Exercise affects tissue lymphocyte apoptosis via redox sensitive and FAS-dependent signalling pathways

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Abstract:

Intensive and exhaustive exercise induces an activation of blood T-lymphocytes which seems to be terminated by apoptotic processes in the post-exercise period. Here, we report that exercise induced T-lymphocyte apoptosis is a systemic phenomenon occurring in various lymphoid and non-lymphoid tissues. Apoptosis rate could be related to exercise intensity and type. While in some tissues such as the spleen and Peyer’s patches an early start of apoptosis (1-3h post-exercise) could be detected, a delayed apoptosis (24h post-exercise) was observed in lung, bone marrow, and lymph nodes. Further analysis showed a similar apoptosis distribution among lymphocyte subpopulations.

We tested whether components of the extrinsic or the intrinsic apoptotic pathways or both were involved in these processes. Elevated levels of lipid peroxidation-product malondialdehyde (MDA) indicating an increased production of reactive oxygen species (ROS) were found after exercise in Peyer’s patches, lung and spleen but not in lymph nodes. Application of N-acetyl-cysteine (NAC) prevented exercise induced T cell apoptosis completely in spleen and bone marrow, partially in lung and Peyer’s patches while it was ineffective in lymph nodes. Additionally, exercise addressed the Fas-mediated apoptosis. The percentage of Fas-receptor (Fas+) and Fas-ligand positive (FasL+) lymphocytes was enhanced in Peyer’s patches after exercise. Moreover, FasL+ T cells were increased in the lung, while in lymph nodes Fas+ cells were increased. The critical role of Fas-signalling in exercise-induced apoptosis was supported by using Fas-deficient MRL/Lpr-mice. In Fas-deficient mice exercise induced T-lymphocyte apoptosis was prevented in spleen, lung, bone marrow and lymph nodes but not in Peyer’s patches.

These data demonstrate that exercise induced lymphocyte apoptosis is a transient systemic process with tissue-type specific apoptosis-inducing mechanisms whose relevance for the adaptive immune competence remains to be shown.
Introduction:

Lymphocyte apoptosis is an important regulatory mechanism of the immune system involved in diverse processes like induction of self-tolerance, maintenance of cell homeostasis and termination of an immune response (1). Apoptotic processes are defined by a series of biochemical events and morphological changes, which include membrane shrinkage, phosphatidylserine externalization, chromatin condensation, DNA-fragmentation and the appearance of apoptotic bodies (29). Apoptosis of peripheral T cells can be induced by several pathways. The so called extrinsic pathway is triggered by signals emanating from cell surface death receptors (e.g. Fas receptor (Fas)) that are activated by specific ligands (e.g. Fas ligand (FasL)) followed by the activation of caspases, a cascade of intracellular proteases. In contrast, the intrinsic apoptotic pathway can be activated by various cellular stress signals like UV-radiation and DNA damage, oxidative stress etc. resulting in an imbalance between pro- and anti-apoptotic proteins. This, in turn, induces a permeabilization of the outer mitochondrial membrane, a release of cytochrome c and eventually a convergent activation of the executioner caspases. And finally, the caspase independent cell-death pathway is characterized by the activation of cathepsins which are located in the lysosomes (2,14,15). Which particular cell-death signal is operative under which particular circumstance remains to be shown for the various lymphoid and non-lymphoid compartments.

Intensive exercise is followed by a substantial decline of lymphocytes in peripheral blood. Recent studies indicate that the peripheral lymphopenia is a consequence of both lymphocyte redistribution and apoptosis (16,18,19). These processes seem to run parallel and their relative magnitude seems to depend on exercise intensity, since moderate exercise did not - or only marginally - alter either lymphocyte trafficking or apoptosis (16,19). However, the blood compartment provides only little insight into exercise induced immune cell alterations since most lymphocytes reside in lymphatic organs. Using the mouse model, Hoffman-Goetz et al.
(1999) were the first to investigate the effect of exercise on tissue lymphocyte apoptosis. According to them, strenuous treadmill running was followed by increased lymphocyte apoptosis in the intestinal epithelium and the thymus (11,27). These data suggest that exercise-induced lymphocyte apoptosis might not be restricted locally but might be a general process widely spread throughout various tissues.

Several death-inducing mechanisms and signals such as enhanced oxidative stress and DNA damage, hormonal alterations, e.g. of glucocorticoids and tumor-necrosis-factor $\alpha$ (TNF-$\alpha$), and up-regulation of death-receptors and –ligands have been proposed as being involved in exercise-induced lymphocyte apoptosis (11, 19, 25, 31). So far, most research focused on the role of oxidative stress since exercise is known to increase the formation of reactive oxygen species (ROS) (6). Strenuous exercise was followed by increased levels of lipid peroxidation markers in blood, thymocyte DNA fragmentation in rats and glutathione (GSH) depletion (5, 27,31).

ROS can influence lymphocyte apoptosis at certain stages and different pathways (33). On the one hand ROS can reduce cellular Bcl-2 content and directly depolarize the outer layer of the mitochondrial membrane (8). On the other hand it has been shown that ROS crosslink with the extrinsic apoptosis pathway by modulating FasL expression. Likewise, exercise has been shown to affect both cellular Bcl-2 content in thymic lymphocytes and expression of FasR/FasL on human T cells (19,26).

Therefore, the aim of the current study was to investigate the effect of exercise on systemic T cell apoptosis in several lymphoid and non-lymphoid tissues over a period of 48 hours by using exercise protocols of different intensities and types. In order to characterize the role of oxidative stress we determined both the exercise induced formation of malondialdehyde (MDA), an intermediate product of lipid peroxidation, and the effect of the ROS scavenging agent N-acetylcystein (NAC) on tissue T cell apoptosis. Moreover, we tried to characterize the influence of Fas signalling by determining the surface expression of FasR and FasL on
lymphocytes after exercise and by exercising MRL/Lpr mice, which carry a mutation in the CD95 gene, resulting in a lack of Fas surface expression (33). We hypothesize that exercise induces T cell apoptosis in several lymphoid organs in an intensity-dependent manner which depends on different apoptosis regulating and signalling pathways.
Methods

Experimental animals

The study was performed with a total of 235 male C57BL/6 mice, aged 8-12 weeks and weighing 27.5 ± 3.5g, bred at the animal facility of the Department of Sports Medicine (Justus-Liebig University, Giessen). Additionally, 18 MRL/lpr mice, aged 8-16 weeks and weighing 25.6 ± 4.5g, were used. MRL/lpr mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed 4-6 per cage at 21 ± 1°C in standard cages with free access to food and tap-water. All experiments were approved by the Local Animal Care and Use Committee.

Aerobic capacity

Aerobic capacity was determined by using a treadmill spirometry (custom made). All animals were first acclimatized to the treadmill. Maximal oxygen consumption (VO$_{2\text{max}}$) and maximal running speed (Vmax) of mice were tested at least 4 days before starting the experiments. After 10 min of acclimatization in the treadmill chamber, mice performed a continuous, progressive exercise test until exhaustion. The test uptake started at 0.20 m/sec., every 3 min the speed was increased by 0.05 m/sec.

Exercise protocols and NAC application

Mice of the control group (CON) were exposed to treadmill noise without running while mice of the intensive exercise test group (IET) performed a running test at 80% VO$_{2\text{max}}$ corresponding to 0.33 ± 0.06 m/s until exhaustion. Average running time of IET was 40.5 ± 5.0 min. Mice were sacrificed by cervical dislocation at various points of time after the exercise tests (immediately, 3h, 24h and 48h).

Additional groups of mice performed a moderate exercise test (MET) and a swimming exercise test (SET). Animals of the MET group were subjected to a treadmill running test at
60% VO\textsubscript{2max} for 20 min corresponding to 0.20 ± 0.03 m/s, while the SET group performed a swimming exercise test until exhaustion. The SET was conducted by placing mice in a swimming container (height: 30 cm, diameter: 20 cm) containing water at 22°C. Average swimming time was 41.5 ± 6 min. Mice of the MET and the SET groups were sacrificed 24h after exercise as described above. Mice of the NAC-group performed IET 30 min after receiving an intraperitoneal injection of 1g x kg\textsuperscript{-1} of the antioxidant N-acetylcystein (NAC; Sigma Chemical, St. Louis, USA) dissolved in saline. It has been shown previously that this dosage maintains intracellular glutathione levels and prevents apoptosis in intestinal intraepithelial lymphocytes and thymocytes (26,27). Again, mice of the NAC group were sacrificed only 24h after exercise.

**Blood samples and tissue collection**

All organs were excised immediately after sacrificing the animals and placed in culture dishes containing cold PBS. After cutting organs into small pieces, cell suspensions were created by pressing the minced tissues gently through a fine nylon mesh (pore size: 100\textmu m).

For preparation of bone marrow cells, skin and tissues of legs were removed. Hip and knee joints were cut carefully to expose the femur bone. After removing the muscles, bone ends were cut and bone was flushed with 1 ml of PBS using a syringe and a 27G needle. Bone marrow pieces were homogenized by pulling through the needle repeatedly.

**Lymphocyte isolation**

For lymphocyte separation, cell suspensions were layered over a Ficoll-density gradient separation solution (density: 1.077x, Sigma, St. Louis, USA) and centrifuged for 15 min at 2000 rpm. Lymphocyte layer was removed and cells were washed by centrifugation at 1200 rpm for 10 minutes. Cell viability was assessed by trypan blue exclusion.
Determination of oxidative stress

The extent of oxidative damage done to spleen, lung, bone marrow, lymph nodes and Peyer’s patches by the exercise-associated generation of free radicals was assessed by measuring levels of malondialdehyde (MDA), an intermediate product of lipid peroxidation. For MDA determination we used a modified method of Khoschsorur et al. (2000) and Wong et al. (1987). Spleen, lung, lymph nodes and Peyer’s patches of control and exercise mice were removed and weighed quickly as well as homogenized under nitrogen and derivatized with thiobarbituric acid. Measurement of MDA (TBA-MDA) was performed spectrofluorimetrically using a luminescence spectrometer (Perkin Elmer LS55) at wavelength 532 nm (excitation) and 553 nm (emission). MDA values were repeatedly verified by HPLC measurements. Because stability of MDA values was extremely sensitive during sample preparation, values of control tissues were set to 100% and data are presented as percentage changes compared to CON.

Flow cytometric assessment of lymphocyte subpopulations and apoptosis parameters

Lymphocyte suspensions were incubated either with monoclonal antibodies anti-CD3, anti-CD4 and anti-CD8 (PE-conjugated, Immunotools, Friesoythe, Germany) and appropriate isotype controls. For detection of apoptosis, cells were additionally labelled with Annexin-V antibodies (FITC-conjugated, Immunotools, Friesoythe, Germany) for 30 min in the dark. Cells were centrifuged again for 10 min at 1200 rpm and prepared for flow cytometry using an EPICS XL Flow Cytometer (Beckman Coulter). A minimum of 5000 events, mostly 10000 events were obtained for all samples.

For detection of death surface receptors and ligands lymphocyte suspensions were stained either with CD95FasR or CD95FasL (PE-conjugated, Immunotools, Friesoythe, Germany and
BD Biosciences) and with mAb against CD3 (FITC-conjugated). Cells were incubated for 30 min at RT in the dark. Finally, cells were washed again and analyzed via flow-cytometry.

Statistical analysis

Data are means ± SEM unless indicated otherwise in the figure legends. Differences between pre-exercise values and values at the post-exercise time points were compared with repeated-measures ANOVA. If significant main effects were observed, post hoc analysis was conducted by using the Bonferroni test. In all cases $P < 0.05$ was accepted as being significant. Data were analyzed using the SPSS statistical analysis program.
Results

Systemic T cell apoptosis after intensive exercise

IET was followed by a significant increase in apoptotic cells in all lymphoid and non-lymphoid tissues investigated. However, marked differences were observed regarding kinetics and extent of cell death. In the spleen IET was followed by a significant increase in apoptosis of CD3$^+$ and CD4$^+$ cells immediately after exercise. 3h after IET, apoptosis was evident in all lymphocyte subpopulations lasting for at least 48h after exercise (Fig. 1 A). In the Peyer’s patches T cell apoptosis showed a bimodal pattern. An early increase of apoptosis was found immediately after the IET in all subpopulations followed by a second, significant peak 24h later (Fig. 1 E). In contrast, in lung, lymph nodes and bone marrow we observed a delayed effect of exercise on lymphocyte apoptosis. Fig. 1 F illustrates that apoptosis of pulmonary lymphocytes did not increase until 24h after exercise compared to CON. Likewise, percentage rates of apoptotic CD3$^+$, CD4$^+$ and CD8$^+$ cells in lymph nodes and bone marrow increased 24h after IET (Fig. 1 C and D). In all organs, except for the spleen, the rate of apoptosis returned to baseline values within 48h after exercise. Although the individual running time was quite variable, it could not be related to the rate of apoptotic cells.

Effect of intensity and type of exercise on T cell apoptosis

Next we investigated the effect of different exercise intensities and types on T cell apoptosis by exposing mice either to MET or SET. In this section we focused on measurements 24h after exercise, since at that point of time T cell apoptosis showed significant increases in all organs investigated. Interestingly, MET did not affect T cell death in any organ (Fig. 2 A-E). In contrast, SET seems to affect apoptosis selectively. In spleen only a slight increase of apoptotic CD3$^+$ was observed (Fig. 2 A), while in lung and lymph nodes apoptosis of CD3$^+$ and CD4$^+$ cells was enhanced (Fig. 2 B+D). In Peyer’s patches apoptosis of all subpopulations was increased, while in bone marrow T lymphocytes were not affected by SET (Fig. 2 C+E).
Increased oxidative stress affects lymphocyte apoptosis

In order to determine whether lymphocyte apoptosis could be related to oxidative stress, we were first interested in investigating the kinetics and distribution of MDA tissue levels after exercise. Therefore, mice were subjected to IET and organs were harvested immediately, 3h and 24h after exercise. We found that MDA levels were differentially affected by exercise. Exercise induced increases of MDA were found to be highest in Peyer’s patches with an almost 3.5-fold change immediately and 3h after IET (Fig. 3). Increases of MDA were substantially smaller in lung and spleen, while no significant changes were observed in lymph nodes (Fig. 3). We were unable to detect any changes of MDA levels in bone marrow cells due to technical reasons. MDA changes could not be related to the alterations of T cell apoptosis in any organ. However, we could demonstrate that there were no significant changes of MDA after subjection to MET corresponding to the unchanged apoptosis levels under this condition (Tab. 1; Figure 2).

Then we tested the effect of NAC pre-treatment on MDA levels and exercise-induced apoptosis after we had confirmed that this procedure was effective in preventing an increase of MDA in Peyer’s patches and spleen (Tab. 1). As shown in Fig. 4, NAC effectively abolished the induction of T cell apoptosis in spleen and bone marrow 24h after IET. Similarly, NAC-application prevented apoptosis of CD3$^+$ and CD8$^+$ T cells in lung, and of CD3$^+$ and CD4$^+$ in Peyer’s patches. However, in lymph nodes NAC application failed to affect the rate of apoptotic T lymphocytes after IET.

Exercise and Fas-mediated apoptosis

In the final section we studied the involvement of Fas/FasL signalling on exercise induced tissue apoptosis. Therefore we first determined the expression of both Fas and FasL on tissue CD3$^+$ cells 24h after IET. Expression of both Fas and FasL was significantly up-regulated in
CD3+ T cells from Peyer’s patches (Fig. 5E). Fas expression was increased in T-lymphocytes from lymph nodes, while FasL expression was enhanced on pulmonary CD3+ T cells (Fig. 5 B and D). In contrast, we observed no changes in either Fas or FasL expression on splenic or bone marrow T cells (Fig. 5A and C).

In order to confirm the role of Fas-signalling in exercise-induced lymphocyte apoptosis, MRL/Lpr-mice, which lack a FAS receptor surface expression, were subjected to IET. Due to the fact that this mutation causes lymphoproliferation and autoimmune disease, basal levels of apoptotic tissue T cells may vary as compared to wildtype mice. Therefore, we initially determined T cell apoptosis in MRL/Lpr mice under control conditions. We found that exercise-induced apoptosis of CD3+, CD4+, and CD8+ cells was completely abolished in spleen, lung and bone marrow of MRL/Lpr-mice (Fig. 6 A-C). In lymph nodes the exercise-induced increase of apoptotic CD3+ cell was attenuated, while in the CD4+ and CD8+ subpopulations the increase of apoptosis was completely abolished (Fig. 6D). Conversely, exercise was still effective in inducing apoptosis of T cell populations from Peyer’s patches of MRL/Lpr mice (Fig. 6E).
Discussion

The current study presents evidence that exercise induced T cell apoptosis is a systemic process involving several lymphoid and non-lymphoid tissues. Interestingly, marked differences with respect to kinetics and amount of lymphocyte apoptosis were detected with early alterations in spleen and Peyer’s patches and delayed changes in lung, bone marrow and lymph nodes.

These data upgrade previous studies showing an increased rate of T cell apoptosis in the vascular compartment after strenuous exercise such as marathon runs and exhaustive treadmill exercises (18,19,25,24). But only a small fraction of T lymphocytes can be found transiently in the circulation since the immune system is a complex network distributed throughout the body. Specialized compartments (de-)centralize the immune functions in the lymphoid organs from which immune cells migrate and patrol into non-lymphoid tissues. Recently, we could demonstrate that exercise affects lymphocyte homing and induces a redistribution of cells within the different tissues (16). It cannot be excluded that these processes might also have affected the distribution of apoptotic T cells as shown in the present study.

Our results corresponded closely to previous studies showing an intensity-dependent regulation of peripheral lymphocyte apoptosis (19,24). More precisely, Navalta et al. (2007) increased exercise intensity stepwise and observed an increase of apoptotic cells at exercise intensities beyond 60%VO\textsubscript{2max} in human subjects. Similarly, the MET, which corresponded to 60% VO\textsubscript{2max} was unable to induce any apoptosis. In contrast, the SET enhanced T cell apoptosis in spleen, lung, lymph nodes and Peyer’s patches increased, while bone marrow lymphocytes were not affected. One reason for these divergent effects could be the different metabolic and hormonal response of this type of exercise as indicated by lactate and catecholamine levels (16). Enhanced lymphocyte apoptosis in lymphatic compartments is in agreement with other studies on exercise and stress research. Increased apoptosis of splenic lymphocytes and thymocytes was observed after chronic restraint stress as well as after
several models of physiological stress like exercise, sepsis and burn injury (9,12,26,35). Intensive treadmill running induced a massive apoptosis of thymocytes and intestinal lymphocytes 24h later (11,27). Increased T cell death in bone marrow 24h after exercise, as shown in our study, might temporarily affect the thymus’s supply with naive T cells and therefore affect adaptive immune competence. Similarly, the increased T cell apoptosis in lung and Peyer’s patches 24h after exercise might destabilize these major defence barriers of the body. The relevance of these processes for immune competence has to be confirmed in future studies using suitable infection models. Otherwise the enhanced tissue T cell apoptosis might indicate an important termination of the exercise associated immune cell activation.

*Increased oxidative stress differentially affects tissue T cell apoptosis*

Differences in kinetics and magnitude of exercise-induced T cell apoptosis between organs might be the result of both an increased production of apoptosis-inducing factors and an enhanced susceptibility to apoptosis of resident cells in these compartments. Exercise is associated with enhanced oxidative stress, which is an effective inducer of apoptosis in T cells (11,25,26,27). Therefore, we first determined levels of MDA as a product of lipid peroxidation which can serve as an indicator of increased free radical formation in peripheral tissues (21).

Our data suggest that the load of oxidative stress is considerably different for the various tissues. The highest increases of MDA levels were found in PP immediately after exercise persisting up to 3h after IET. An increase of MDA was also evident in lung and spleen after IET, although changes were considerably lower than in PP. And finally, no increases of MDA levels were found in lymph nodes. These data enable the setup of a systemic map of exercise-induced oxidative stress leading to the assumption that perfusion changes are major determinants for the generation of ROS. On the one hand, it can be proposed that the high MDA levels in Peyer’s patches are primarily a result of the exercise-induced reduction of
Splanchic blood flow (22). Thereby cessation of blood flow seems to be sufficient to increase free radical generation without any anoxia present (17). A further mechanism might involve the free radical generation during an ischemia-reperfusion injury. Likewise, Muia et al. (2005) observed a significant increase of MDA in the gut after splanchic artery occlusion. On the other hand, recently published data suggest that the rapid onset of perfusion or a variable perfusion is also able to induce free radical generation (7,30). This may be one reason for the enhanced pulmonary MDA levels during exercise since lung perfusion is substantially enhanced due to the increased cardiac output. In contrast, one can assume that lymph node perfusion does not vary considerably during exercise resulting in unaltered MDA levels as determined in the present study.

The experiments using NAC suggest the relevance of exercise associated ROS formation for tissue T lymphocyte apoptosis. NAC rapidly enters the lymphocyte and is hydrolyzed to release cysteine, the rate limiting substrate in GSH synthesis (28). Maintenance of GSH concentrations affects ROS-induced apoptosis because GSH is the major determinant of intracellular redox potential. We were able to show that NAC efficiently prevented both the exercise induced increase of MDA and the increase of tissue T cell apoptosis except for the lymph nodes. Likewise, NAC has been shown previously to inhibit intestinal lymphocyte GSH depletion and phosphatidylserine externalisation after treadmill running in mice (11,26). In lymph nodes we detected neither an increase of exercise induced MDA levels nor an effect of NAC treatment on lymphocyte apoptosis. Therefore, we suggest that alternative apoptosis mediating mechanisms are operative.

*Increased apoptosis after exercise is a Fas-mediated process*

Apoptosis in T cells can be induced by a variety of signals using either the extrinsic or the intrinsic pathway (14). Oxidative stress predominantly acts via the intrinsic pathway. However, some crosslinks to the extrinsic system, represented e.g. by the Fas/Fas-ligand
pathways, have been shown (23). After activation T cells become progressively more sensitive to apoptosis mediated by increased expression of Fas and FasL (2). Gabriel et al. (1993) found that endurance exercise increased CD45RA^-45RO^ cells, which most probably reflect an activation of T cells. Consequently we were recently able to demonstrate that human blood T lymphocytes express increased levels of Fas and FasL following intensive exercise (19). Our current results confirm that exercise regulates Fas/FasL expression of T cells in some but not all lymphatic and non-lymphatic tissues. Enhanced expression of FasL in lung lymphocytes 24h after exercise corresponds with the onset of apoptosis. These results are consistent with the finding that exercise did not affect pulmonary T cell apoptosis in MRL/Lpr mice. In lymph nodes an increased expression of Fas was found, while T cell apoptosis after IET was attenuated in MRL/Lpr mice. Both Fas and FasL expression of T cells from PP increased significantly following exercise. However, loss of functional Fas receptor in MRL/Lpr mice did not prevent an exercise induced T cell apoptosis suggesting that Fas-mediated apoptosis is not the main pathway in this tissue type.

For the spleen and bone marrow our results were inconsistent. While exercise neither affected the expression of Fas nor of FasL on splenic or bone marrow T lymphocytes, exercise-induced apoptosis of T cells was prevented in MRL/Lpr mice. One explanation might be that apoptosis is controlled by a paracrine expression or release of FasL of endothelial or reticular origin (4). Studies concerning the role of Fas-expression in splenic lymphocytes after stressful conditions are inconsistent. While Yin et al. (2000) observed an increased Fas expression after restraint stress, apoptosis was still evident in FasL deficient mice after burn injury (9).

Altogether these data suggest that T cell apoptosis is mediated by different mechanisms depending on the tissue compartment and the type of stress. While the ROS-mediated apoptosis pathway seems to be of major importance in PP after exercise, Fas-mediated apoptosis prevails in lymph nodes. Both pathways eventually seem to be operative in spleen, bone marrow and lung, which raise the question about interactions of both pathways (Table
2). One critical mediator between ROS and the Fas-pathway might be the transcription factor NF-κB. NF-κB activation and translocation into the nucleus in response to external stressors, oxidative stress and sepsis have been shown to occur rapidly (3). Moreover, NF-κB activation has been related to the up-regulation of numerous cytokines and mediators such as FasL (8). Finally, it has to be considered that further death inducing mechanisms may be operative such as glucocorticoid –dependent pathways. Serum glucocorticoid concentrations are enhanced especially after strenuous exercise protocols (27).

**Perspectives and Significance**

These findings indicate that exercise-induced T cell apoptosis is not limited to the vascular compartment but is of general significance in a number of lymphoid and non-lymphoid tissues such as spleen, lung, lymph nodes, bone marrow and Peyer’s patches. Furthermore, both the extent and kinetics of apoptosis and the inducing mechanisms show tissue specific patterns. The physiological impact and clinical relevance of these transient systemic alterations of T cell apoptosis remain to be clarified. We suppose that exercise-induced apoptosis might be a regulatory mechanism to remove activated and potentially autoreactive immune cells. Whether these alterations have consequences for post-exercise immune competence is unclear at the moment and has to be addressed in future studies.

Acknowledgements:

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**Figure legends:**

**Figure 1:**
Effect of intensive exercise test (IET) on apoptosis of CD3⁺, CD4⁺ and CD8⁺ cells immediately, 3h, 24h and 48h after exercise. The results show critical differences regarding extent and kinetic of apoptosis in lymphatic/non-lymphatic tissues. In spleen (A) and Peyer’s patches (B) IET was followed by a significant increase of apoptosis of CD3⁺ and CD4⁺ cells immediately after exercise. In contrast, in lung (B), bone marrow (C) and lymph nodes (D) apoptosis did not increase until 24h after exercise. Figure 1 F illustrates percentage changes of apoptotic of CD3⁺ cells leading to a classification of both tissues with an early and prolonged apoptosis and tissues with a delayed T cell apoptosis (24h after exercise). * indicates significant differences compared to control group levels (P < 0.05). All values are mean ± SEM for 8-11 animals/group. PP = Peyer’s patches, SPL = spleen, LU = lung, BM = bone marrow, LN = lymph nodes.

**Figure 2:**
Effect of different exercise intensities on apoptosis of CD3⁺, CD4⁺ and CD8⁺ cells in lymphatic organs 24h after exercise. Mice of the control group (CON) were exposed to treadmill noise without running, the moderate exercise test group (MET) performed a 20 min treadmill run at 60 % VO₂max, and the intensive exercise test group (IET) performed a treadmill run at 80% VO₂max until exhaustion, while the swimming exercise test group (SET) was subjected to a swimming exercise until exhaustion. * indicates significant differences compared to control group levels (P < 0.05). All values are mean ± SEM for 8-11 animals/group.
Figure 3:
Percentage (%) changes of MDA levels in spleen, lung, PP and lymph nodes immediately, 3h and 24h after IET. MDA-levels of control mice were set to 100%. * indicates significant differences compared to control group levels (P < 0.05). All values are mean ± SEM for 6-8 animals/group. PP = Peyer’s patches

Figure 4:
Effect of NAC application 30min before IET on lymphocyte apoptosis in spleen, lung, bone marrow, lymph nodes and Peyer’s patches. Analysis was performed 24h after exercise. CON control group, IET intensive exercise test group, IET-NAC n-acetylcysteine/intensive exercise test group. * indicates significant differences compared to control group levels (P < 0.05), # indicates significant differences compared to IET (P < 0.05). All values are mean ± SEM for 8-11 animals/group.

Figure 5:
Effect of IET on percentage changes of Fas (A) and FasL (B) expression in CD3⁺ cells of different tissues 24h after IET. Control group levels were set to 100%. * indicates significant differences compared to control group levels (P < 0.05). All values are mean ± SEM for 7-9 animals/group. PP = Peyer’s patches, SPL = spleen, LU = lung, BM = bone marrow, LN = lymph nodes.

Figure 6:
Effect of IET on apoptosis of CD3⁺, CD4⁺ and CD8⁺ cells in MRL/Lpr mice compared to wildtype mice (WT). Control group of wildtype mice (CON/WT) and control group of MRL/LPR mice (CON/LPR) were subjected to control conditions, while IET group of
wildtype mice (IET/WT) and IET group of MRL/LPR mice (IET/LPR) were subjected to a treadmill run at 80% VO2max until exhaustion. *indicates significant differences of either CON/WT to IET/WT and of CON/LPR compared to IET/LPR (P < 0.05). All values are mean ± SEM for 8-11 animals/group.

Table 1:
Percentage (%) changes of MDA levels in PP and spleen immediately after MET, IET and after IET-NAC conditions. MDA-levels of control mice were set to 100%. * indicates significant differences compared to control group levels (P < 0.05). All values are mean ± SEM for 6-8 animals/group.

Table 2:
Involvement of oxidative stress and Fas-mediated pathways on exercise-induced apoptosis of T cells. + mediates apoptosis, ( ) means partially mediates apoptosis, - means does not mediate apoptosis.
References


Figure 1

A. Spleen T cells

B. Lung T cells

C. Bone marrow T cells

D. Lymph node T cells

E. Peyer's patches T cells

F. Increase of apoptosis of CD3 positive cells

Legend:
- CD3
- CD4
- CD8

Early/late apoptosis
Late apoptosis

Bars represent post/3h and 24h
Figure 2

A) Spleen T cells

B) Lung T cells

C) Bone marrow T cells

D) Lymph node T cells

E) Peyer's patches T cells

Annexin positive cells [%]

CON MET IET SET

CD3 CD4 CD8
Figure 3

Change of malondialdehyde level [%]

CON                            POST                             3h                             24h

Spleen                          Lung                             PP                             Lymph nodes
**Figure 4**

A. **Spleen T cells**

B. **Lung T cells**

C. **Bone marrow T cells**

D. **Lymph node T cells**

E. **Peyer's patches T cells**
Figure 5

A

Fas expression

B

FasL expression
Figure 6

A

Spleen T cells

Annexin positive cells [%]

B

Lung T cells

Annexin positive cells [%]

C

Bone marrow T cells

Annexin positive cells [%]

D

Lymph node T cells

Annexin positive cells [%]

E

Peyer's patches T cells

Annexin positive cells [%]
<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>condition</th>
<th>Change of malondialdehyde level [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>spleen</td>
<td>MET</td>
<td>111± 17 n.s.</td>
</tr>
<tr>
<td></td>
<td>IET-NAC</td>
<td>106 ± 24 n.s.</td>
</tr>
<tr>
<td>Peyer's Patches</td>
<td>MET</td>
<td>112 ± 12 n.s.</td>
</tr>
<tr>
<td></td>
<td>IET-NAC</td>
<td>132 ± 32 n.s.</td>
</tr>
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Tab. 1
<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Oxidative stress</th>
<th>FAS-signalling</th>
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</thead>
<tbody>
<tr>
<td>Peyer's patches</td>
<td>(+)</td>
<td>(-)</td>
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<tr>
<td>Bone marrow</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>(+)</td>
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</tr>
<tr>
<td>Lymph nodes</td>
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Tab. 2