Does prolonged cycling of moderate intensity affect immune cell function?

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Background: Prolonged exercise may induce temporary immunosuppression with a presumed increased susceptibility for infection. However, there are only few data on immune cell function after prolonged cycling at moderate intensities typical for road cycling training sessions.

Methods: The present study examined the influence on immune cell function of 4 h of cycling at a constant intensity of 70% of the individual anaerobic threshold. Interleukin-6 (IL-6) and C-reactive protein (CRP), leucocyte and lymphocyte populations, activities of natural killer (NK), neutrophils, and monocytes were examined before and after exercise, and also on a control day without exercise.

Results: Cycling for 4 h induced a moderate acute phase response with increases in IL-6 from 1.0 (SD 0.5) before to 9.6 (5.6) pg/ml 1 h after exercise and CRP from 0.5 (SD 0.4) before to 1.8 (1.3) mg/l 1 day after exercise. Although absolute numbers of circulating NK cells, monocytes, and neutrophils increased during exercise, on a per cell basis NK cell activity, neutrophil and monocyte phagocytosis, and monocyte oxidative burst did not significantly change after exercise. However, a minor effect over time for neutrophil oxidative burst was noted, tending to decrease after exercise.

Conclusions: Prolonged cycling at moderate intensities does not seem to seriously alter the function of cells of the first line of defence. Therefore, the influence of a single typical road cycling training session on the immune system is only moderate and appears to be safe from an immunological point of view.

METHODS

Subjects

Twelve male competitive athletes (nine road cyclists, three triathletes; age 26 (SD 7) years, height 179 (5) cm, weight 71 (5) kg, body fat 11.6% (3.8%), heart volume 13.9 (1.6) ml/kg), who had been cycling for 6.5 (3.3) years and had spent 11 (3) h per week road training during the last season, were recruited for the study after giving their written informed consent. In addition to a physical examination, routine blood parameters were determined in each participant to exclude acute or chronic inflammatory diseases. An ECG at rest and during cycle ergometry as well as an echocardiography were performed to exclude cardiovascular abnormalities (ergometric data: maximal power output 365 (SD 35) W, maximal heart rate 191 (12) beats/min, maximal oxygen uptake 66 (6) ml/min/kg, power output at the IAT 261 (32) W and 3.7 (0.5) W/kg, respectively).

General design

After an incremental stage test to determine the IAT, a constant load trial and an examination on a control day without exercise were performed in randomised order.

Cycle ergometry

To determine the IAT by the method of Stegmann et al.,4 an incremental multi-stage cycle ergometric test was started at a workload of 100 W and increased by 50 W every 3 min until exhaustion. Blood samples were taken from the hyperaemised earlobe at the end of each stage and 1, 3, 5, and 10 min after cessation of exercise to determine lactate concentrations (Super GL, Greiner Biochemica, Flacht, Germany). In addition, VO2max was measured by direct mixing chamber spirometry (Cortex MetaMax I, Leipzig, Germany).

Constant load trial and control day

The constant load trial of 4 h duration at an intensity of 70% IAT given in Watts was carried out on a 400 m track. Heart rate (Polar, Kempele, Finland) and oxygen consumption (Cortex MetaMax I) were recorded continuously. Subjects used their own bicycles which were equipped with an SRM powermeter (Schorberer, Jülich, Germany) to monitor workload. Blood samples from the hyperaemised earlobe to determine lactate concentrations were taken at rest and at the end of cycling after 4 h. Fluid intake was permitted every 30 min and registered exactly; only mineral water with no carbohydrates or energy content was supplied. The tests took place in a temperature conditioned environment.

Abbreviations: AU, arbitrary unit; CRP, C-reactive protein; fMLP, formylised-1-methionyl-1-leucyl-1-phenylalanin; IAT, individual anaerobic threshold; IL-2, interleukin-2; IL-6, interleukin-6; NK cells, natural killer cells; NKCA, NK cell cytotoxic activity
place during the summer season between April and October. Each participant started the trial at 9 am.

In addition, on a separate control day without any exercise an identical blood sampling schedule was performed on the same subjects to monitor the circadian rhythms of the determined parameters. Participants were told to take the same breakfast on the exercise and the control day at about 7 am. Strenuous exercise was forbidden on the days before the tests.

**Immunological parameters**

Blood samples were collected from an antecubital vein into tubes containing K₂, EDTA (2.7 ml) and lithium heparin (7.5 ml) while subjects were in a supine position. Samples were obtained before (before), immediately after cessation of exercise (end), and 1, 2, and 19 h after exercise (1 h post, 2 h post, and 1 day post, respectively) for analysis of haematological parameters (whole blood cell counts, haemoglobin, haematocrit) using a cell counter (Sysmex K-1000, Sysmex, Langenfeld, Germany). Leucocyte and lymphocyte subpopulations were determined by flow cytometry (FACSScan, Becton Dickinson, Heidelberg, Germany) at the following time points: before, end, 1 h post, 2 h post, and 1 day post. NK cells were defined as CD3⁺CD16⁺CD56⁺ lymphocytes, and CD94 and CD158a receptors were determined on CD3⁺CD16⁺ lymphocytes.

The activity of NK cells, neutrophils, and monocytes was also determined by flow cytometry. The NK cell cytotoxic activity (NKCA) was measured before and immediately after exercise. Neutrophil and monocyte phagocytic activity as well as their formylated-1-methionyl-l-leucyl-l-phenylalanin (fMLP) stimulated oxidative burst (intracellular oxidation of dihydrorhodamine 23 to rhodamine 23) were determined before, 1 h post, and 1 day post exercise. All cell counts were adjusted for changes in plasma volume using the formula of Dill and Costill.¹

**NKCA**

NKCA was measured with NKTEST (Orpegen Pharma, Heidelberg, Germany) using fluorescently labelled K562 target cells. Heparinised blood (5 ml) was diluted with phosphate buffered saline (PBS; 1:2) and layered onto 15 ml Histopack 1077 (Sigma, Deisenhofen, Germany).

After centrifugation for 20 min at 700 g, the mononuclear cell layer was resuspended in 12 ml PBS, vortexed, and centrifuged (10 min, 250 g). The supernatant was then aspirated, cells were resuspended in 1 ml Complete Medium (Orpegen Pharma) and the cell concentration adjusted to 1 x 10⁶ cells/ml. Rapidly thawed K562 target cells were suspended in 50 ml prewarmed (37°C) Complete Medium, vortexed, and centrifuged (5 min, 120 g). The supernatant was aspirated, cells were resuspended in 1 ml Complete Medium, and the cell concentration adjusted to 1 x 10⁶/ml. Effector cells were then mixed with K562 target cells at ratio of 50:1 and incubated in a final volume of 200 μl of effector and target cell suspension and Complete Medium. In the high control samples 30 μl interleukin-2 (IL-2) were added prior to the addition of the target cell suspension. In the negative control sample, no effector cells were added. All tubes were then centrifuged (3 min, 120 xg), incubated (120 min, humidified CO₂ incubator), and placed on ice until flow cytometric analysis. To label permeabilised target cells, 50 μl DNA Staining Solution (Orpegen Pharma) were added to each sample and incubated (5 min, 0°C) after vortexing. Dead and live target cells were determined by flow cytometry (FACSScan), which was performed within 30 min after addition of DNA Staining Solution. Per cent specific lysis was determined by subtracting the percentage of dead cells in the control sample tube from the percentage of killed target cells in the test samples (normal sample and high control sample with IL-2), and the number of NK cells needed to lyse one target cell were then calculated (needed NK cells = effector cells x% NK cells measured by flow cytometry/target cells x% specific lysis).

**Neutrophil and monocyte phagocytosis**

Neutrophil phagocytic activity was determined using a commercial test (PHAGOTEST; Orpegen Pharma), which allows the quantitative determination of fluorescein labelled oposionised Escherichia coli bacteria. Heparinised whole blood was vortexed and 100 μl were aliquoted on the bottom of a 5 ml tube and incubated in an ice bath for 10 min. Afterwards, 20 μl precooled E.coli bacteria were added and mixed. While the control samples remained on ice, the test samples were incubated for 10 min at 37°C in a water bath. After incubation, samples were put on ice and 100 μl of ice cold Quenching Solution (Orpegen Pharma) and vortexed. Washing Solution (3 ml; Orpegen Pharma) was added and cells were spun down (5 min, 250 xg, 4°C) twice and the supernatant discarded. After 20 min at room temperature with 2 ml of prewarmed Lysing Solution (Orpegen Pharma), cells were spun down (5 min, 250 xg, 4°C) and the supernatant discarded. After washing the samples with 3 ml of Washing Solution and centrifugation (5 min, 250 xg, 4°C), 200 μl of DNA Staining Solution (Orpegen Pharma) were added, mixed, put on ice (light protected), and measured within 60 min by flow cytometry (FACSScan) collecting 10 000 leukocytes per sample. After gating in the FSC versus SSC, percentages of neutrophils and monocytes having performed phagocytosis were analysed as well as their mean fluorescence intensity in the green fluorescence histogram, representing the number of ingested bacteria. The control sample was used to set a marker for fluorescence so that less than 1% of the events were positive.

**Neutrophil and monocyte oxidative burst**

As described by Rothe,² 3 ml heparinised peripheral blood (10 IU/ml Na⁺ heparin) were layered onto Histopack 1077

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**Table 1**

<table>
<thead>
<tr>
<th>Metabolic Parameters</th>
<th>Concentrations (mmol/l)</th>
<th>Corresponding Values of Control Day Corresponding to Time Point After Exercise (After co-d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.7 (1.0)</td>
<td>Before ex: 1.5 (1.0)</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.4 (1.1)</td>
<td>After ex: 1.5 (0.5)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.57 (0.66)</td>
<td>NS</td>
</tr>
<tr>
<td>FFA</td>
<td>0.15 (0.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glyceral</td>
<td>0.06 (0.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.41 (0.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.55 (0.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cortisol</td>
<td>2.4 (1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>604 (214)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>731 (296)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>503 (244)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>362 (113)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>298 (146)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviations. *At the end of exercise; †1 h after exercise.
(Sigma) to allow sedimentation of erythrocytes at room temperature without centrifugation within 50 min. The leukocyte enriched supernatant was carefully harvested and put on ice. A volume of 1 ml Hank’s balanced salt solution (Sigma) containing 2 x 10^6 leucocytes was incubated at 37°C with 10 μl dihydrodrolamine (100 μm/l), and 10 μl FMLP (10 μm/l) were added after 5 min. After 25 min of incubation samples were put on ice to stop the reaction and stained with propium iodide to differentiate living from dead cells. Flow cytometric measurements for gated neutrophils and monocytes, respectively, were made within 60 min after sample assessment.

CRP, IL-6, metabolic parameters, and hormones
CRP was determined turbidimetrically (Biomed, Oberschleißheim, Germany) and IL-6 by an enzyme linked immunosassay (R&D Systems, Minneapolis, MN, USA). Lactate, glucose (Super GL, Greiner Biochemica), glycerol, and triglycerides were determined enzymatically (Vitalab Lyte Analyzer, CIBA-Corning Diagnostics, Fernwald, Germany), and free fatty acids by photometry (Photometer 1101M, Eppendorf, Germany). Cortisol was determined by chemoluminescence (Magic Lyte Analyzer, CIBA-Corning Diagnostics, Fernwald, Germany), and epinephrine and norepinephrine radioenzymatically.7

Statistics
All data are presented as means (SD). Differences between the constant load trial and the control day were tested using a two factor analysis of variance (mode x time), and the corresponding time points. Newman-Keul’s test was used for post hoc testing. Differences between pre- and post-exercise values for metabolic parameters and hormones were tested using a paired Student’s t test. Pearson’s coefficient of correlation was used to test correlations between selected variables. An α error < 0.05 was considered as significant.

RESULTS
Metabolic parameters and hormones
All 12 cyclists finished the constant load trial of 4 h duration without any problems. The mean power output was 181 (SD 23) W, corresponding to a mean percentage of 72% (SD 5%) of the maximal heart rate and to a mean percentage of 59% (SD 6%) of the maximal oxygen consumption. Mean fluid consumption was 1.8 (SD 1) l mineral water. The results for metabolic parameters and hormones before and after exercise and the corresponding values of the control day are shown in table 1.

IL-6 and CRP
Significant increases in IL-6 from 1.0 (SD 0.5) before to 9.6 (5.6) pg/ml 1 h after exercise (fig 1A) and in CRP from 0.4 (0.3) before to 1.8 (1.3) mg/l 1 day after exercise (fig 1B) were noted. Significant correlations between IL-6 and the following parameters were found: CRP (r = 0.71, p < 0.01), epinephrine (r = 0.70, p = 0.01), norepinephrine (r = 0.63, p = 0.03), cortisol (r = 0.71, p = 0.01), and neutrophils (r = 0.74, p = 0.006). Furthermore, IL-6 correlated inversely to glucose (r = −0.60, p = 0.04). IL-6 did not correlate to

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Table 2  Leukocyte, neutrophil, monocyte, lymphocyte, and NK cell concentrations before and immediately, 1 h, 2 h, and 1 day (Before, End, 1 h post, 2 h post, 1 day post) after 4 h of cycling at an intensity of 70% IAT and at the corresponding time points on the control day without exercise

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mode</th>
<th>Before (SD)</th>
<th>End (SD)</th>
<th>1 h post (SD)</th>
<th>2 h post (SD)</th>
<th>1 day post (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>70% IAT</td>
<td>4958 (1294)</td>
<td>11 186 (3497)</td>
<td>10 609 (3717)</td>
<td>10 746 (3576)</td>
<td>5479 (1460)</td>
</tr>
<tr>
<td>(cells/μl)</td>
<td>Control day</td>
<td>5133 (1061)</td>
<td>5331 (1193)</td>
<td>5650 (924)</td>
<td>6024 (1228)</td>
<td>5180 (1215)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>70% IAT</td>
<td>2949 (1122)</td>
<td>8716 (2863)</td>
<td>8847 (3483)</td>
<td>8389 (3035)</td>
<td>3340 (1096)</td>
</tr>
<tr>
<td>(cells/μl)</td>
<td>Control day</td>
<td>3186 (930)</td>
<td>3276 (894)</td>
<td>3450 (701)</td>
<td>3739 (898)</td>
<td>3140 (935)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>70% IAT</td>
<td>361 (144)</td>
<td>309 (115)</td>
<td>364 (73)</td>
<td>392 (110)</td>
<td>368 (72)</td>
</tr>
<tr>
<td>(cells/μl)</td>
<td>Control day</td>
<td>3276 (894)</td>
<td>3450 (701)</td>
<td>3739 (898)</td>
<td>3140 (935)</td>
<td>3140 (935)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>70% IAT</td>
<td>1226 (467)</td>
<td>1568 (604)</td>
<td>1038 (272)</td>
<td>1182 (387)</td>
<td>1182 (387)</td>
</tr>
<tr>
<td>(cells/μl)</td>
<td>Control day</td>
<td>1175 (256)</td>
<td>1523 (382)</td>
<td>1430 (321)</td>
<td>1510 (397)</td>
<td>1218 (382)</td>
</tr>
<tr>
<td>NK cells</td>
<td>70% IAT</td>
<td>167 (81)</td>
<td>294 (133)</td>
<td>57 (28)</td>
<td>103 (55)</td>
<td>157 (72)</td>
</tr>
<tr>
<td>(cells/μl)</td>
<td>Control day</td>
<td>170 (50)</td>
<td>220 (88)</td>
<td>171 (56)</td>
<td>203 (64)</td>
<td>159 (47)</td>
</tr>
</tbody>
</table>

See text for statistics.

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*Table 2* Leukocyte, neutrophil, monocyte, lymphocyte, and NK cell concentrations before and immediately, 1 h, 2 h, and 1 day (Before, End, 1 h post, 2 h post, 1 day post) after 4 h of cycling at an intensity of 70% IAT and at the corresponding time points on the control day without exercise.
concentrations of free fatty acids, triglycerol, or glycerol or to activities of NK cells, neutrophils, or monocytes.

**Leukocyte and lymphocyte subpopulations**

Exercise induced changes of leukocytes, neutrophils, monocytes, lymphocytes, and NK cells are shown in [table 2](#). Significant increases were found for leukocytes and neutrophils after cessation of exercise (End), and 1 and 2 h after exercise (1 h post and 2 h post, respectively), which were 2-fold for leukocytes and 3-fold for neutrophils. The neutrophil count was significantly correlated to IL-6 (r = 0.74, p = 0.006) and cortisol (r = 0.54, p = 0.07). Furthermore, monocytes were significantly elevated after cessation of exercise as well as 2 h after exercise.

Lymphocytes were raised significantly on both the exercise day and the control day at the time exercise ended but were diminished significantly at 1 and 2 h after exercise (table 2). Counts of NK cells were raised significantly after cessation of exercise but were significantly decreased at 1 and 2 h after exercise (table 2). The rise in NK cells was strongly correlated to epinephrine (r = 0.74, p = 0.005) and ephrine (r = 0.60, p = 0.02) and IL-6 (r = 0.61, p = 0.04). Although CD$^+$CD16$^+$CD94$^+$ and CD$^+$CD16$^+$CD158a$^+$ lymphocytes were raised significantly at the end of exercise (p<0.05), they had dropped to significantly under pre-exercise values at 1 h and 2 h after exercise (p<0.05): CD94$^+$ lymphocytes/μl: 102 (SD 40) (before), 160 (77) (end), 30 (16) (1 h post), 50 (42) (2 h post), 94 (54) (1 day post); CD158a$^+$ lymphocytes/μl: 53 (19) (before), 100 (44) (after), 19 (8) (1 h post), 31 (23) (2 h post), 45 (10) (1 day post). The mean fluorescence intensities of CD$^+$CD16$^+$CD94$^+$ and CD$^+$CD16$^+$CD158a$^+$ lymphocytes remained unchanged.

**NK cytotoxic activity (NKCA)**

The number of NK cells needed to lyse one K562 target cell did not significantly change. This was also true for the interleukin-2 stimulated NKCA (fig 2).

**Neutrophil and monocyte phagocytosis**

The relative number of phagocytising neutrophils and monocytes did not significantly change. This was also true for the absolute number of phagocytising monocytes did not change (fig 3A and B). Mean fluorescence intensities of phagocytising neutrophils and monocytes did not change significantly.

**Figure 2** Unstimulated and interleukin 2 stimulated (+IL-2) NKCA on a per cell basis (shown as NK cells needed to lyse one K562 target cell) before (Before) and immediately after exercise (End) at an intensity of 70% as well as on the control day without exercise at the corresponding time points.
of 70% IAT, which corresponded to 59% V̇O₂max in the present study, was chosen as cyclists usually spend about 80% of their training time below 65% V̇O₂max. Therefore, the present findings are representative for immune reactions in well trained and competitive cyclists during their training sessions.

**Acute phase response**

In the present study, a 3- to 4-fold increase in CRP and an almost 10-fold increase in IL-6 was observed, indicating a moderate acute phase response. This observation is in accordance with the results of previous studies which reported 3- to 10-fold increases in CRP after exercise. The increase in IL-6 is comparable to the increase after 2–2.5 h of cycle ergometric testing at 75% V̇O₂max reported recently, and is higher than after a single maximal or repetitive anaerobic cycle ergometric test. The higher increases in IL-6 reported for runners (up to 100-fold and more after a marathon race) can be explained by the higher mechanical muscular strain.

According to previous reports, the increase in IL-6 results from exercise induced decrease in blood glucose, as IL-6 is produced in the contracting muscle to regulate substrate delivery and especially to maintain the glucose supply to glycogen depleted muscles. Furthermore, the close relation between IL-6 and CRP in the present study supports exercise induced release of CRP through hepatocytes, which is induced by IL-6. But in contrast to Pedersen and Keller, we did not find a relation between the increase in IL-6 and an increase in free fatty acids, glycerol, or triglycerides caused by the lipolytic effects of IL-6. Furthermore, although IL-6 has additional anti-inflammatory properties and induces an increase in plasma cortisol, we did not find a relation to immune cell function.

**Exercise induced leukocytosis and immune cell function**

In a typical response to exercise, a more than 2-fold increase in the numbers of circulating leukocytes was observed, dominated by an almost 3-fold increase in neutrophils which resulted from IL-6 and cortisol mediated recruitment from the bone marrow as described previously. Although lymphocytes were not elevated significantly at the end of exercise, a significant increase in NK cells was noted. This increase is thought to be induced by the catecholamine mediated down regulation of adhesion molecules. The post exercise reductions observed in lymphocytes and NK cells, however, were negatively correlated with both epinephrine and IL-6 mediated increases in cortisol and were in accordance with previous reports.

But beyond alterations in absolute cell numbers, an attenuated immune function of different leukocyte subsets has been discussed in the literature, which might be responsible for greater susceptibility to opportunistic infections within the first hours after exercise and therefore are termed the "open window". As NK cells and macrophages represent the first line of defence, they have attracted particular interest.

**NK cell activity (NKCA)**

No differences in single cell NKCA were observed between before and after exercise, nor between the day of exercise and the control day. This was also true for the in vitro IL-2

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**Figure 3** Absolute numbers of phagocytising neutrophils and monocytes (A and B) and absolute numbers of Rh123⁺ neutrophils and Rh123⁺ monocytes (C and D) before (Before), 1 h and 1 day after (1 h post, 1 day post) 4 h of cycling at an intensity of 70% IAT as well as on the control day without exercise at the corresponding time points.
stimulated single cell NKCA, which, given that exercise increases the level of activity in part through IL-2 by an enhanced IL-2 receptor expression on NK cells, could have been more sensitive. Also the unchanged mean fluorescence intensities of CD3 CD16 CD94 and CD3 CD16 CD158 lymphocytes suggest that exercise had no influence on the expression levels on CD94 or CD158 receptors, which either activate or inhibit NK cell function.89 Taken together, single cell NKCA was unaffected. The present observations accord well with previous reports on single cell NKCA using Cr2+ labelled K562 target cells.90 Nevertheless, they must be differentiated from an intensity dependent increase in total NKCA (reflecting the overall cytotoxic activity of a given population of peripheral blood mononuclear cells with an exercise induced higher proportion of NK cells) during or immediately after exercise.90 NK cell function expressed on a per cell basis has been reported to remain unchanged immediately after exercise in runners after 45 min to 2.5 h of treadmill running at intensities ranging from 50 to 85% VO2max or after repeated cycle ergometric testing.91 Therefore, these previous findings can be extended to even longer exercise bouts by the present results. As single cell NKCA may not be affected even by prolonged exercise, it seems that augmented or attenuated immunological responses presumably resulted from the IL-6 and cortisol release after exercise than before or the day after. This temporary immuno-competence of NK cells is more a matter of numerical redistribution than of single cell NKCA.

CONCLUSION

Prolonged cycling at moderate intensities induces a moderate acute phase response and possibly induces a moderate affection of neutrophil oxidative burst, whereas neutrophil and monocyte phagocytosis remain unaffected. In conclusion, prolonged cycling at moderate intensities does not seem to seriously alter the function of cells in the first line of defence. Therefore, the influence of a single typical road cycling training session on the immune system is only moderate and appears to be safe from an immunological point of view.

REFERENCES


REFERENCES
