Reduction in Resting Plasma Granulysin as a Marker of Increased Training Load

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ABSTRACT

Granulysin is a cytolytic granule protein released by natural killer cells and activated cytotoxic T lymphocytes. The influence of exercise training on circulating granulysin concentration is unknown, as is the relationship between granulysin concentration, natural killer cell number and natural killer cell cytotoxicity. We examined changes in plasma granulysin concentration, natural killer cell number and cytotoxicity following acute exercise and different training loads. Fifteen highly trained male cyclists completed a baseline 40-km cycle time trial ($TT_{40}1$) followed by five weeks of normal training and a repeat time trial ($TT_{40}2$). The cyclists then completed four days of high intensity training followed by another time trial ($TT_{40}3$) on day five. Following one final week of normal training cyclists completed another time trial ($TT_{40}4$). Fasting venous blood was collected before and after each time trial to determine granulysin concentration, natural killer cell number and natural killer cell cytotoxicity. Granulysin concentration increased significantly after each time trial ($P<0.001$). Pre-exercise granulysin concentration for $TT_{40}3$ was significantly lower than pre-exercise concentration for $TT_{40}1$ (-20.3 ± 7.5%, $P<0.026$), $TT_{40}2$ (-16.7 ± 4.3%, $P<0.003$) and $TT_{40}4$ (-21 ± 4.2%, $P<0.001$). Circulating natural killer cell numbers also increased significantly post-exercise for each time trial ($P<0.001$), however there was no significant dif-

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ference across TT_{40} ($P>0.05$). Exercise did not significantly alter natural killer cell cytotoxicity on a per cell basis, and there were no significant differences between the four time trials. In conclusion, plasma granulysin concentration increases following moderate duration, strenuous exercise and is decreased in response to a short-term period of intensified training.

INTRODUCTION

Natural killer (NK) cells are involved in cell mediated cytotoxicity by ligand pathways and granule exocytosis. Granulysin is one of the cytolytic granule proteins released by NK cells and activated cytotoxic T lymphocytes, although gene expression of granulysin is greater in NK cells. Granulysin lyses extracellular pathogens, targets intracellular mycobacterium and can increase membrane permeability of *Escherichia coli* (11). Granulysin contributes to both innate and adaptive immune responses, acting locally as a cytotoxic granule and peripherally as a chemoattractant. *In vitro* culture of human monocytes with granulysin increases mRNA expression of chemokines such as monocyte chemotactic protein-1 and regulated on activation, normal T cell expressed and secreted (RANTES) (9).

A decrease in T helper 1 (Th1) cytokines, and in turn cell-mediated immunity, may play a role in exercise induced immunosuppression (14) and increased risk of illness (17). Granulysin concentration may reflect cell mediated immunity (25), and also serve as a potential indicator of exercise training load, because cell-mediated immunity is reportedly reduced following high-intensity exercise (3). Low circulating concentrations of granulysin are linked with poor prognostic outcome in gastric cancer patients (25); granulysin expression of CD8+ T cells is also reduced in tuberculosis lesions (1). Good lifestyle habits increase the intracellular granulysin of NK cells and increased peripheral NK cell counts (15), however the relationship between NK cell count and circulating granulysin concentration remains unclear.

NK cell counts rise immediately after intense exercise, and then decline below pre-exercise counts in the hours following (20). In combination with decreased NK cell counts, periods of intense training also suppress NK cell cytotoxicity (NKCC) (28). Whereas some studies have reported that NKCC decreases immediately following intense exercise (22, 29), when NKCC is expressed on a per cell basis there is little or no change (16, 18, 27) or possibly an increase (20). NKCC per cell may not change, but lower numbers of circulating NK cells reduce peripheral NKCC. Chronic stress reduces NKCC (6), and lower NK cell counts are associated with poor clinical outcomes in B cell lymphoma patients (24). Decreased peripheral NKCC may increase the risk of illness during recovery or the 'open window' period following strenuous exercise.

Recently Zhang and colleagues (31) reported that serum granulysin concentration remains unchanged following exhaustive exercise, despite an increase in circulating NK cell number. Connolly et al (7) have reported that high-intensity exercise (80% $VO_{2\,max}$) sustained for a period of 30 min increases granulysin gene expression in peripheral blood cells by 2.3-fold, Whether this increase translates to a rise in circulating concentrations of granulysin is currently uncertain. To date, no studies have examined the relationship between circulating granulysin concentrations, peripheral NK cell number and NKCC following high-intensity exercise.

Considering that NK cells are the primary source of granulysin, and that NKCC may vary with exercise intensity, granulysin concentrations may vary with alterations in training load. The present study aimed to determine if granulysin concentrations are altered in response to acute exercise and an increase in training load, and if changes are related to alterations in NK cell number and cytotoxicity. We hypothesised that granulysin concentration would increase following moderate duration, strenuous exercise, and that resting granulysin concentration would decrease following a period of high-intensity training, in combination with a reduction in NKCC and NK cell number.

METHODS

Subjects. Fifteen highly trained, male road cyclists (VO_{2max}: 69.3 ± 1.3 ml·kg^{-1}·min^{-1}, age: 27 ± 2 years, body mass: 77.6 ± 2.2 kg, training volume: 422 ± 5 km·wk^{-1}; mean ±SE) volunteered to participate in the present investigation. They had been racing competitively for at least two seasons and had maintained consistent training volumes for at least two months prior to the study. Prior to acceptance into the study, the cyclists completed a medical history questionnaire and gave their written consent. The experimental protocol was approved by the Medical Research Ethics Committee at The University of Queensland.

Experimental overview. All testing was completed in the Exercise Physiology Laboratory at The University of Queensland. Cyclists completed one week of familiarization testing that included a VO_2 max test and a 40-km time trial (TT_{40}). The VO_2 max test and TT_{40} were repeated again the following week and the data from this week were used as a baseline measure (i.e., $TT_{40}1$). Following five weeks of regular training, cyclists returned to the laboratory to complete another TT_{40} (ie. $TT_{40}2$). During the week following, the cyclists completed five consecutive days of high-intensity training (HIT) (which included a 40-km time trial, i.e. $TT_{40}3$) followed by another TT_{40} the following week (ie. $TT_{40}4$). Blood was collected before and immediately following each TT_{40} (ie. $TT_{40}1$, $TT_{40}2$, $TT_{40}3$, $TT_{40}4$). Cyclists avoided all strenuous physical activity for 24 hours prior to each TT_{40}.

$VO_{2\ max}$ test. Height and body mass were measured when the cyclists first arrived at the laboratory. Then, following a warm-up up at a self-selected pace for 5 minutes, cyclists completed a graded exercise test to exhaustion. An electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands) modified with clip-in pedals and low profile racing handlebars was used for exercise testing; the saddle and handle bar positions of the cycle ergometer were adjusted to resemble each cyclist's own bike. Each cyclist wore a heart rate monitor (Vantage NV, Polar Instruments Inc., Finland) for the duration of the $VO_{2\ max}$ test. The test commenced at an initial workload of 100 W; workload thereafter increased by 15 W every 30 s (30 W/min) until volitional fatigue. Oxygen consumption and carbon dioxide production were measured continuously during exercise; expired air was analyzed for F_EO_2 and F_ECO_2 every 30 s during exercise

(Ametek; SOV S-3A11 and COV CD3A, Pittsburgh, PA, USA), while minute ventilation (V_E) was recorded every 30 s using a turbine ventilometer (Morgan, Model 096, Kent, England). The gas analyzers were calibrated immediately before and verified after each test using a certified beta gas mixture (Commonwealth Industrial Gas Ltd., Brisbane, Australia); the ventilometer was calibrated before and verified after each test using a 1-l syringe in accordance with the manufacturer's instructions (Vitalograph, Buckingham, England). Oxygen consumption was calculated with an online data-acquisition and analysis program (South Australian Sports Institute, Adelaide, Australia). $VO_{2\ max}$ was recorded as the highest value averaged from two consecutive readings, and peak power output was recorded as the highest 30-s power output completed during the incremental test. $VO_{2\ max}$ was defined using the following criteria: 1) the oxygen consumption ceased to increase linearly with a rising workload and approached a plateau or dropped slightly, with the last two values agreeing within 2 $ml \cdot kg^{-1} \cdot min^{-1}$; 2) 90% of age predicted HR_{peak} attained; 3) respiratory exchange ratio greater than 1.10. Ventilatory threshold was established during the $VO_{2\ max}$ test, and was defined as the point at which a first clear breakpoint in the V_E/VCO_2 was observed (4).

Laboratory simulated 40-km time trial. Cyclists were instructed to avoid any physical activity for 24 hours before each laboratory simulated 40-km time trial (TT_{40}) and to arrive at the laboratory well hydrated and fasted (for a period of eight hours). The cyclists rode their own road bicycle mounted to a stationary windtrainer (Cateye – Cyclosimulator CS-1000). The rear tyre was inflated to 120 pounds per square inch, and placed against the friction device before it was secured; the spring-loaded release brake was removed placing a wind-regulated friction load against the rear wheel. The cyclists' same rear wheel was used for each TT_{40}. Performance time was blinded to the cyclist during the TT_{40} and revealed to them on completion of the ride. Cyclists were allowed to consume water *ad libitum* during exercise; intake was monitored and recorded.

High-intensity training. Following the testing in week eight, cyclists completed five consecutive days of high-intensity training (HIT). All training sessions involved cycling in the laboratory at or above ventilatory threshold. The first day of HIT involved 20 x 1-min efforts at peak power output with two minutes recovery at 50 W in between each effort. Training on day two involved 60 min of cycling at 100% ventilatory threshold (comparable to the intensity of a maximal effort TT_{40}). Day three involved 12 x 30-s sprints at 175% of peak power output with 4.5 min cycling at 50 W in between sprints. HIT on day four involved 30 min cycling at 80% of the cyclist's ventilatory threshold followed by 45 min at 100% of their ventilatory threshold. On the final day of HIT, the cyclists performed a TT_{40} (ie. $TT_{40}3$). Exercise at or greater than ventilatory threshold was approximate to an intensity greater than 83% VO_{2max}, ensuring that each session was strenuous.

Blood collection. Subjects arrived at the laboratory fasted and rested. Blood was sampled from a forearm vein by venipuncture prior to the start of TT_{40} (following 5 min of rest) and immediately following TT_{40}. 10 ml of blood was collected and separated into different collection tubes: 5 ml was placed into a tube containing

EDTA for cell counts, and 5 ml was placed into a heparinized tube for the measurement of NKCC, phenotyping of lymphocytes and determination of granulysin concentrations.

Measurement of NK cell number. Three colour flow cytometry was used to determine the total number of NK cells ($CD3^-CD16^+CD56^+$). Briefly, 50 µl of whole blood was incubated at room temperature for 20 min with 10 µl of antibody cocktail in a polypropylene tube. Antibodies were purchased from Becton-Dickinson (San Jose, CA, USA). Erythrocytes were then lysed with 1 ml FACSlyse™ and incubated for a further 9 min. Finally, the tube was centrifuged at 2500 rpm for 3 min before the supernatant was removed, and cells were then resuspended in 250 µl 1% formalin until analysis. Samples were analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) located in a commercial pathology laboratory (Sullivan Nicolaides, Taringa, Queensland, Australia). The flow cytometer was calibrated daily by using different microbeads [QC Windows and Quantum 1000 (Flow Cytometry Standards, San Juan, PR, USA) and CaliBRITE (Becton-Dickinson, San Jose, CA, USA)], as per usual methods for quantifying cellular antigens by flow cytometry. A total of 20,000 events were collected and the data recorded as percentage of positive cells. The total number of lymphocytes determined from a full blood count was multiplied by the percentage of $CD3^-CD16^+CD56^+$ cells.

Determination of NKCC. Peripheral blood lymphocytes (effector cells) were separated from whole blood by Ficoll-Hypaque separation. Cells were resuspended in RPMI at 1 x 10^7 mL. 5 µL of a fluorescent probe, DiI (Sigma Aldrich), was added per ml of cell suspension and cells were incubated at 37°C for 20 min. Cells were then centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and cells were washed twice and resuspended at a final concentration of 1 x 10^7 cells/ml. K562 cells (target cells), an erythroleukemia cell line, were thawed rapidly in a water bath at 37°C, washed twice with phosphate-buffered saline/bovine serum albumin and suspended in RPMI at a concentration of 1 x 10^5 cells/ml. Effector cells were added to target cells at a concentration of 50:1 and then centrifuged at 1500 rpm for 5 min prior to incubation at 37°C in 5% CO_2 for 4 h. Following incubation, cells were centrifuged at 1500 rpm for 5 min and resuspended in 50 µl of Annexin V buffer (Becton-Dickinson, San Jose, CA). 50 µl of 1/50 dilution of Annexin V was then added to each tube. 10 µl of 7-amino actinomycin D (7aad) was then added and cells were incubated at 4°C for 10 min. The assay was performed in duplicate and controls (target cells only) were included with each run. A gate was set on forward scatter and DiI to exclude effector cells. Target cell death and apoptosis were determined on Annexin V versus 7aaD quadrants. Four populations of cells could be determined: negative cells for both fluorochromes (R1) which represented living target cells; annexin V positive - 7aaD negative cells (R2) which represented early apoptotic cells; annexin V positive – 7aaD positive cells (R3) which represented late stage apoptotic cells (necrotic cells) and annexin V negative – 7aaD positive cells (R4) which represent cells with permeabilized membranes only. Natural killer cell activity was determined using the following formula: the sum of cells in R2, R3 and R4 divided by the total number of cells (sum of R1-R4) which was then divided by the sum of

spontaneous cell death in the control condition (R2, R3 and R4) divided by the total number of cells, multiplied by 100 (2).

Plasma granulysin. Plasma granulysin was measured by an in-house ELISA according to the following method. Plates (Nunc, Roskilde, Denmark) coated with RB1 mAb at 5 μg/ml were blocked with 10% FBS in washing buffer (PBS containing 0.1% Tween-20) and serially reacted at room temperature with the following materials with washing steps between each reaction: samples or standards in blocking buffer for 2 h, 0.1 μg/ml of biotinylated RC8 mAb in blocking buffer for 1 h, and 0.05 U/ml of β-galactosidase-conjugated streptavidin (Roche Diagnostics) in washing buffer. The plates were finally incubated with 0.4 mM of 4-methylumbelliferyl-β-D-galactoside in 10 mM sodium phosphate buffer (pH 7.0) containing 0.02% BSA, 100 mM NaCl and 1 mM $MgCl_2$ at 37°C for 17 h. Then, the enzyme reaction was stopped with 100 mM glycine-NaOH (pH 10.3), and the fluorescence intensity was measured with Cyto-Fluor 4000 fluorescence multiwell plate reader (Applied Biosystems, Foster City, CA) with excitation and emission wavelength of 360 nm and 460 nm, respectively. Standard granulysin was purified partially from the serum free culture supernatant of pGrn-FLAG-transfected COS-7 cells by affinity chromatography with heparin Sepharose CL-6B (Amersham Biosciences) followed by gel filtration with Superdex-75 (Amersham Biosciences). Purity of granulysin in the standard sample was determined by SDS-PAGE followed by Coomassie Brilliant Blue staining, and the gel image analysis performed with Intelligent Quantifier densitometer (Bio Image, Ann Arbor, MI). Concentration of granulysin in the standard sample was calculated from the purity and the total protein concentration. Our past experience generated an interassay ELISA CV of 5.67%. The CV of the intraassay test was 3.01%.

Circulating NK cell numbers and plasma granulysin measures were adjusted to account for changes in plasma volume according to the methods of Dill and Costill (10)

Statistical analysis. All statistical analyses were performed using SPSS version 10.0 for Windows (SPSS, Chicago, IL, USA). Data were first tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. All data were normally distributed. To examine changes in variables across the training period a 2-way repeated measures ANOVA was applied to assess the main effects of time (pre- and post-exercise) and trial (TT_{40}). When a significant main effect or interaction was found, t-tests determined where differences occurred. $P < 0.05$ was accepted as being statistically significant. To determine possible relationships between plasma granulysin concentration, NK cell number and NKCC Pearson's product correlation coefficient was calculated. $P < 0.05$ was accepted as statistically significant. Data are expressed as mean ± SE.

RESULTS

Training time. Total training time (in minutes) spent at a heart rate above 95% ventilatory threshold was significantly greater for each subject during the HIT period when compared to the previous week of training (256 ± 8%, $P < 0.001$).

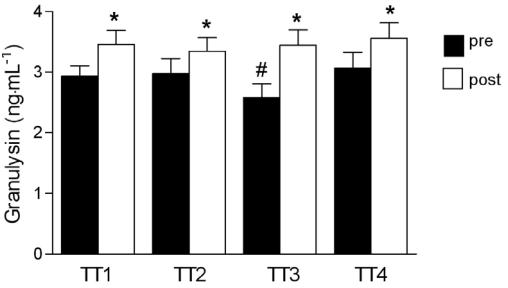

Figure 1. Granulysin concentration pre- and post-exercise for each 40-km cycle time trial (TT_{40}). * = significantly different from pre-exercise, # = significantly different from pre-exercise $TT_{40}1$, $TT_{40}2$ and $TT_{40}4$ ($P < 0.05$). $TT_{40}3$ was completed at the end of the high-intensity training period.

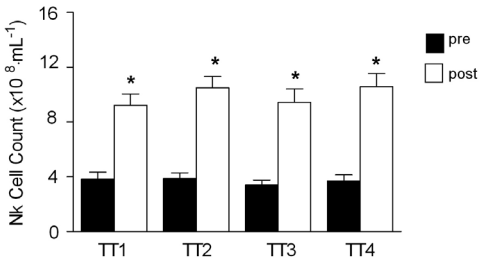

Figure 2. Total peripheral NK cell number pre- and post-exercise for each 40-km cycle time trial (TT_{40}). * = significantly different from pre-exercise ($P < 0.05$).

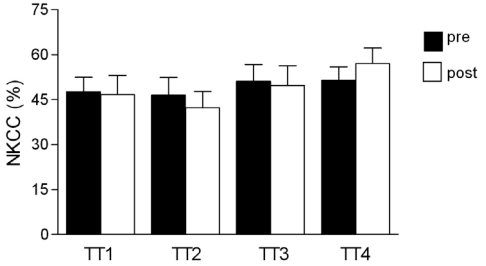

Figure 3. Percentage of target cells (K562) lysed by natural killer cells pre- and post-exercise for each 40-km cycle time trial (TT_{40}).

Plasma granulysin. There was a significant main effect of time (pre- to post-exercise) ($P < 0.001$) and a significant interaction of trial x time ($P < 0.05$) for plasma granulysin concentration. Granulysin increased significantly post-exercise for each TT_{40} ($P < 0.03$) (Figure 1). Pre-exercise values for $TT_{40}3$, performed at the end of the HIT period, were significantly lower than pre-exercise values for $TT_{40}1$ (-20.3 ± 7.5%, $P<0.026$), $TT_{40}2$ (-16.7 ± 4.3%, $P<0.003$) and $TT_{40}4$ (-21 ± 4.2%, $P<0.001$).

NK cell number. There was a significant main effect of time (pre- to post-exercise) ($P < 0.001$) for NK cell count. NK cell count ($CD3^{-}CD16^{+}CD56^{+}$) increased significantly post-exercise for each TT_{40} ($P < 0.001$) (Figure 2). NK cell counts did not differ between the time trials ($P > 0.05$).

NKCC. NKCC did not change significantly in response to acute exercise, or over the experimental period ($P > 0.05$) (Figure 3).

Correlations. There was no significant correlation between plasma granulysin, NKCC and NK cell number ($P > 0.05$).

DISCUSSION

The present study aimed to determine if granulysin concentrations are altered in response to acute exercise and an increase in training load, and if changes are related to alterations in NK cell number and cytotoxicity. This is the

first investigation to report a significant increase in plasma granulysin concentration following strenuous exercise, however there was no significant relationship between granulysin and NKCC or absolute NK cell number. One novel finding was that resting (pre-exercise) granulysin level was significantly reduced following consecutive days of high-intensity cycle training, suggesting that granulysin may serve as a potential indicator of exercise training load.

Zhang and colleagues (31) have previously shown that incremental treadmill exercise to exhaustion (approx. 19 mins) does not significantly alter serum granulysin concentrations. The authors concluded that circulating granulysin concentrations reflect the total body pool of NK cells, as opposed to the distribution of NK cells in the peripheral circulation. Granulysin gene expression in peripheral blood mononuclear cells is up-regulated 2.35 fold following 30 mins of exercise at 80% $VO_{2\ peak}$ (7). However, this increase possibly reflects an increase in the number of NK cells in the peripheral circulation, similar to the magnitude of increase observed in the present study. Granulysin concentrations in the present study increased following exercise in combination with elevated circulating NK cell counts. Alterations in circulating granulysin concentrations may depend on the exercise stimulus. The average time taken to complete the 40-km time trial in the present study was 52 min, at an average intensity of 83% $VO_{2\ max}$. In contrast, the exercise intensity in the study by Zhang et al (31) was incremental and of a shorter duration. The combined influence of exercise duration and intensity on alterations in circulating concentrations of granulysin requires further investigation. However, the current data suggest that moderate duration, strenuous exercise increases plasma granulysin concentration.

Granulysin is released from cytotoxic NK cells. Accordingly, we hypothesised that an increase in NKCC or circulating NK cells would raise peripheral granulysin concentrations. NKCC was unchanged after exercise, although circulating NK cell numbers significantly increased. Intense exercise increases NK cell counts immediately post-exercise; cell counts then decrease to below pre-exercise counts in the following hours (20). Some studies have reported that NKCC decreases immediately following exercise (22, 29), yet it appears that when NKCC is expressed on a per cell basis, there is little or no change (16, 18, 27) or possibly an increase (20). Periods of intense training reduce NKCC (28), in combination with decreased NK cell counts (27). In the present study, resting plasma granulysin concentrations decreased following consecutive days of high-intensity exercise. Despite this significant decline in granulysin, circulating NK cell number or NKCC did not decrease in the same manner. If the total pool of NK cells in the body remains relatively constant over an acute bout of exercise then the decrease in granulysin may reflect a decrease in the production of granulysin per cell.

NK cells comprise two populations based on the intensity of surface expression of CD56. $CD56^{dim}$ cells have a greater cytolytic activity against K562 cells (5) and are believed to differentiate from immature $CD56^{bright}$ cells which have an immunoregulatory function through the production of cytokines (5). Maintenance of NKCC post-exercise despite an increase in peripheral NK cell count could be explained by an increase in the proportion of $CD56^{bright}$ cells in the circulating population. We did not determine the proportion of $CD56^{bright}$ and $CD56^{dim}$, however Suzui et al (28) have shown an increase in $CD56^{bright}$ cells following 10 days

of intensive volleyball training. It is possible that the reduction in granulysin observed in the present study following a period of strenuous training may indicate a reduction in the cytotoxic subset of NK cells. Although a constant number of PBMC were isolated as effector cells to determine NKCC, we may have isolated a greater number of NK cells post-exercise, as NK cells increased significantly following each time trial. This should be a consideration for future investigations using a similar isolation procedure and for interpreting the results of previous studies using the same method (19, 28, 30). The relationship between circulating NK cell number and granulysin concentration remains uncertain. The biological significance of a decrease in resting granulysin concentration among athletes awaits further investigation, however it may reflect alterations in immune status.

The reduction in resting granulysin following short-term, intensified training may represent a reduction in cell mediated immunity (21). When compared with healthy controls, cancer patients present with significantly lower plasma granulysin expressed by NK cells (12). In contrast, elevated granulysin mRNA expression in urine is predictive of acute rejection following renal allograft (13) and is associated with elevated Th1 cells (8). In healthy females, granulysin concentrations also correlate with the percentage of cells staining positive for Th1 cytokines (26). Infants suffering from respiratory syncytial virus infection demonstrate impaired Th1 immunity (23). A decrease in Th1 cytokines, and in turn cell-mediated immunity, may contribute to exercise induced immunosuppression (14) and increased risk of illness (17). Moderate to good lifestyle habits, such as regular exercise, raise peripheral NK cells and increased intracellular granulysin in NK cells, when compared to poor lifestyle habits (15). Plasma granulysin is reduced following an increase in training load and may prove potentially useful as a marker of overreaching, however further training studies are needed to elucidate the relationship between training load and granulysin.

In summary, circulating granulysin concentration is increased following moderate duration, strenuous exercise, and is reduced at rest in response to short-term high-intensity training. Acute changes in granulysin may reflect changes in the circulating NK cell number, while alterations in granulysin over a period of intensified training may reflect changes in granulysin content per NK cell.

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