Exercise-induced leukocyte apoptosis

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ABSTRACT

Physical exercise is well known to affect leukocyte numbers and function. While regular exercise training has been shown to enhance specific immune functions, acute bouts of intensive exercise often lead to a pro-inflammatory response accompanied by a transient lymphocytopenia and neutrophilia. It can be assumed, that lymphocytopenia can be attributed at least partially to an enhanced lymphocyte apoptosis. In contrast, regulation of neutrophil apoptosis after exercise remains controversial since studies demonstrated both an up-regulation as well as a down-regulation of cell death. However, these discrepancies may be due to differences in exercise protocols, subjects' fitness levels, and to different methodological approaches.

Two major signalling pathways of exercise induced apoptosis have been identified. First the external receptor mediated pathway using death receptors, and second the internal, oxidative-mediated pathway which encompasses the mitochondria. Potential apoptosis modulating mediators are reactive oxygen species (ROS), glucocorticoids and cytokines which are part of the systemic inflammatory response evoked after acute intensive exercise.

Finally, the physiological impact and clinical relevance of leukocyte apoptosis will be discussed. On the one hand, exercise-induced apoptosis might be a mechanism to remove activated and potentially autoreactive immune cells. On the other hand, apoptosis might be a regulatory mechanism which is necessary for tissue reorganization and adaptational training processes.

Keywords: cell death, lymphocytes, neutrophils, extrinsic apoptosis pathway, intrinsic apoptosis pathway, reactive oxygen species, glucocorticoids.

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1. Apoptosis

Apoptosis is of fundamental importance to guarantee a balance between the generation of new cells and removal of damaged or aged cells. Thereby, apoptosis is a complex process of cell death that allows cells to die in a well controlled fashion. Apoptotic processes play an important role during development and in maintaining tissue homeostasis. Besides physiological cell turnover, the role of apoptosis has also been demonstrated during a variety of pathophysiologicalconditions, for example: during inflammatory response, after cellular damage or in preventing neoplastic diseases (9). The process of apoptosis is highly orchestrated and cells have specific intracellular signalling pathways necessary for cell death. The specific induction-pathways of apoptosis strongly depend on the cell type, the presence or absence of specific death promoting or cell stabilizing signals, and the extent of cell damage or dysfunction (29).
A common characteristic of apoptosis is the elimination of cells without lysis or necrosis which is known to result in local inflammatory reactions. The typical morphological changes during apoptosis include membrane blebbing, DNA fragmentation and cellular component degradation.
Therefore, apoptosis offers the opportunity to remove cells without any collateral damage in adjacent tissues. Based on the nature of the apoptotic stimuli, two main pathways for apoptosis induction have been differentiated: an extrinsic pathway which is initiated by the binding of a ligand. These ligands are more frequently expressed after specific physiologic stimuli and bind to a death promoting receptor. The second main pathway is known as the intrinsic pathway, which is mainly triggered by damage to the cell or single components (23).

1.1 The extrinsic pathway of apoptosis

The extrinsic pathway of apoptosis is initiated by specific ligands which bind todeath receptors. Death receptors are membrane receptors which transduce apoptotic signals from the extracellular space into the cytoplasm. The ligands for these receptors form a family of related cytokines collectively named as the TNF family. The ligands are known to act in both an autocrine and a paracrine way to induce the trimerization of their respective cell surface receptors (65).

A major signalling pathway for the extrinsic induction of apoptosis is the Fas-receptor (FasR)/Fas ligand (FasL) pathway (64). After binding of FasL to FasR, the receptor is trimerized. The Fas adaptor protein, Fas-associated death domain protein (FADD), binds to the trimerized Fas cytoplasmatic region through the interaction of the death domains. Next pro-caspase-8, which is an important executing protease, is recruited to FADD. The Fas-receptor, FADD and pro-caspase-8 form a functional death-inducing signalling complex (DISC). This process induces self activation of caspase-8, which is released into the cytosol and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis (32,50).

1.2 The intrinsic pathway of apoptosis

Besides the extrinsic pathway, a second route can initiate apoptosis in response to specific stimuli. Because one of the first steps in this pathway is the permeabiliza-

tion of the outer membrane of the mitochondria, this pathway is also known as the mitochondrial pathway. Several stimuli like ionizing radiation, oxidative or heat stress, osmotic changes or cytotoxic drugsare able to provoke induction of the intrinsic pathway. In this framework, the life and death of cells is largely controlled by proteins of the Bcl-2 family. Within this family of proteins, three subfamilies exist represented by Bcl-2 like molecules, Bax like molecules and BH3-molecules (7). It is assumed that the Bcl-2 proteins mediate their effects at least in part by regulating mitochondrial morphology and/or function. The pro-apoptotic Bax protein may form channels in the outer mitochondrial membrane, whereas Bcl-2 interferes with that mechanism and maintains the integrity of mitochondria (1). As previously mentioned, mitochondria are central regulators of the intrinsic apoptosis pathway. After permeabilization of the mitochondrial membrane, cytochrome c is released into the cytoplasm which recruits the caspase adaptor molecule Apaf-1 and the apoptosis initiator molecule procaspase-9 (22). Consequently, cytochrome c, Apaf-1 and caspase-9 form a holoenzyme complex called an apoptosome. They proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed (14).

In many cell types both apoptosis pathways are not fully separated. Instead, there is some crosstalk between the extrinsic and intrinsic apoptosis pathway. The crosstalk is known to be caspase-8-mediated which induces a cleavage of the Bcl-2 family member Bid (38). Bid mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cleaved Bid activates Bax and causes oligomer formation on the mitochondrial membrane, loss of mitochondrial transmembrane potential and triggers the intrinsic pathway of apoptosis (71).

2. Leukocyte apoptosis

Apoptosis is a central mechanism in all leukocyte subclasses to govern their lifespan and to support their cellular function. In the case of an inflammatory response cells develop a certain apoptosis resistance to increase their cell population (clonal expansion), while after termination of the immune reaction these cells have to be deleted effectively through controlled cell death (clonal contraction) (32). Because the topic of exercise and leukocyte apoptosis was up to now mainly focused on lymphocytes and neutrophils, this review will also focus on these cell types as central themes.

2.1 Lymphocyte apoptosis

T and B cells are the central actors of the adaptive immune response. During physiological conditions, apoptosis plays an important role in the maintenance of lymphocyte homeostasis and the elimination of aged or potentiallyself-reactive cells.In the thymus, apoptosis is a part of the immature T cell development. Thereby, immature thymocytes expressing either self-reactive (TCRs) or non-functional T cell receptors are eliminated. Cells with functional TCRs migrate out

of the thymus as naïve T cells. During infection some of these cells recognize foreign antigens, become activated and rapidly expand. Some of their descendants differentiate into effector cells. Mature T cells can further differentiate into cytotoxic T cells, helper T cells, or regulatory T cells. Mostly, proliferation and differentiation are accompanied by a significant shortened lifespan (71). Hence, the expression of death receptors is a general feature of activated lymphocytes rendering them susceptible to apoptosis. This homeostatic mechanism ensures that all cellular components of the adaptive immune system are submitted to control through a specific form of the extrinsic apoptosis pathway, the activation-induced cell death (AICD) (32,34). After peak expansion phase, it is estimated that roughly 90% of T cells are eliminated by AICD. While earlier studies concluded that there is a major role for CD95 in AICD, recent studies proposed that Bcl-2-interacting mediator of cell death (BIM) is also a main regulator in the contracton phase of the T cell response. However, it is assumed that a failure in this system could predispose to autoimmunity due to the persistence of potentially cross-reactive activated T cells (32).

2.2 Neutrophil apoptosis

Neutrophils are known to be key regulators of inflammatory responses. During inflammatory conditions, cytokines such as G-CSF mobilize neutrophils from bone marrow. Activated neutrophils adhere to the vascular wall and transmigrate to the extracellular space along concentration gradients of chemokines. Locally, neutrophils initiate immune reactions by phagocytosis of pathogens, the production of ROS and the release of protease enzymes. Moreover, neutrophils amplify the inflammatory response and direct other immune cells to inflammatory sites by production of various cytokines and chemokines (6). Therefore, they are inflammatory effectors as well as immunoregulatory cells. Since ROS and proteases damage cells, the removal of neutrophils from inflamed tissue is recognized as a cardinal step in the resolution of inflammation. Physiological cell death of neutrophils occurs by both, necrosis as well as apoptosis. While necrosis leads to a release of cellular contents into extracellular space, this process ampilfies inflammation. By contrast, apoptosis induces the stepwise release of cell fragments which are cleared by neighbouring phagocytic cells.

Similarly to lymphocytes, caspases are crucial for the initiation and execution of cell death. Activaton of caspases in neutrophils is also induced by both the extrinsic and intrinsic apoptosis pathway (12). In general, several mechanisms and signalling pathways involved in the regulation of neutrophil apoptosis have many similaritieswith those described in lymphocytes. However, apoptosis in neutrophils shows also some peculiarities. In neutrophils, apoptosis is an invitable and early event in unstimulated cells, but their progression can be delayed by several cytokines and other inflammatory mediators. LPS, CRP, leukotriene B4, IL-8, GM-CSF and G-CSF are known to inhibit or delay neutrophil apoptosis, although the complete mechanisms of these effects are unclear. It is assumed that this process is PI 3-kinase and ERK-dependend. Recent studies suggest that a reduced expression of pro-apoptotic Bax and a stabilisation of Mcl-1 expression play crucial roles in delay of apoptosis during inflammatory conditions (12). A transient delay of apoptosis enables the cells to initate their immune response effectively. In the absence of an inflammatory stimulus they remain after maturation 2-6 days before undergoing spontaneous apoptosis (12,15).

Beside the common apoptosis pathways recent studies described an additional neutrophil-specific cell death process described as NETosis. Thereby, dying cells generate neutrophil extracellular traps (NETs) which are extracellular structures composed of chromatin and granule proteins that bind or kill pathogens. This form of cell death depends strongly on the generation of ROS by NADPH oxidase. On the one hand, NETosis seems to be an important defence mechanism of the innate immunity. On the other hand, evidence suggests that NET formation participates in pathogenesis of several autoimmune and inflammatory disorders, with proposed involvement in chronic lung disease, sepsis, and vascular disorders. However, the biological significance of NETs is just beginning to be explored (24).

3. Exercise and apoptosis

Exercise is a type of physiological stress which has a substantial effect on leukocyte life span. The amounts of several hormones, cytokines and other factors which might influence cellular survival are increased or decreased in organs, tissues and peripheral blood during exercise (20,32,42). However, the initiation of apoptosis depends on both the critical balance between pro-survival and pro-apoptotic factors as well as the intracellular protection systems contributing to apoptosis resistance (5).

3.1 Exercise and apoptosis of circulating lymphocytes

Exercise has a marked impact on numbers and functions of lymphocytes in blood. While numbers of circulating lymphocytes significantly increase during exercise, it is followed by a post-exercise lymphopenia (58). It is believed that lymphopenia is the result of at least two different processes. On the one hand lymphocytes are redistributed into various tissues and organs (29,30). On the other hand cells die by apoptosis (42,46). These processes are thought to run in parallel and their relative magnitude seemsto depend on the mode of exercise.

Exercise intensity is assumed to be a main effector of exercise induced lymphocyte apoptosis. It has been repeatedly demonstrated that intensive exercise significantly increases both percentage as well as total numbers ofcirculating apoptotic lymhocytes (28,46). Regarding endurance exercise, an increase of apoptosis was observed after ultra-marathon (2), marathon run (46,47), intensive treadmill running (47), intensive ergometer cycling (70), and triathlon (35). In contrast, moderate exercise did not or only marginally affected lymphocyte apoptosis (28,46). It was further found that an increase of lymphocyte apoptosis occurs after exceeding a threshold of 40-60% of VO_{2max} (49). It can be speculated, that the concentration of potential apoptosis mediators are gradiently expressedwith increasing exercise intensity and that they induce apoptosis after exceeding a specific threshold (47,51). Similarly, the effect of exercise duration can be postulated. After the athlete exceeds a specific duration of exercise, expression of several potential mediators is amplified and might exceed a death inducing threshold (70,71).

Besides endurance exercise, it was also shown that lymphocyte apoptosis increased also after resistance exercise (27,61). Similarly to endurance exercise, an increased rate of apoptosis was mainly related to the intensity of resistance exercise. In this regard, intensity means weight loads of 75% of the one repetition

maximum or above, while rest-interval length seemed to have only minimal effects (27,61).
There is less information available about lymphocyte apoptosis after short bouts of exercise. Only a study by Friedman et al. (2012) found no increase of apoptotic cells after repeated high-intensity "Wingate cycle bouts". Therefore, it can be suggested that not only exercise intensity, but also a minimum of exercise duration has to be exceeded to induce a significant increase of apoptosis (51,77).

3.2 Exercise and apoptosis of tissue lymphocytes

Because the blood compartment represents only a small fraction of total lymphocytes, some studies assessed lymphocyte apoptosis inside organs or tissues. Using mouse models, increased lymphocyte apoptosis was presented for several lymphphatic organs. At first, the group of Hoffman-Goetz demonstrated increased apoptosis of both intestinal lymphocytes (ILs) as well as for lymphocytes in thymus and in spleen after 90 min of intensive treadmill running in mice (20,21,63). Similarly, our group showed that exercise-induced lymphocyte apoptosis is a systemic phenomenon which was also observed in lung, lymph nodes, bone marrow and Peyer's patches after treadmill running at an intensity corresponding to 80% of VO_{2max}. Thereby, the extent of apoptosis, the kinetics and the inducing mechanisms seemed to have a certain tissue specificity. While an early and strong increase of apoptosis was observed in Peyer's patches, delayed smaller changes were found in lung, bone marrow and lymph nodes after exercise. As shown for apoptosis in human circulating lymphocytes, no increase of lymphocyte tissue apoptosis was found at moderate exercise intensities (28).

3.3 Effect of trainings status on lymphocyte apoptosis

Apoptosis sensitivity of lymphocytes seems to be inversely related to the athlete's training status (47,60). Mooren et al. (2004) analyzed subgroups of athletes after a marathon run and found that programmed cell death occurred only in less trained, but not in well trained athletes. Similarly, Peters et al. (2006) did not find changes of apoptosis in well trained athletes after prolonged exercise despite a significant lympopenia. In support of these data similar observations were published about tissue lymphocytes in mice. Accordingly, the number of intestinal CD4 lymphocytes decreased after an acute bout of exercise in non-trained mice, but not in mice with 4 month access to a running wheel (10).
Regarding potential mechanisms Avula et al. (2001) demonstrated that lymphocytes seem to decrease apoptosis sensititvity in response to repeated stress (3). In detail, it was demonstrated that mouse splenic lymphocytes from trained mice were less sensitive to H_2O_2 induced apoptosis compared to cells from non-trained mice indicating the up-regulation of cellular anti-oxidative defence mechanisms by regular exercise training (3). These observations support the hypothesis that regular physical activity may prevent stress-induced suppression of the immune system (17).

3.4 Exercise and neutrophil apoptosis

There are conflicting data about the effects of exercise on neutrophil apoptosis. Syu et al. (2011) demonstrated that acute incremental exercise test induced an oxidative state in neutrophils which resulted in acceleration of spontaneous neu-

trophil apoptosis (74). The same group presented evidence that repeated moderate exercise (30 min a day, 5 days a week at 60% of maximal workload) delayed neutrophil apoptosis (72). The latter observation was proved by a recent study of our group. Here a significant delay of neutrophil apoptosis after marathon run, intensive endurance and downhill running as well as intensive resistance exercise was presented (49). In this regard, it has to be considered that acute exercise mobilizes immature non-segmented neutrophils from the bone marrow which might affect relative numbers of apoptotic cells in the circultation (79). However, also the *"in vitro"* spontaneous apoptosis was demonstrated as being significantly delayed after intensive exercise protocols. In contrast, the delay of apoptosis was not observed after moderate exercise protocols suggesting also an intensity dependent mechanism (49).

Regarding NETosis or neutrophil NET formation, Syu et al. (2013) demonstrated an acute bout of severe exercise facilitated NET formation in inactive subjects, while they did not observe changes in trained subjects (75). In sedentary subjects increased NET formation was accompanied by increased ROS production and a reduced mitochondrial membrane potential. A comparison of these results to previous data is difficult since subjects completed a short incremental exercise protocol in this study. However, these data implicate that apoptosis regulation also depends on subjects' training status indicating an effect of training adaptation on neutrophils cell death (75).

Only limited data were available regarding the effect of exercise on tissue neutrophils. Lagranha et al. (2004) obtained neutrophils of exercised rats by intraperitoneal lavage after injection of oyster glycogen solution. Here an increase in DNA fragmentation, chromatin condensation, and phosphatidylserine externalization was demonstrated. In addition, oral glutamine supplementation partially prevented the exercise-induced apoptosis in neutrophils. However, the differing results to human studies might be dueto the pre-treatment and source of the cells (33).

3.5 Apoptosis signalling pathways during exercise

There is evidence that exercise induces lymphocyte apoptosis by both, the intrinsic as well as the extrinsic pathways. For peripheral human lymphocytes, it was shown that intensive treadmill running, marathon running and intensive resistance training lead to an up-regulation of CD95 receptors (28,46,47) and partly of CD95 ligands (47). Furthermore, it is assumed that exercise also induces a shift to lymphocyte subpopulations with a higher density of CD95-receptors on their surface (46). Further evidence for Fas induced apoptosis comes from animal studies. After intensive treadmill running in mice the increase of apoptotic lymphocytes in tissues was accompanied by increased expression of either FasR or FasL, respectively. The critical role of Fas signaling in apoptosis induction was supported by the absence or reduction of apoptosis signalling after treadmill running of Fas-deficient MRL/lpr-mice (28).

Experiments with intestinal lymphocytes suggest that also the intrinsic pathway of apoptosis is addressed by exercise. Here apoptosis after exercise was accompanied by mitochondrial membrane depolarization, an increase of cytosolic cytochrome c, and a significant reduction of Bcl-2 protein content (62).

A possible crosslink of both pathways is indicated by a recent study of our group. It was found that increased lymphocyte apoptosis after resistance exercise was

accompanied by an up-regulation of FasR as well as a significant reduction in cellular Bcl-2, followed by a loss of mitochondrial membrane potential. However, further evidence for a crosslink of the intrinsic and extrinsic apoptosis pathway remains to be shown (27).
Regarding the mechanisms of neutrophil apoptosis modulation after exercise only little information is available. Mooren et al. (2012) showed that delayed neutrophil apoptosis was neither accompanied by changes of mitochondrial membrane potential nor by death receptor/ligand expression. Instead, apoptosis delay was accompanied by enhanced intracellular calcium transients and decreased glutathione levels. Further details for potential signaling pathways come from Su et al. (2011) who showed a collective up-regulation of the iNOS-NO-cGMP-Mcl-1 pathway after exercise (72). Taken together, it can be assumed that both the extrinsic as well as the intrinsic pathways are involved in exercise induced leukocyteapoptosis. Their predominant role seems to depend on the compartment, the exercise protocol and of the mediators of apoptosis induction.

3.6 Mediators of exercise-induced apoptosis

Intensive exercise is accompanied by changes of the expression of numerous cytokines, hormones, growth factors and the oxidative status. All these factors are known to potentially mediate either accelerated death or prolonged survival of leukocytes (Figure 1). Often apoptosis regulation depends on the proper balance between pro-apoptotic and anti-apoptotic factors and on the cell type and tissue.

3.6.1 Reactive oxygen species (ROS)

Intensive exercise is known to affect the balance between the production of free radicals and their depletion by antioxidant defense mechanisms. A major source of free radicals during exercise might be the high turnover rate of the mitochondrial transport chain due to higher energetic demands, ischemia reperfusion injury and the mobilisation or activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) carrying cells (11). ROS are known to affect certain pathways of lymphocyte apoptosis. On the one hand, ROS reduce cellular Bcl-2 and depolarize the outer layer of the mitochondrial membrane (11). On the other hand, ROS crosslink the intrinsic with the extrinsic apoptosis pathway by increasing Fas expression (11,15).
The role of oxidative stress in mediating exercise induced apoptosis mainly comes from animal studies. Here it was repeatedly demonstrated that exercise-induced lymphocyte apoptosis was accompanied by markers of oxidative stress including decreased intracellular glutathione concentrations (63) or formation of malondialdehyde (MDA), an intermediate product of lipid peroxidation (25). More direct evidence for a major role of ROS for apoptosis comes from studies which used the application of antioxidants like the ROS scavenging agent N-acetylcysteine (NAC). Lin et al. (1999) demonstrated that antioxidant administration inhibits exercise-induced thymocyte apoptosis in rats (37). Similarly, Quadrilatero and Hoffman-Goetz (2004) showed that application of NAC prevented exercise-induced intestinal lymphocyte apoptosis by maintaining intracellular glutathione levels and reducing mitochondrial membrane depolarization. However, further studies indicated that apoptosis inducing mechanisms during exercise

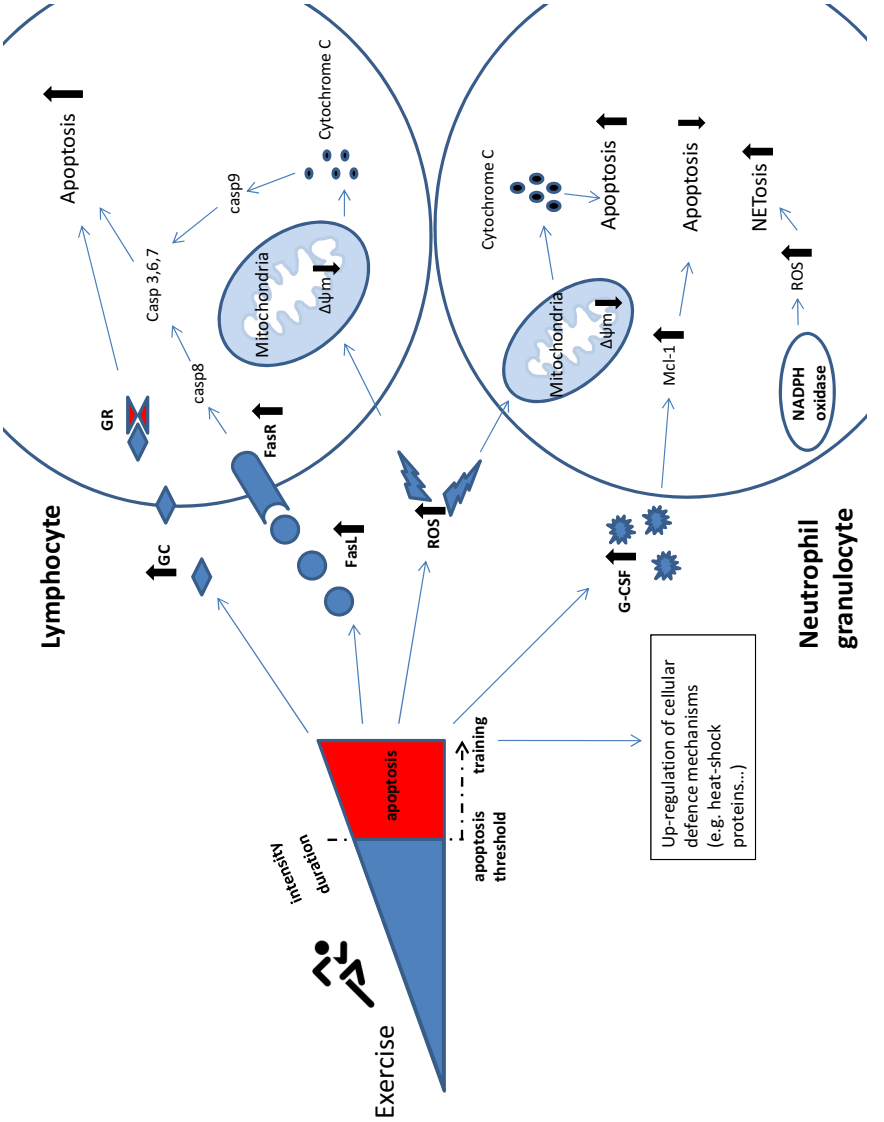

Figure 1: Schematic figure of pathways of exercise-induced lymphocyte and neutrophil apoptosis (GC=glucocorticoids, GR = glucocorticoid receptor, casp = caspase, Δψm = change in mitochondrial membrane potential, ROS = reactive oxygen species, FasL = fas ligand, FasR = fas receptor).

might be tissue specific. While application of NAC prevented exercise-induced T cell apoptosis in spleen and bone marrow, it was ineffective in lymph nodes (28). It can be speculated that the increased apoptosis resistance of trained individuals might be the result of an increased antioxidant defence. In this regard, it was demonstrated that cells initiate the expression of antioxidant enzymes and heat shock proteins (HSPs) in response to repeated exercise stress which exhibit protective effects against cellular stressors induced by acute bouts of exercise (16).
There is some evidence that ROS have divergent roles in neutrophils' cell cycle-physiology. On the one hand ROS production mediates the process of NETosisafter intensive exercise (75). On the other hand, it was demonstrated that ROS production was associated with the activation of pro-survival signals (43). Neutrophils have a specific antioxidant enzyme activity profile, probably related to the lower numbers of mitochondria and the production of high amounts of ROS during the oxidative burst (12). Accordingly, Fadeel et al. (1998) demonstrated that activated neutrophils showed an increased ROS production and suppressed caspases (15). Additionally, in neutrophils increased ROS generation is followed by NF-κ-B activation and translocation, which is a strong anti-apoptotic signal through the expression of proteins of the Bcl-2 family. Otherwise, if ROS production exceeds the neutrophil's natural defences, sustained perturbation of this balance may result in apoptotic cell death via the intrinsic pathway of apoptosis (15).

3.6.2 Glucocorticoids

Glucocorticoids (GC) are known to induce apoptosis in monocytes, macrophages and T cells. (13). Thereby, GCs act via binding to their intracellular receptors known as the glucocorticoid receptors (GRs). Prior to activation by interaction with its ligand, GRs are associated with heat shock protein 90 (HSP90). Upon hormone binding, a conformational change occurs where HSP90 dissociates from the receptor molecule and allows the receptor hormone complex to migrate into the nucleus. Here, it can transactivate genes that are involved in apoptosis. A first step in apoptosis induction is mitochondrial dysfunction followed by the relase of cytochrome c into the cytosol. Further steps in GC induced apoptosis involved the activation of the caspase cascade (13). Our group demonstrated that lymphocyte apoptosis after resistance training correlated with levels of plasma cortisol. Furthermore, an *in vitro* approach elucidated that exercise-concentrations of hydrocortisone induced lymphocyte apoptosis which can be attenuated by adding mifepristone (MIF), a glucocorticoid receptor blocker. Simultaneously to apoptosis induction, a significant decrease of mitochondrial membrane potential, a reduction of Bcl-2, and an upregulation of FasR were observed. Therefore, it can be speculated whether the extrinsic and intrinsic pathways were crosslinked after cortisol induced apoptosis during exercise (27). For intestinal lymphocytes Quadrilatero et al. (2005) demonstrated no major role for GCs for intestinal lymphocyte apoptosis in mice. In contrast, for thymocytes in rats it was demonstrated that DNA fragmentation after exercise was decreased in MIF treated rats after exercise (8).
Considering the role for GCs on peripheral lymphocyte apoptosis, a possible effect of the time of day when exercise is performed has to be discussed. Because cortisol levels follow a circadian rhythm, time of day might have an impactupon the rate of lymphocyte apoptosis (26). However, up to now there are no data available regarding circadian rhythm and apoptosis.

3.6.3 Further signalling pathways

Catecholamines and oestrogen hormones were considered to affect exercise-induced lymphocyte apoptosis. Marra et al. (2005) found that both adrenaline (epinephrine) and noradrenaline (norepinephrine) application in mice resulted in fewer apoptotic intestinal lymphocytes compared to control mice given saline. Similarly, it was demonstrated that neither alpha- nor beta-antagonism prevented exercise-induced cell loss in the intestine (41). Therefore, it can be assumed that catecholamines are not responsible for apoptosis in tissue lymphocytes.

It is known that oestrogen hormones (E2) protect lymphocytes from apoptosis *in vitro*. Therefore, it was tested if gender or menstrual cycle phase influences exercise-induced lymphocyte apoptosis. However, up to now neither gender specifty nor menstrual cycle effects for exercise-induced lymphocytes apoptosis have been found (53).

Since intensive exercise induces an intensity dependent systemic inflammatory response, Neubauer et al. (2008) investigated if exercise-induced apoptosis or DNA damage might be a consequence of inflammatory processes (58). In order to test this hypothesis, they tried to find relationships between DNA damage in lymphocytes and different systemic inflammatory markers in athletes after an Ironman triathlon race. However, no clear relationship was found. In addition, our group analysed the effect of exercise concentrations of inflammatory mediators on *"in vitro"* lymphocyte apoptosis. Both C-reactive protein as well as IL-6 failed to affect apoptosis of isolated lymphocytes (27).

It was previously demonstrated that the immune perturbations during exercise can be reduced by nutritional supplementation of carbohydrates. However, supplementation of carbohydrates failed to alter the apoptotic response after 60min of cycling on an ergometer at 80% of VO_{2max} (52).

Regarding inflammatory factors, granulocyte-colony stimulating factor (G-CSF) has been identified to have a major role in delaying neutrophil apoptosis after exercise (49). More precisely, it was found that a delay of spontaneous apoptosis *in vitro* could be induced by incubation of neutrophils in post-exercise serum. Addition of anti-G-CSF antibody to post-exercise serum was effective in reversing its apoptosis delaying effect (49). A role of G-CSF in apoptosis modulation is also supported by Su et al. (2011). They recently showed that exercise up-regulated Mcl-1 proteins in neutrophils which are known to be stabilized by G-CSF (72).

4. Methodological aspects of apoptosis measurement

There are some variations in percentage and total numbers of apoptotic cells in studies on exercise-induced leukocyte apoptosis. However, besides the use of subjects of different training status and the use of various exercise protocols, some of these variations might be the result of sensitivity issues related to the methodology used to assess cell death or apoptosis. Therefore, some methodological aspects have to be discussed.

It was described that the morphological characterization by microscopy to be the gold standard for identification of apoptotic cells (18). Using this method, characteristics of apoptotic cells such as nuclear condensation, nuclear fragmentation, and membrane blebbing were used to quantify cell death. However, this method is described as time consuming and error prone since there is a lack of objectivity

and reproducibility. In this context, Navalta et al. (2011) investigated the intra- and inter-rater reliability of morphological apoptosis evaluation in trained and untrained observers (53). It was found that a single trained observer get the highest reliability in apoptosis assessment.

Morphological analyses make it difficult to identify changes in large populations. Most exercise studies measured apoptosis by flow cytometry using different fluorescence markers which target hallmarks of apoptosis. Common methods are represented by the TUNEL (Terminal Deoxynucleotide Transferased UTP Nick End Labeling) assay for detection of DNA fragmentation, the Annexin V assay for surface phosphatidylserine (PS) exposure, and fluorogeniccaspase substrates to detect caspase activation (19).

Comparing the different methods, it is assumed that morphological methods yield a greater apoptotic index compared to those employing biochemical markers (55). Therefore, it can be proposed that an objective evaluation by morphological method in conjunction with biochemical marker assessment might be the better strategy compared to using a single method (19). A similar conclusion was drawn by Navalta et al. (2010) who described an image-based morphological approach by which computer software assesses the characteristics associated with lymphocyte apoptosis (55). Here automated analysis of the cellular morphology helps to overcome the lack of objectivity. They also compared morphological, image-based, and biomarker methods and got remarkble differences in percentage of apoptotic cells after an exercise intervention. Therefore, they recommended an *in vivo* method to be the best way to measure apoptosis after exercise conditions because exercise rapidly changes the internal cellular environment. However, such a method is currently not available. As a conclusion they discussed the possibility of combining methods such as morphological analysis with current biochemical marker methods. In this regard, a promising method is represented by the usage of multispectral image-based flow cytometry which couples the quantitative advantage of flow cytometry with the accuracy of morphology-based algorithms (18,19).

Recently, Navalta et al. (2011) presented a rapid and minimally invasive procedure for the analysis of the actual status lymphocyte apoptosis in athletes. In detail, whole blood taken from a finger stick sample is added to an antibody panel (56). Following the incubation period, red blood cells are lysed and samples are analysed. The usefulness, validity and reliability of this method has to be evaluated in future studies using morphological and biochemical methods in parallel.

5. Physiological relevance of apoptosis during exercise conditions

Finally, the physiological function of exercise-induced leukocyte apoptosis has to be discussed. In early studies lymphocyte apoptosis is often considered to contribute to post-exercise lymphopenia. In this regard, it was often assumed that increased apoptosis is associated with a loss of immunological competence. This idea is supported by studies which demonstrated a transient increase of upper respiratory tract infections (URTIs) after prolonged intensive exercise (59). However, the precise role of apoptosis in a clinical context remains to be shown. Similarly, the contribution of apoptosis to post-exercise lymphopenia is still discussed. While some studies demonstrated that lymphopenia was directly accompanied by

increased apoptosis (46), others found a decrease of lymphocytes without changes of total numbers of apoptotic cells (66,68). As previously mentioned, it is assumed that lymphopenia is the result of both lymphocyte migration as well as apoptosis. Therefore, their relative magnitude might depend on the different exercise protocols applied in these studies (29,30).

Beside detrimental effects on immunity researchers also considered that lymphocyte apoptosis is a regulatory mechanism to remove senescent, activated or potentially autoreactive lymphocytes. In this context, Simpson et al. (2010) noted that lymphocytes, which are mobilized into blood during exercise, are mainly senescent cells. Given that these cells mainly die by apoptosis, it can be speculated that exercise-induced lymphocyte apoptosis creates "vacant space" for newly functional lymphocytes to occupy and expand the naïve T-cell repertoire (68). A similar conclusion came from Goon et al. (2008) who demonstrated that long term Tai Chi exercise increased both lymphocyte apoptosis as well as proliferation (22). Therefore, it can be assumed that exercise promotes both cell death as well as cell production. The idea that exercise increases not only cell death, but cell turnover in general, is also supported by different studies which showed that exercise mobilizes hematopoietic progenitor cells from bone marrow (4). It can be speculated that an increased turnover of cells might be part of exercise-induced adaptation processes.

The physiological function of neutrophil apoptosis during exercise is also still unclear. From several acute and chronic inflammatory conditions it is known that a delay in neutrophil apoptosis contributes to the development of neutrophilia (43). However, it remains to be shown whether such a process is also operative during neutrophilia after exercise conditions. Further it can be speculated that life span is prolonged by the inflammatory mileu to take part in the damage repair processes as part of the adaptation to regular exercise training (39). In this regard, it is known that especially after eccentric exercise protocols neutrophils are rapidly mobilized into the circulation and migrate into the damaged muscle tissue (60). Therefore, it can be speculated that neutrophils survival is prolonged in order to amplify the repair process in muscle tissues.

Taken together, it is clearly demonstrated that exercise modulates leukocyte apoptosis depending on the exercise intensity. Thereby, apoptosis seemed to be induced by both the extrinsic as well as the intrinsic pathway depending on the mediators and the cell compartment. In case of lymphocytes, it was shown that ROS and glucocorticoids are major mediators of increased cell death, while neutrophils' life span seems to be affected by the inflammatory reaction evoked by exercise. In future studies, researchers should focus more on the physiological impact and clinical relevance of the transient alterations of leukocyte apoptosis. Additionally, the methods employed in future studies should be improved, for example, by using morphological methods in addition to biochemical markers.

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