant difference from baseline ($p<0.05$) indicated with an asterisk (*).

observed at rest (1.2±1.9). The statistical analysis of difference between the ratio [(Post/Pre)*100] of neutrophils infiltration percentage in both groups showed that percentage of neutrophils in symptomatic group was significantly higher than asymptomatic group ($p<0.0001$). These findings reinforced both the information reported by the athletes and the blind clinical examination performed by an otolaryngologist.

Alteration in cytokine levels before and after the marathon race in the sera and upper-airway mucosa.

According to the results obtained in the Two-way ANOVA analysis, we obtained a significant effect of time and time-symptoms interaction for IL-6 and IL-10 cytokines. Based on this observation, was performed the Post-hoc comparisons between groups. Baseline analysis of serum IL-6 concentrations (Figure 2A) did not show differences between the groups (symptomatic = 8.2±20.8 pg/mL and asymptomatic = 14.9±32.3 pg/mL). However, the evaluation of IL-10 levels (Figure 2B) showed that in the asymptomatic group (17.4±34.0 pg/mL), the concentration was statistically higher ($p<0.03$) than the symptomatic group (0.76±0.1 pg/mL) at rest. Immediately after the marathon, the IL-6 (symptomatic = 40.9±30.9 pg/mL and asymptomatic = 39.2±26.5 pg/mL, Figure 2A) and IL-10 (symptomatic = 57.2±32.7 pg/mL and asymptomatic = 30.7±25.3 pg/mL, Figure 2B) levels were significantly increased in both groups ($p<0.01$), when compared to basal concentrations. Seventy-two hours after the marathon, IL-6 (symptomatic = 10.6±20.4 pg/mL and asymptomatic = 16.9±34.3 pg/mL, Figure 2A) and IL-10 (symptomatic = 1.1±0.9 pg/mL and asymptomatic = 4.5±11.4 pg/mL, Figure 2B)

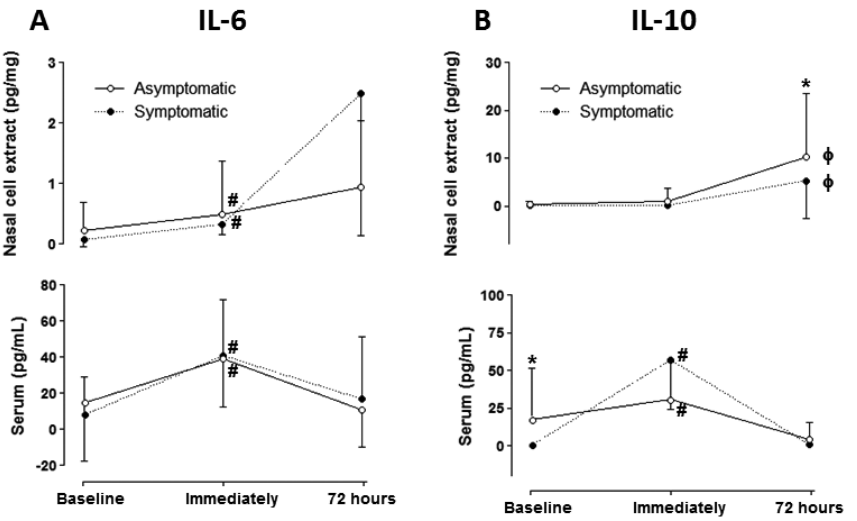

Figure 2. Concentrations of cytokine IL-6 (A) and IL-10 (B) in serum (pg/mL) and extracts from nasal cells (pg/mg total protein) from marathon runners in asymptomatic and symptomatic groups on three different occasions: at rest (baseline), immediately after and 72 hours after running a marathon. The significance level was $p<0.05$ and * denotes differences between the values obtained for the groups at the same moment; # denotes differences in values obtained at baseline moment and immediately after the marathon; φ denotes differences in values obtained at immediately and 72 hours after the marathon

levels returned to basal values in both groups. In relation to cytokine concentrations in nasal cell extracts, previously normalised by the Bradford method to pg/mg, we observed differences between the groups, as shown in Figure 2A and Figure 2B. Analysis of IL-6 concentrations at rest (Figure 2A) did not show a difference between the groups (symptomatic = 0.07±0.11 pg/mg and asymptomatic = 0.22±0.47 pg/mg). Immediately after the marathon, the IL-6 levels (Figure 2A) increased significantly compared with levels at rest in both groups (symptomatic = 0.33±0.17 pg/mg, $p<0.005$ and asymptomatic = 0.49±0.89 pg/mg, $p<0.01$) and remained elevated 72 hours after the marathon (Figure 2A, symptomatic = 2.49±2.35 pg/mg, $p<0.001$ and asymptomatic = 0.94±1.1 pg/mg, $p<0.001$). The IL-10 levels (Figure 2B) in both groups were significantly increased 72 hours after the marathon (symptomatic = 5.33±8.00 pg/mg, $p<0.05$ and asymptomatic = 10.26±13.31 pg/mg, $p<0.001$) in relation to levels at rest (symptomatic = 0.22±0.30 pg/mg and asymptomatic = 0.28±0.69 pg/mg) and immediately after the marathon (symptomatic = 0.15±0.15 pg/mg and asymptomatic = 1.00±2.68 pg/mg). Furthermore, the elevation of IL-10 levels observed 72 hours after the marathon in the asymptomatic group was statistically higher ($p<0.05$) than the symptomatic group (Figure 2B).

DISCUSSION

In this study we were able to observe significant differences in the kinetics of IL-10 in the sera and mucosa of symptomatic and asymptomatic groups of athletes after a marathon. Our results on serum IL-6 behaviour are in accordance with the literature, and the study of the kinetics in the nasal mucosa showed an increase of IL-6 concentration immediately after the marathon that persisted until 72 hours but without statistical differences between the groups.

The serum cytokines studied behaved according to previous reports in the literature; however the kinetics of IL-10, both in the serum and nasal mucosa extract suggests new insights in the comprehension of the upper-airway disease of the athlete.

An interesting finding was that sera IL-10 was significantly increased in the asymptomatic group even before the marathon, suggesting that these athletes were prone to activate anti-inflammatory mechanisms to cope with the inflammatory response expected due to the exhaustive exercise and hyperventilation that occurs during the marathon, as consequences of mucosal dryness and excessive exposure to air pollutants. This group of athletes probably developed this adaptive behaviour during their training period.

The results obtained in the mucosa were not remarkable except for the behaviour of IL-10, whose levels were significantly increased in the extract of nasal mucosa obtained from the group that did not present symptoms compared to the symptomatic group. This finding suggests that asymptomatic individuals must actively induce a local immune response directed to inhibit the inflammatory response. Although levels of IL-10 were increased in both groups 72 hours after the marathon, the values obtained for the asymptomatic group were significantly higher, so that we can infer that levels of cytokines in the extract of nasal mucosa are not simply a reflection of the changes serum cytokine concentrations, which is a finding that, as we will see below, has great importance.

"Mucosal immunity has the function of maintaining homeostasis on exposed mucosal surfaces. It is noteworthy that this system is anatomically and functionally distinct from its blood-borne counterpart of the systemic (or peripheral) immune system and is strategically located at the portals by which most microorganisms enter the body. The development of this specific branch of the immune system alongside and separate from the peripheral immune system may have been necessitated by the size of the mucosal surfaces, which cover an area of ~400 m^2 in the adult human, as well as the large number of exogenous antigens to which these surfaces are exposed"(4). In light of these facts we must take into account that the study of mucosal responses cannot be correlated only on results obtained from the systemic immune response, although both branches of the immune system are directed towards the same goals, in some instances they can act in different ways.

The upper-airway mucosa is continuously in contact with different types of antigenic substances and so it is of extreme importance to have tight control of the

mechanisms that can modulate the response to these antigens. This can be done by inhibition of pro-inflammatory cytokines or by increased secretion of IL-10 by epithelial cell-conditioned dendritic cells. Moreover, epithelial cell-conditioned T lymphocytes showed increased differentiation towards IL-10-producing type 1 T-regulatory (Tr1) cells, suggesting that epithelial cells induce non-inflammatory microenvironments that regulate the local immune response (12). Awasthi et al. (1) discuss the importance of T regulatory cells in maintaining the balance of immune homeostasis and the potential of Tr1 cells in regulate T-cell function by demonstrating that dendritic cells modified by Treg cells induced the generation of IL-10-producing Tr1 cells.

Many other studies in the literature have highlighted the importance of IL-10 in blocking the airways´ inflammatory responses (13, 14, 18, 21) so, the elevated levels of IL-10 that we found in the asymptomatic group can be a factor of protection to avoid mucosal inflammation.

Bazzoni et al (2), in a review about the interaction between IL-10 and the innate immune system, showed that neutrophils, a key cellular target for IL-10, act in response to the cytokine and its actions on macrophages and tissue chemoattractants, modulating their responsiveness and their capacity to produce and release cytokines, thus inducing an anti-inflammatory response. In light of these facts, the percentage of neutrophils obtained from nasal mucosa of athletes at rest and after the race assumed great importance, as we found in the symptomatic group a significant increase of cells after the marathon that was not seen in the asymptomatic individuals, a mucosal environment that suggests a blockade of the inflammatory response. Although IL-10 acts on many others types of immune cells, we chose to study neutrophils because we are dealing with an acute response.

Regarding to salivary SIgA we found decreased concentrations in both groups. According to Walsh et al (20) falls in SIgA levels can occur during intensive periods of training, and these authors reinforce the idea that there is reasonable evidence to indicate that a reduced concentration of saliva SIgA is associated with an increased risk of URTI. In agreement on the fact that reduction of the levels of SIgA is a key event in triggering upper respiratory symptoms, we believe that cytokines in serum and especially those that are locally produced play important role to determine whether the athlete will or not became symptomatic.

Based on our findings on the behaviour of IL-10 in the serum and nasal mucosa of asymptomatic and symptomatic groups of athletes, we suggest that the research on upper-airway disease of athletes must, as often as possible, include the study of local factors, and that after exhaustive exercises associated with hyperventilation and usually with inhalation of substances that can cause an inflammatory response of the upper airway mucosa, the maintenance of a non-inflammatory status is an actively induced process in which both, systemic and locally released IL-10 has a key action.

REFERENCES

1. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, Kuchroo VK, Oukka M, and Weiner HL. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol 8: 1380-1389, 2007.
2. Bazzoni F, Tamassia N, Rossato M, and Cassatella MA. Understanding the molecular mechanisms of the multifaceted IL-10-mediated anti-inflammatory response: lessons from neutrophils. Eur J Immunol 40: 2360-2368, 2010.
3. Bermon S. Airway inflammation and upper respiratory tract infection in athletes: is there a link? Exerc Immunol Rev 13: 6-14, 2007.
4. Blumberg RS SP, MacDonald TT;. Principles of mucosal immunology. London: Taylor & Francis Group, 2012.
5. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
6. Cox AJ, Gleeson M, Pyne DB, Callister R, Hopkins WG, and Fricker PA. Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. Clin J Sport Med 18: 438-445, 2008.
7. Cox AJ, Pyne DB, Saunders PU, Callister R, and Gleeson M. Cytokine responses to treadmill running in healthy and illness-prone athletes. Med Sci Sports Exerc 39: 1918-1926, 2007.
8. Daele J, and Zicot AF. Humoral immunodeficiency in recurrent upper respiratory tract infections. Some basic, clinical and therapeutic features. Acta Otorhinolaryngol Belg 54: 373-390, 2000.
9. Gleeson M. Mucosal immunity and respiratory illness in elite athletes. Int J Sports Med 21 Suppl 1: S33-43, 2000.
10. Harriss DJ, and Atkinson G. Update--Ethical standards in sport and exercise science research. Int J Sports Med 32: 819-821, 2011.
11. Luna LA, Jr., Bachi AL, Novaes e Brito RR, Eid RG, Suguri VM, Oliveira PW, Gregorio LC, and Vaisberg M. Immune responses induced by Pelargonium sidoides extract in serum and nasal mucosa of athletes after exhaustive exercise: modulation of secretory IgA, IL-6 and IL-15. Phytomedicine 18: 303-308, 2011.
12. Mayer AK, Bartz H, Fey F, Schmidt LM, and Dalpke AH. Airway epithelial cells modify immune responses by inducing an anti-inflammatory microenvironment. Eur J Immunol 38: 1689-1699, 2008.
13. McCusker CT. Use of mouse models of allergic rhinitis to study the upper and lower airway link. Curr Opin Allergy Clin Immunol 4: 11-16, 2004.
14. Nakagome K, Imamura M, Kawahata K, Harada H, Okunishi K, Matsumoto T, Sasaki O, Tanaka R, Kano MR, Chang H, Hanawa H, Miyazaki J, Yamamoto K, and Dohi M. High expression of IL-22 suppresses antigen-induced immune responses and eosinophilic airway inflammation via an IL-10-associated mechanism. J Immunol 187: 5077-5089, 2011.
15. Nieman DC. Exercise, infection, and immunity. Int J Sports Med 15 Suppl 3: S131-141, 1994.
16. Nieman DC. Special feature for the Olympics: effects of exercise on the immune system: exercise effects on systemic immunity. Immunol Cell Biol 78: 496-501, 2000.

17. Peters EM, and Bateman ED. Ultramarathon running and upper respiratory tract infections. An epidemiological survey. S Afr Med J 64: 582-584, 1983.
18. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, and Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 212: 28-50, 2006.
19. Spence L, Brown WJ, Pyne DB, Nissen MD, Sloots TP, McCormack JG, Locke AS, and Fricker PA. Incidence, etiology, and symptomatology of upper respiratory illness in elite athletes. Med Sci Sports Exerc 39: 577-586, 2007.
20. Walsh NP, Gleeson M, Shephard RJ, Woods JA, Bishop NC, Fleshner M, Green C, Pedersen BK, Hoffman-Goetz L, Rogers CJ, Northoff H, Abbasi A, and Simon P. Position statement. Part one: Immune function and exercise. Exerc Immunol Rev 17: 6-63, 2011.
21. Wang H, Liu Y, and Liu Z. Clara cell 10-kD protein in inflammatory upper airway diseases. Curr Opin Allergy Clin Immunol 13: 25-30, 2013.