Thieme

Acute Exercise Increases the Expression of KIR2DS4 by Promoter Demethylation in NK Cells

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ABSTRACT

Positive effects of exercise on cancer prevention and progression have been proposed to be mediated by stimulating natural killer (NK) cells. Because NK cell receptors are regulated by epigenetic modifications, we investigated whether acute aerobic exercise and training change promoter DNA methylation and gene expression of the activating KIR2DS4 and the inhibiting KIR3DL1 gene. Sixteen healthy women (50–60 years) performed a graded exercise test (GXT) and were randomized into either a passive control group or an intervention group performing a four-week endurance exercise intervention. Blood samples (pre-, post-GXT and post-training) were used for isolation of DNA/RNA of NK cells to assess DNA promoter methylation by targeted deep-amplicon sequencing and gene expression by qRT-PCR. Potential changes in NK cell subsets were determined by flow cytometry. Acute and chronic exercise did not provoke significant alterations of NK cell proportions. Promoter methylation decreased and gene expression increased for KIR2DS4 after acute exercise. A high gene expression correlated with a low methylation of CpGs that were altered by acute exercise. Chronic exercise resulted in a minor decrease of DNA methylation and did not alter gene expression. Acute exercise provokes epigenetic modifications, affecting the balance between the activating KIR2DS4 and the inhibiting KIR3DL1, with potential benefits on NK cell function.

Abbreviations

NK cells	natural killer cells
KIR	killer immunoglobulin-like receptor
IG	intervention group
CG	control group
PBMCs	peripheral blood mononuclear cells
GXT	graded exercise test
LSM	lymphocyte separation medium
dCT	delta CT
ddCT	delta delta CT
TDBS	targeted deep amplicon bisulfite sequencing
CpG	cytosine guanine dinucleotide
BSP	bisulfite sequencing PCR
HCMV	human cytomegalovirus

Introduction

Epidemiologic studies reported associations between increased physical activity levels and decreased cancer risk and progression [13]. It has been hypothesized that these positive effects are mediated by an exercise-induced activation of natural killer (NK) cells [1, 20]. As part of the innate immune system, NK cells play a major role in eliminating virus-infected and tumor cells [27]. The regulation of NK cell function is mediated by a balance of signals from activating and inhibiting receptors on the NK cell surface. One large group of NK cell receptors is the killer immunoglobulin-like receptor (KIR) family [15]. KIRs are categorized by their number of immunoglobulin domains (2D and 3D) and length of their cytoplasmatic tail (S for short and L for long) [5]. The latter also determines the function of the KIRs. Short cytoplasmatic tails transduce activating signals, whereas long cytoplasmatic tails transduce inhibitory signals [5]. The development and regulation of NK cells is at least partially driven by epigenetic modifications (for review read [25]) that also account for NK cell receptors involved in NK cell activation. Gene expression of KIR genes is regulated by promoter DNA methylation, with hypomethylation for expressed KIRs and hypermethylation for non-expressed KIRs [4, 10, 24].

A vast body of literature suggests that NK cell functions (e. g., cytotoxicity) and numbers are sensitive to acute bouts of exercise (reviewed by [30]). In view of chronic (training) effects, findings of studies are inhomogeneous. Against this background, it was hypothesized that positive effects of exercise on NK cell function are the sum of acute effects rather than chronic or long-term changes in NK cell properties [30].

We recently showed that acute exercise evokes global histone modifications (hyperacetylation at H4K5) in NK cells and a concurrent increase in the expression of the activating NK cell receptor NKG2D [29]. Fernandez-Sanchez and colleagues have previously demonstrated that NKG2D expression is also regulated by DNA promoter methylation [8]. Moreover, gene expression of the activating KIR2DS4 and the inhibiting KIR3DL1 have been shown to be affected by exercise [15, 21, 22], although it is not yet clear whether this is due to changes in DNA methylation.

Against this background, this study aims to examine (i) whether a single bout of exercise and a four-week aerobic exercise intervention leads to alterations in promoter DNA methylation of the KIR2DS4 and KIR3DL1 genes, and (ii) whether these changes are associated with alterations in gene expression.

Materials and Methods

This study was performed in accordance with the Declaration of Helsinki, under consideration of the standards for ethics in sport and exercise research [12] and approved by the ethics committee of the University Hospital of Cologne. Sixteen healthy women between the ages of 50 and 60 were recruited by regional newspaper announcement between December 2014 and September 2015. Healthy was defined as no acute or chronic diseases. Moreover, regular drug intake was an exclusion criterion. Participants were chosen to reflect a population with enhanced cancer risk. All participants provided written consent. To determine acute effects of exercise on changes in promoter DNA methylation and gene expression of KIR2DS4 and KIR3DL1, venous blood samples were collected before (t0) and 1 min after (t1) a graded exercise test (GXT, procedure described below) by a venipuncture at each measurement time point. Subsequently, participants were randomized into either an intervention group (IG) or a passive control group (CG). After the IG completed a four-week aerobic exercise program (see below), resting blood samples from both groups were collected again (t2). The GXT was repeated to investigate if the exercise program was successful in terms of increasing cardio-respiratory fitness.

Graded exercise test (GXT)

The GXT was performed on a bicycle ergometer (ergoline GmbH, Bitz, Germany). Spirometry analysis (Cortex Biophysik GmbH, Leipzig, Germany) began with a 1-min rest measurement, followed by a 3-min warm-up phase at 50 watts of power output and an increase of 25 watts every 2 min until exhaustion (RER > 1). Moreover, heart rate (Promedia Medizintechnik, Siegen, Germany) and self-perceived exhaustion (Borg scale) were assessed at the beginning of the test and after each intensity interval.

Aerobic exercise program

The IG performed a four-week endurance interval exercise program, consisting of 2–3 exercise sessions per week. Each session started with a 3-min warm-up period at low intensity. Subsequently, participants performed four bouts of 4 min at 70–75 % of maximum heart rate (assessed during GXT) on a cross trainer. Participants rested for 1 min between bouts. The intensity of the exercise was automatically adjusted to maintain heart rate at the desired level.

NK cell isolation

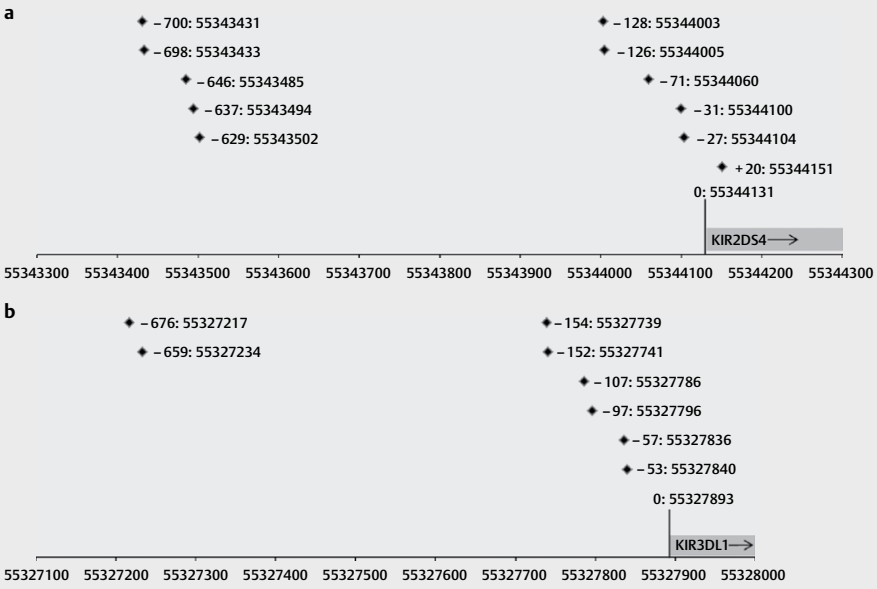
Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation with lymphocyte separation medium for 30 min at 800 x g (LSM; Promo Cell, Heidelberg, Germany). Isolated PBMCs were used for magnetic bead-mediated negative separation (all non-NK cells were labeled with magnetic antibodies) to isolate NK cells (EasySep™ Human NK Cell Enrichment Kit; STEMCELL Technologies Germany GmbH, Cologne, Germany) according to the manufacturer's protocol.

DNA and RNA isolation

DNA and RNA were isolated by phenol chloroform isolation with TRIzol® reagent (Thermo Fischer Scientific, Dreieich, Germany). NK cells were lysed with TRIzol® reagent, and chloroform was added for phase separation. For RNA isolation, isopropanol was added to the aqueous phase to precipitate the RNA. RNA was subsequently washed with ethanol, precipitated, dried and dissolved in RNAse-free water. For DNA isolation, ethanol was added to the interphase and phenol phase to precipitate the DNA. DNA was then washed twice with 0.1 M sodium citrate in 10 % ethanol. DNA was precipitated, dried and dissolved in 0.8 M sodium hydroxide.

Targeted deep amplicon bisulfite sequencing (TDBS)

The methylation status of KIR3DL1 and KIR2DS4 was investigated by TDBS on an Ion Torrent PGM platform. KIR2DS4 ranges from position 55344131 to 55360024 and KIR3DL1 from 55327893 to 55342233 on chromosome 19 (genome build Hg19). ▶ **Figure 1** represents the genomic location of both genes with the analyzed CpG dinucleotides. The 10 CpGs of the KIR2DS4 promoter were analyzed together with an eleventh CpG in the beginning of the gene.



▶ **Fig. 1** Gene loci of KIR2DS4 and KIR3DL1. Transcription initiation sites of both genes are marked with 0 and positions of the analyzed CpGs are in relation to the transcription initiation site. In addition to the position relative to the transcription initiation site, the absolute positions of CpGs on chromosome 19 are presented.

The KIR3DL1 promoter contains 8 CpGs in the promoter regions, which have been analyzed.

MethPrimer [16] was used to design the primer pairs. Primer design resulted in 6 assays with a length of 152–326 bp. Primer pairs were designed for bisulfite sequencing PCR (BSP) to amplify the genomic regions independently of the methylation status. Each region covered between 6 and 13 CpGs (average: 8). An EZ-96 DNA Methylation Kit (Zymo Research Europe GmbH, Freiburg, Germany) was used to deaminate the samples according to manufacturer's recommendations. Target enrichment was done by qPCR in single PCR reactions, followed by pooling of the 6 targets sample by sample. Library preparation and targeted sequencing for the Ion Torrent PGM was completed according to the manufacturer's protocol. Briefly, after DNA end repair to generate blunt ends (NEBNext End Repair Enzyme Mix contained in NEBNext Fast DNA Library Prep Set for Ion Torrent; New England Biolabs GmbH, Frankfurt am Main, Germany), individual barcodes and sequencing adapters (Xpress Barcode Adapters, Thermo Fisher Scientific) were attached to the pooled targets (n = 6) of each sample and purified with AMPure XP magnetic beads (Beckman Coulter GmbH, Krefeld, Germany). The samples were divided into 2 batches of 8 samples each. For each batch, the barcoded samples were pooled equimolar for the template preparation by emulsion PCR with the Ion PGM HI-Q OT2 KIT on the Ion One Touch 2 Instrument (ThermoFisher Scientific, Dreieich, Germany). The 318 Chip Kit v2 (Thermo Fisher Scientific, Dreieich, Germany) was used to sequence the two sample batches with the Ion PGM Hi-Q Sequencing Kit. A special workflow, recently published by Pabinger et al. [19], was used to map the readings from the Ion Torrent PGM to the reference genome.

Quantitative real-time PCR

Gene expression was analyzed by qRT-PCR using the GoScript Reverse Transcription System and GoTaq qPCR Mastermix (Promega, Mannheim Germany). The primer pair for KIR2DS4 reported by Gallez-Hawkings et al. [9] was used for qRT-PCR. Primer pairs for KIR3DL1 and β-actin were designed to span exon-intron junctions. Primer pairs are presented in ▶ **Supplement Table 1S**. qRT-PCR was performed with an initial 2 min at 95 °C followed by 45 cycles with 15 s denaturation at 95 °C and 60 s annealing and extension at 57 °C. PCR results were controlled by melting curves and agarose gel electrophoresis.

Flow cytometry

Isolated PBMCs were stained with anti-CD3 APC-Cy7 and anti-CD56 PE (BD Bioscience, Heidelberg, Germany). NK cells were gated as CD3-and CD56 + . Further NK cell subpopulations ($CD56^{bright}$ and $CD56^{dim}$) were separated by CD56 density. Flow cytometry was conducted on a BD FACS Array (BD Bioscience, Heidelberg, Germany). $CD56^{bright}$ and $CD56^{dim}$ NK cells are reported as a percentage of the NK cell pool.

Statistics

All statistical analyzes were performed using SPSS software version 22.0 (IBM Software and Systems, Armonk, NY, USA). Descriptive data are presented as means and standard error of the mean. Acute effects of exercise (t0 vs. t1) on DNA methylation, gene expression and NK cell subsets were analyzed using Wilcoxon tests for paired samples. Before analyzing chronic effects of the exercise intervention, baseline (t0) characteristics of the IG and the CG were compared using a Mann-Whitney U test. Subsequently, changes over

time (deltas: t2–t0) were calculated for both groups. As with a time-versus-group interaction in ANOVA, a Mann-Whitney U test was used to compare deltas between groups. Because multiple testing was conducted, α-error correction was performed using the Bonferroni-Holm method. To determine potential associations between DNA methylation and gene expression, a spearman correlation analysis was conducted. All analyses were two-tailed with an alpha level set at $p \leq 0.05$.

Results

Participant characteristics

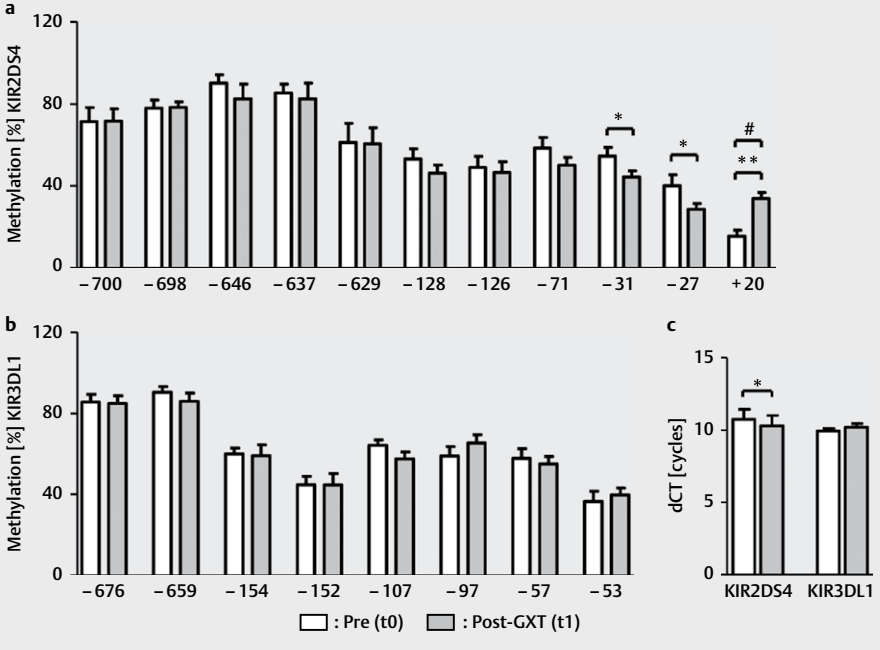
Participant characteristics are listed in ▶ **Table 1**. In terms of the four-week exercise intervention, BMI was significantly higher in the CG than in the IG. Age and endurance capacity at baseline showed no differences. Mean training frequency was 10.5 training sessions.

Acute exercise promoter methylation and gene expression

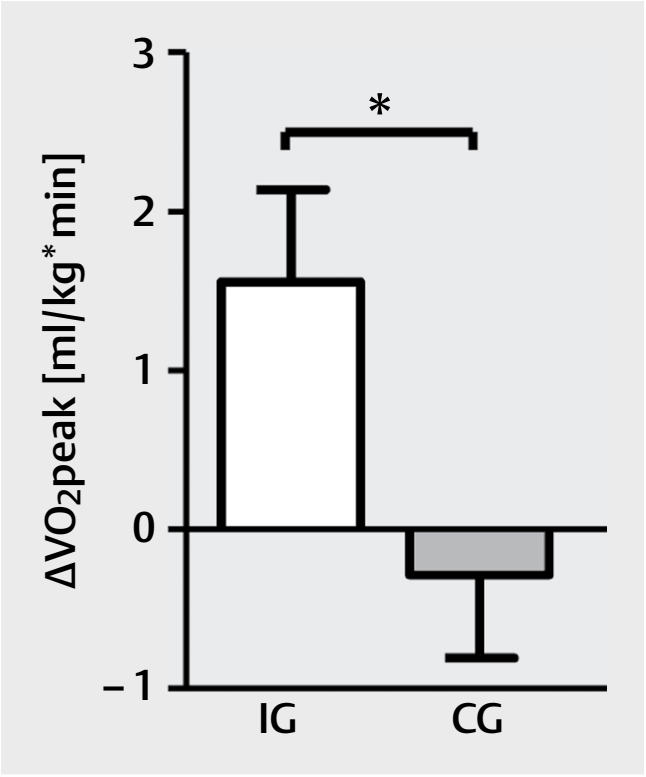
KIR2DS4 showed the highest DNA methylation between –700 and –630 bp in the promoter region, a decline in methylation between –630 and –27, and the lowest methylation at –27 bp (▶ **Fig. 2a**). Accumulated methylation of KIR2DS4 promoter CpGs was 64 % at baseline (t0) and 59 % after GXT (t1). The Wilcoxon test revealed a significant decrease in promoter methylation for CpGs, which are located close to the gene body of the KIR2DS4 gene (at –31 bp: $p = 0.026$, at –27 bp: $p = 0.020$), after GXT. Moreover, a hypermethylation of the first CpG within the gene body of KIR2DS4 was detected (+ 20 bp: $p = 0.005$) (▶ **Fig. 2a**). After α-error correction

▶ **Table 1** Participant characteristics. Values are given as mean ± SE.

	Total sample n = 16	Intervention group n = 9	Control group n = 7	p-value
Age [years]	53.44 ± 0.55	53.33 ± 0.99	53.57 ± 0.20	0.329
BMI [kg/m^2]	22.29 ± 0.68	20.87 ± 0.41	24.11 ± 1.16	0.034
VO_{2peak} [ml/min/kg]	32.94 ± 1.70	34.67 ± 2.07	30.71 ± 2.78	0.167
RER	1.20 ± 0.02	1.21 ± 0.04	1.19 ± 0.03	0.837
HR_{max}	171.62 ± 2.70	170.67 ± 3.58	172.86 ± 4.39	1.000
Smoker	2/16	1/9	1/7	1.000
Hyperlipidemia	7/16	3/9	4/7	0.615



▶ **Fig. 2** Acute effects of exercise on DNA methylation and gene expression of KIR2DS4 and KIR3DL1. Percentage of methylation of CpGs in the promoter region of KIR2DS4 **a** and KIR3DL1 **b** are shown before (t0) and after (t1) GXT. Gene expression of these genes **c** is given as dCT, which is the CT value of each gene in relation to the housekeeping gene β-actin at both time points. A lower dCT value depicts a higher gene expression. Bars represent mean values with the standard error. Significance is established at * ($p \leq 0.05$) and * * ($p \leq 0.01$), while # indicates significance after α-error correction.



▶ **Fig. 3** Comparison of endurance capacity development (before (t0) and after training (t2)) between the intervention group (IG) and control group (CG). Endurance capacity (VO_{2peak}) is compared after 4 weeks of training. Bars represent mean values with the standard error. Significance is established at * ($p \leq 0.05$).

using the Bonferroni-Holm method, only the changes of CpG located at +20 remained significant, whereas the CpGs at –27 and –31 still indicated a tendency to be altered by acute exercise (each p-value compared to the corresponding local α-level is presented in ▶ **Supplement Table 2S**). Additionally, gene expression of KIR2DS4 was significantly increased after the GXT, with a delta CT (dCT) relative to the housekeeping gene β-actin of 11.76 ± 1.10 at t0 and 11.03 ± 0.90 at t1 (mean ± SE; p = 0.020) (▶ **Fig. 2c**). The change of dCt for KIR2DS4 revealed a 1.6-fold increase in gene expression after GXT.

For KIR3DL1, the DNA methylation was highest in the area of –700 to –600 bp upstream of the gene. In the area of –150 to –100 bp the DNA methylation showed a U-shape and decreased to the lowest methylation in the area of –50 bp (▶ **Fig. 2b**). Accumulated methylation of analyzed KIR3DL1 promoter CpGs was 62 % at baseline (t0) and 62 % after GXT (t1). In contrast to KIR2DS4, no statistically significant changes in DNA promoter methylation and gene expression of the KIR3DL1 gene were found in response to the GXT (▶ **Fig. 2b and c**). Methylation for each measurement time point and investigated CpGs are listed in ▶ **Supplement Table 2S**.

Chronic exercise promoter methylation and gene expression

The four-week endurance exercise intervention led to a significant increase in endurance capacity within the IG (p = 0.035) (▶ **Fig. 3**).

When comparing changes in DNA methylation within the promoter regions we found a single CpG in the KIR2DS4 promoter to be different between the IG and CG. The CG showed an increase in DNA methylation at the CpG –637 of 18.95 ± 6.70 %, whereas the IG showed a decline of 7.94 ± 10.11 % (p = 0.005) (▶ **Fig. 4a**). This difference remains significant after α-error correction (▶ **Supplement Table 2S**). Because BMI was different between both groups, the correlation of BMI with changes in DNA methylation at CpG –637 was assessed. No correlation was found regarding BMI, indicating that BMI was not a confounder for CpG methylation at –637 (p = 0.102). No differences over time were found when comparing IG and CG in terms of KIR3DL1 promoter methylation (▶ **Fig. 4b**). Furthermore, no statistically significant differences were detected comparing changes in gene expression between the IG and the CG for both analyzed genes (▶ **Fig. 4c**).

Correlation analyses of promoter methylation and gene expression

Correlation analyses revealed strong correlations between promoter methylation at position –31 (r = 0.918, p < 0.001) and –27 (r = 0.745, p = 0.001) and gene expression for KIR2DS4 (▶ **Fig. 5a, b**) after acute exercise (GXT). No correlation was observed between DNA methylation at +20 within the gene body and gene expression of KIR2DS4 (r = 0.495, p = 0.061) (▶ **Fig. 5c**).

Methylation of both promoter CpGs (–31 and –27) correlated with each other (r = 0.692, p = 0.003), whereas no correlation existed between the methylation of these promoter CpGs with methylation of the CpG at +20 within the gene body (–31 vs. +20: r = 0.444, p = 0.085; –27 vs. +20: r = 0.261, p = 0.329). No correlation was found between CpG methylation at –637 and gene expression for KIR2DS4 (▶ **Fig. 5d**) after the four-week exercise training (r = –0.363, p = 0.167).

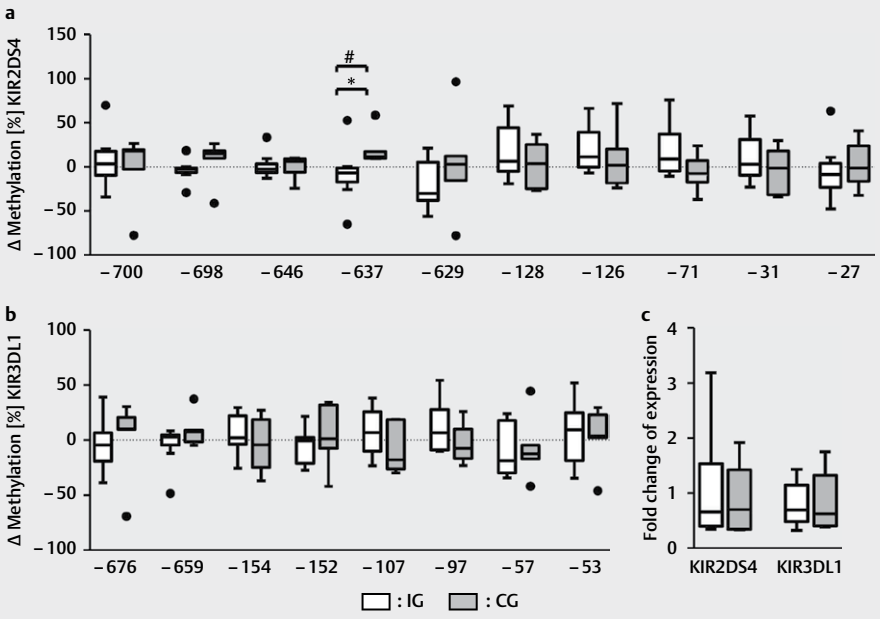
Effects of acute and chronic exercise on NK cell populations

Flow cytometry was conducted to analyze the effect of acute exercise and chronic exercise training on the distribution of the NK cell subsets $CD56^{bright}$ and $CD56^{dim}$. ▶ **Fig. 6a** represents the acute effects on NK cell subsets. The percentages of $CD56^{bright}$ and $CD56^{dim}$ NK cells before and after the GXT show no significant change ($CD56^{bright}$: t0: 20.02 ± 1.21 %; t1: 17.88 ± 2.17 %; p = 0.163; $CD56^{dim}$: t0: 80.11 ± 1.25 %; t1: 82.14 ± 2.33 %; p = 0.179).

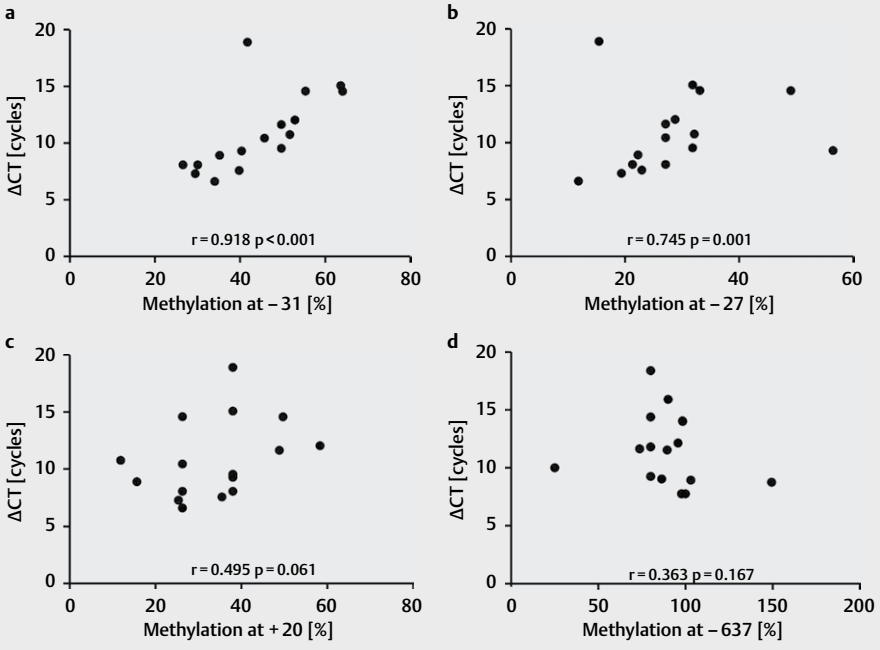
In view of chronic exercise training, proportions of NK cell subsets remain unchanged. Neither subset showed time or time x group interactions ($CD56^{bright}$: time: p = 0.388; time x group: p = 0.737; $CD56^{dim}$: time: p = 0.501; time x group: p = 0.936). The distribution of NK cell subsets for chronic exercise training is presented in ▶ **Fig. 6b**.

Discussion

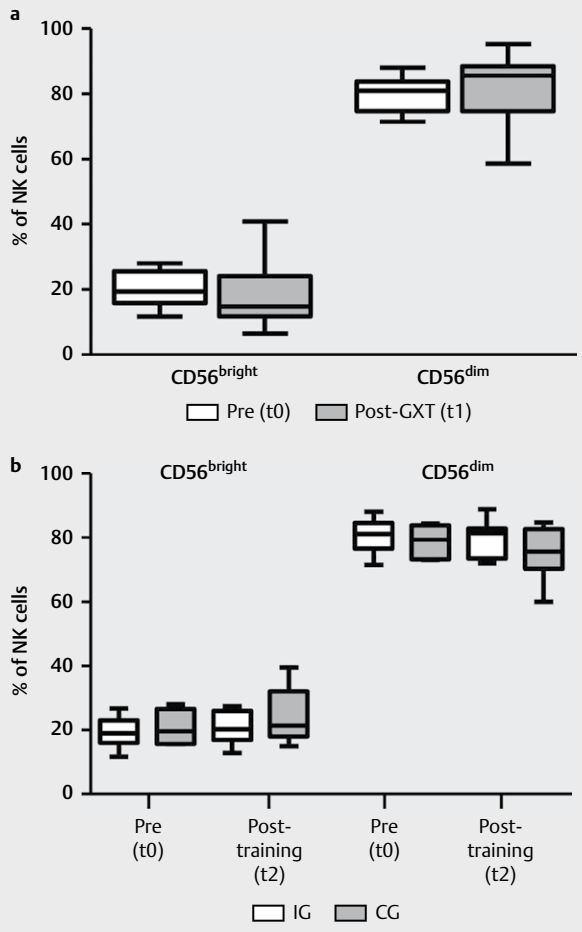
To the best of our knowledge, this is the first study to investigate the acute and chronic effects of exercise on gene-specific alterations in promoter methylation in NK cells. The results suggest that acute exercise is associated with decreased promoter methylation of the activating NK cell receptor gene KIR2DS4 and an increase in its gene expression. Moreover, our results show an increase in DNA methylation of the first CpG within the gene body, which is com-



▶ **Fig. 4** Changes of promoter methylation and gene expression over time (before (t0) and after training (t2)) between the intervention group (IG) and control group (CG). The difference of CpG methylation, in percent, before and after 4 weeks of training is compared between the IG and the CG for KIR2DS4 **a** and KIR3DL1 **b**. The fold change of gene expression of both genes **c** was calculated with the delta delta CT method. Values are given as box plots with whiskers according to Tukey with the whiskers at 1.5 times the interquartile range and outliers as dots to visualize if results are driven by outliers. Bars represent mean values with the standard error. Significance was established at * ($p \leq 0.05$), whereas # indicates significance after α-error correction.



▶ **Fig. 5** Correlations between KIR2DS4 gene expression and CpG methylation at CpGs changed by exercise. Delta CT values are in relation to reference gene, with a low CT value indicating a high gene expression. Delta CT values are given after acute exercise **a–c** with the respective CpGs where methylation changed during exercise. Delta CT values after chronic exercise training **d** are correlated with the respective CpGs that changed during exercise training. .



▶ **Fig. 6** Influence of acute exercise and training on NK cell subpopulations. **a** shows the effect of the acute exercise bout (GXT) on the two NK cell subpopulations CD56^{bright} and CD56^{dim} before (t0, white) and after the GXT (t1, grey). **b** shows the effect of chronic exercise training in the intervention group (IG) and the control group (CG). The figure presents the two NK cell subpopulations CD56^{bright} and CD56^{dim} of the IG (white) and CG (grey) before (t0) and after (t2) the training intervention. Results are given as box plots with whiskers according to Tukey with the whiskers at 1.5 times the interquartile range.

monly thought to be transcriptionally activating. In contrast, neither promoter methylation nor gene expression of the inhibiting KIR3DL1 was affected by exercise.

NK cell subsets

NK cells are known to be strongly affected by exercise with a preferential redistribution of highly differentiated CD56^{dim} NK cells [1, 2, 28]. Consequently, it could be argued that the observed changes in DNA methylation are attributed to exercise-dependent alterations of circulating NK cell subsets. However, flow cytometry data showed only a slight and non-significant increase of the CD56^{dim} NK cell subset. As described by Campbell and Turner [3] acute exercise lasting 45–60 min is characterized by lymphocytosis, which is driven by a preferential increase of NK cell numbers. In the present investigation, the GXT lasted 15.5 min (min: 12 min, max: 18 min) on average. This short duration may explain the comparable small effects on NK cell subset mobilization and redistribution. Furthermore, Rooney et al. described the kinetics of the egress of lymphocytes after acute exercise, with a fast egress of lymphocytes immediately on cessation of exercise [23]. Therefore, timing of blood sampling after acute exercise was standardized to 1 min after cessation of the GXT. Nevertheless, the lymphocyte redistribution affects the composition of lymphocytes and it is conceivable that the changes in blood lymphocyte populations could lead to changes in methylation by redistributing NK cells with a specific epigenetic pattern. Moreover, infections with human cytomegalovirus (HCMV) were described to impair exercise-induced NK cell mobilization [26]. A higher risk of HCMV infection due to the advanced age of the study population may also contribute to an alleviated NK cell response. Finally, infections with human cytomegalovirus (HCMV) are known to induce memory-like NK cells, known as NKG2C^{hi}, with a specialized epigenetic pattern. [14] and receptor repertoire [11]. Because our study used a within-participant design, it is not assumed that the HCMV status would affect the results. However this aspect should be considered in studies with a between-participant design.

Methylation of KIR genes

As reported by Gao et al. [10] and Cichocki et al. [4], unexpressed KIR genes possess dense promoter methylation, whereas expressed KIR genes display unmethylated promoters. Santourlidis et al. [24] further specified the methylation pattern of KIR genes with a promoter methylation of 70–100 % for unexpressed KIR genes and < 70 % for expressed KIR genes. Our results are in line with those of Santourlidis et al. [24] with accumulated promoter CpG methylation of both genes below 70 % accompanied by gene expression.

Further, Santourlidis et al. [24] state that gene expression is not based on DNA methylation of specific CpGs but rather CpG methylation throughout the whole KIR gene. Because acute exercise (GXT) stimulated a demethylation of the KIR2DS4 promoter and gene expression of KIR2DS4 increases, no change of DNA methylation at the KIR3DL1 promoter occurred and gene expression was not changed. Nevertheless, the acute bout of exercise stimulated predominant demethylation at two contiguous CpGs near the transcription start site, accompanied by increased gene expression. Our results suggest that demethylation of specific CpGs of expressed KIR genes could stimulate an increase in gene expression. The decrease of DNA methylation of both CpGs became statistically non-significant with correction for multiple testing but still showed a tendency. Nevertheless, the strong correlations that were found between the methylation of these CpGs and gene expression support the role of these two CpGs within the context of corresponding gene expression.

Besides acute exercise, training interventions have also been discussed to induce regulatory and functional changes in NK cells. However, studies investigating the effect of chronic exercise training on NK cell function reveal inconsistent results (reviewed by [30]). The data of this study show a difference in methylation of a single CpG of the KIR2DS4 promoter after the four-week training intervention between the intervention and control group. This difference seems to be driven mainly by an increase in DNA methylation within the control group and therefore cannot be attributed to exercise effects.

Moreover, this difference in DNA methylation is not accompanied by a difference in gene expression between both groups.

Balance of activating and inhibitory receptors

Because NK cell function is based on the balance of inhibitory and activating signals [7], a higher expression of activating receptors could promote a higher activitability and may represent a link between regular exercise and a decreased cancer risk. Our results reveal a change in the balance of gene expression between the activating KIR2DS4 and the inhibitory KIR3DL1 in favor of KIR2DS4 after acute exercise. It is speculative whether this change could promote an elevated ability for activation and further contribute to improved NK cell function. Because only two KIR genes were analyzed, a prediction of the proportion of activating and inhibitory receptors is not permissible, but analysis of a wider range of receptors should be investigated in future studies. Furthermore, gene expression analysis was conducted to reveal the overall gene expression of KIR2DS4 and KIR3DL1 without distinguishing between distinct transcript variants [17, 18]. Subsequent studies should also analyze the gene expression of different transcript variants to gain a deeper understanding of the impact of exercise on NK cell regulation.

Limitations

The results of this study should be considered within the context of its limitations. Targeted deep amplicon bisulfite sequencing represents a state-of-the-art method to determine alterations in specific CpGs. Further research may combine this method with another that allows the analysis of a wider range of genes, such as whole genome bisulfite sequencing (WGBS) or CHIP analysis and transcriptome analysis. Although using isolated NK cells provides more detailed information than analyzing PBMCs, future studies may consider focusing on specific NK cell subsets. Because $CD56^{dim}$ and $CD56^{bright}$ NK cells are stated as distinct lymphocytes due to their regulation and differentiation [6], future studies should concentrate on precise analyses on a per-phenotype (separated NK cell subtypes) or a per-cell basis. However, the amount of DNA that is needed may represent a bottleneck for such an attempt. Moreover, the timing of blood sampling upon cessation of exercise affects the composition of leukocytes in the blood stream and therefore was standardized to one minute after cessation of exercise. Nevertheless, by using venipuncture, each blood sampling could be subject to a minor delay. Therefore, future studies should use peripheral venous catheters for precise timing of blood sampling. Furthermore, HCMV infection status was not assessed in this study. HCMV infection could impair the exercise-induced NK cell response and further provoke changes in the NK cell pool by expanding NK cells with an activated phenotype. Therefore, the status of infection should be assessed in future studies and the use of cell sorting for analysis on a per-phenotype or per-cell basis could help to differentiate between NK cell populations. In order to gain a deeper understanding of the underlying mechanisms of exercise-induced epigenetic NK cell regulation, potential mediators (such as epinephrine, cortisol, serotonin and some cytokines, such as IL-6) and their downstream pathways, including interactions with transcription factors and the recruitment of epigenetic modifiers (DNA methyltransferases, DNA demethylases, and histone-modifying enzymes) should be considered. Furthermore, additional measurement time points should be included to gain a deeper understanding of the kinetics of exercise-induced epigenetic alterations in NK cells. Functional changes of NK cells (e. g., cytotoxicity) should be included to examine the relevance of exercise-induced changes in the functional genome or epigenome. Finally, gene expression data should be confirmed on the protein level (e. g., western blot).

Conclusion

In conclusion, this study provides initial evidence that acute exercise provokes gene-specific epigenetic alterations with a change in the corresponding gene expression. These changes may be associated with an activation of NK cells. Because these results could not be confirmed for a training intervention, the results fit with the hypothesis that positive long-term effects of exercise on NK cell function are the sum of acute effects rather than chronic alterations in cell function. More research is needed to develop a comprehensive understanding of exercise-induced alterations in immune function in order to provide detailed exercise recommendations.

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Conflict of Interest

The authors declare no conflict of interest.

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