Original Article
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Obesity

Decreased NK Cell Functions in Obesity can be Reactivated by Fat Mass Reduction

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Objective: Natural killer (NK) cells are the first defense against malignant cells, and their functions are severely impaired in individuals with obesity. However, it is not known whether functions can be re-activated after weight loss. The alterations of NK cell functions after fat mass reduction were investigated.
Methods: Thirty-two healthy adults with obesity were divided into control and experimental groups. Participants of the experimental group performed a 3-month program of exercise training and nutrition. Anthropometric, physiological, and metabolic parameters and plasma adipocytokines were determined. Peripheral blood mononuclear cells were analyzed by means of flow cytometry and Western blot assay for various NK cell-specific functional parameters and leptin signaling components. NK cell-mediated cytotoxicity assay with leptin stimulation was performed.
Results: Male participants significantly decreased their body fat mass ($P < 0.05$) and increased physical fitness ($P < 0.05$). Plasma leptin levels were significantly reduced ($P < 0.05$) and intracellular interferon gamma (IFN-γ) expression in $CD56^{dim}$ NK cells was significantly increased ($P < 0.001$) 3 months after study end. Stimulation of NK-92 cells with different leptin dosages revealed a significant dose-dependent decrease of specific tumor cell lysis.
Conclusions: The present study demonstrates a reactivation of NK cell functionality after body fat mass reduction in persons with obesity.

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Introduction

The Organization for Economic Co-operation and Development (OECD) reports that more than half (53%) of the adult population in the OECD countries are overweight or obese (1). Obesity is an imbalance between energy intake and energy expenditure, whereby an excess of body fat develops and is defined by a body mass index (BMI) of $\geq$30 kg*m^{-2}. An increased BMI enhances the risk of developing several types of cancer (e.g., kidney, colon, postmenopausal breast cancer) and overall mortality (2).

Natural killer (NK) cells are a central component of innate immunity, secreting different cytokines (e.g., interferon gamma, IFN-γ) to stimulate other immune cells, and are able to directly destroy virus-infected or malignantly transformed cells (3,4). NK cells express a wide range of activating and inhibitory surface receptors for recognizing and binding various targets (5,6). The results of an 11-year follow-up study indicated an association of lower NK cell activity (NKCA) with an increased risk of cancer development (7). Obesity with excessive body fat mass leads to an alteration and elevation of circulating adipokine levels (e.g., leptin, adiponectin, resistin) affecting immune functions (8,9). As recently shown, NK cell functionality can be modulated by adipokines (10,11). In previous studies we have shown impaired NK cell functions with regard to leptin signaling pathway in diet-induced obese rats, as well as a potential reversibility of NK cell dysfunction after weight loss (12,13). Few studies exist demonstrating the effect of weight reduction on NKCA in human obesity. The group of Kelley et al. (14) showed that the number of circulating NK cells is decreased after energy-restricted diet and weight loss in overweight women. Scanga et al. (15) published results of an 8-week study with women with obesity participating either in diet-alone program or diet combined with exercise. They reported a reduced NKCA after weight loss because of diet, but not after diet and exercise training. In the same year, a 12-week study showed no significant differences in NKCA after energy restriction and weight reduction in women with obesity (16).

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Besides diet and physical exercise, bariatric surgery is more and more considered as an effective option (with sustained weight loss) for the management of morbid obesity. Before surgery, patients with obesity showed normal NK cell numbers but decreased NKCA, whereas a significant increase in NKCA was observed after bariatric surgery (17). The present study investigates the effect of a reduction in body fat mass on NK cell functions, adipokine levels, and metabolic parameters in healthy women and men with obesity after a structured 3-month program of physical training and nutrition.

Methods

Study population

The participants in this study were female and male volunteers and recruited through advertisements in an employee magazine, information flyers, and e-mails. Exclusion criteria included pregnancy, breastfeeding, and persons with chronic infections, acute diseases, metabolic/endocrine diseases (e.g., type 1 diabetes, hypo- or hyperthyroidism), or immunosuppressive therapy (e.g., chemotherapy). A total of 32 healthy participants were divided into two groups: experimental and control group. Nineteen participants with obesity underwent a 3-month exercise and nutritional intervention to reduce their body weight. They were compared with 13 participants with obesity, matched for BMI, gender, and age, without any intervention. Each participant signed informed consent and completed a questionnaire including questions on familial, academic, behavioral, sportive, and dietary factors. This study followed the Declaration of Helsinki on medical protocol and ethics and was approved by the ethics committee of the Faculty of Medicine, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany.

Intervention

The participants performed a feasible and appropriate exercise training program and were continuously advised to reduce their daily energy intake. The training consisted of three 1 h exercise sessions per week: one unit of endurance activity and two units of combined invigoration and endurance training. The intensity of training was adapted to the individual level of performance, which was assessed during the physiological measurements before the start of the study. Participants of the control group received no advices of dietary or exercise training and were instructed to continue living as before and to maintain their weight.

Measurements

Before and after the study, the body height and weight with calculation of BMI, fat mass, waist and hip circumferences, bioelectrical impedance analysis, electrocardiogram were determined, as well as measurement of blood pressure, physical work capacity (PWC) and lung function. The PWC of each participant was measured on a cycle ergometer (Ergo Fit Cycle 457 Med, ERGO-FIT, Pirmasens, Germany). Lung function was assessed by spirometry during PWC test. O_2 and CO_2 were detected by mobile spirometry machine Cortex MetaMax 3B (Cortex Biophysik GmbH, Leipzig, Germany) with facemasks worn by the participants. Relative maximal oxygen uptake ($VO_{2\ max}$) was calculated from maximal oxygen uptake at the end of PWC test and body weight by means of MetaSoft Studio software (Cortex Biophysik).

Blood samples

Blood samples of participants were obtained at baseline, at the end of the study and 3 months after the end of the study. All fasted blood samples were collected in the early morning and mixed with heparin, apart from sodium fluoride for glucose determination. For plasma preparation heparinized blood was centrifuged and supernatants were stored at −80°C until further analysis.

Analysis of glucose, triacylglycerol, and cholesterol levels

Sodium fluoride-treated blood was centrifuged immediately to obtain plasma for glucose analysis. Concentrations of triacylglycerol, total cholesterol, and high-density lipoprotein (HDL) cholesterol were determined in heparinized plasma. All analyses were performed using colorimetric enzymatic tests (DiaSys, Holzheim, Germany) and were run in duplicate. The low-density lipoprotein (LDL) cholesterol was estimated indirectly with the Friedewald equation.

Preparation of peripheral blood mononuclear cells

After whole blood dilution with phosphate-buffered saline (PBS) (Biochrom AG, Berlin, Germany), peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation on biocoll separating solution (Biochrom AG). PBMC were collected, washed twice and resuspended in PBS. Then, samples were stained with trypan blue dye (Biochrom AG) to assess cell viability and to count and split them for further analysis.

Extracellular and intracellular staining for flow cytometric analysis

Live, unstimulated PBMC were stained with fluorescence-conjugated mouse anti-human mAb: CD3 (T lymphocytes) conjugated with phycoerythrin (CD3 PE) or phycoerythrin Cy 7 (CD3 PE-Cy 7), CD4 conjugated with allophycocyanin (CD4 APC), CD8 PE, CD14 (monocytes) conjugated with fluorescein isothiocyanate (CD14 FITC), CD20 (B lymphocytes) conjugated with allophycocyanin-Hilite®7-BD (CD20 APC-H7), CD56 (NK cells) APC, CD253 (anti-tumor necrosis factor-related apoptosis-inducing ligand; TRAIL) PE, and suitable isotype controls conjugated with FITC or PE (BD Biosciences, San Diego, USA). Furthermore, a CFS-conjugated mouse anti-human mAb for the leptin receptor (R&D Systems, Minneapolis, USA) was used. Protected from light cells were incubated 30 min at 4°C, washed twice with washing buffer (PBS supplemented with 1% BSA and 0.1% sodium azide) and resuspended in measuring buffer (PBS supplemented with 0.1% BSA and 0.1% sodium azide).

Intracellular markers were detected with mouse anti-human mAb for granzyme A (granzyme A FITC) and mouse anti-human mAb for IFN-γ (IFN-γ PE-Cy 7) antibodies. Additionally isotype controls according to granzyme A FITC and IFN-γ PE-Cy 7 were used. Cells were fixed in 4% paraformaldehyde for 10 min at 4°C in the dark and washed with washing buffer and saponin buffer (aqua dest. containing 0.1% saponin and 0.01 M HEPES), resuspended in saponin buffer including the antibodies mentioned above. After 30 min at 4°C in the dark, cells were washed twice in saponin buffer, once in washing buffer and finally resuspended in measuring buffer to

TABLE 1 Characteristics of the experimental group with anthropometric and metabolic parameters

	Male		Female	
Study population				
Subjects (n)	8		10	
Age (years)	48.8 ± 3.3		46.8 ± 3.4	
	Baseline	End of study	Baseline	End of study
Anthropometric measurements				
Weight (kg)	102.7 ± 3.6	92.6 ± 4.4	92.1 ± 3.3	87.7 ± 3.5
BMI ($kg*m^{-2}$)	32.7 ± 0.8	29.4 ± 1.1	32.3 ± 0.7	30.8 ± 0.8
Waist circumference (cm)	110.6 ± 2.5	100.1 ± 3.4*	105.9 ± 1.6	100.7 ± 2.2
Hip circumference (cm)	113.9 ± 2.4	105.9 ± 2.0*	118.3 ± 1.8	113.9 ± 2.5
Waist-to-hip ratio (WHR)	0.97 ± 0.01	0.94 ± 0.02	0.90 ± 0.01	0.89 ± 0.02
Fat mass (kg)	34.4 ± 2.3	24.2 ± 3.2*	39.3 ± 2.1	34.7 ± 2.1
Lean mass (kg)	68.3 ± 1.8	68.4 ± 1.9	52.8 ± 1.7	52.8 ± 1.8
Metabolic parameters				
Glucose ($mmol*l^{-1}$)	5.88 ± 0.36	4.89 ± 0.30*	5.13 ± 0.15	4.95 ± 0.27
Total cholesterol ($mmol*l^{-1}$)	4.98 ± 0.19	4.49 ± 0.30	5.50 ± 0.54	5.08 ± 0.27
HDL cholesterol ($mmol*l^{-1}$)	1.71 ± 0.09	1.67 ± 0.08	2.13 ± 0.09	1.95 ± 0.10
LDL cholesterol ($mmol*l^{-1}$)	2.60 ± 0.18	2.19 ± 0.24	2.68 ± 0.52	2.42 ± 0.25
Triacylglycerol ($mmol*l^{-1}$)	1.46 ± 0.08	1.39 ± 0.09	1.52 ± 0.12	1.57 ± 0.11

Values are means ± SEM.
*$P < 0.05$ vs. baseline.

analyze all samples by flow cytometry using LSR Fortessa with BD FACSDiva Flow Cytometry Software Version 6.2 (BD Biosciences).

Analysis of plasma adipokines and cytokines

The eBioscience® FlowCytomix™ Human Obesity 9plex Kit (Bender MedSystems GmbH, Vienna, Austria) was used to determine leptin, resistin, and monocyte chemoattractant protein-1 (MCP-1). Plasma samples were incubated with a Bead Mixture, Conjugate Mixture and Streptavidin-PE Solution. After washing and resuspending in assay buffer, samples and standard dilutions were analyzed by flow cytometry as described above.

Protein preparation and Western blot assay

PBMC pellets were lysed in 1x Triton X-100 completed with protease and phosphatase inhibitor cocktail (Sigma–Aldrich, St. Louis, USA). Protein concentrations were determined with Micro BCA™ Assay Kit (Thermo Fisher Scientific, Waltham, USA). SDS-PAGE was performed with equal amounts of protein denatured in Laemmli sample buffer and heated 10 min at 70°C. Samples were separated by a 4-12% SDS-Gel, transferred onto nitrocellulose membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and blocked 1 h at room temperature with 5% nonfat milk in TBS containing 0.1% Tween (TBS-T). After an overnight incubation at 4°C with primary mAb for phospho-STAT3 and phospho-ERK1/2, membranes were washed and secondary horseradish peroxidase-linked antibody (all from Cell Signaling Technology, Cambridge, United Kingdom) were added for 1 h at room temperature. Using ECL Prime Western Blotting Detection Reagent (GE Healthcare Bio-Sciences AB) and Fusion Fx7 (Peqlab Biotechnology GmbH, Erlangen, Germany) positive immunoreactivity was detected. Same membranes were stripped with a combination of Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) and stripping buffer. Subsequently, membranes were washed with TBS-T, reprobed with mAb for STAT3 and ERK1/2 (Cell Signaling Technology), and for correcting loading differences with mAb for β-Actin (Sigma–Aldrich). The amount of STAT3, ERK1/2, and their phosphorylated forms were calculated in relation to a positive control (isolated proteins of hepatoma SK Hep-1 cells) with equal protein amounts on each SDS-Gel.

Cell lines and cultivation

The human colon adenocarcinoma cell line DLD-1 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, both from Biochrom AG), 100 $U*ml^{-1}$ penicillin and 100 $\mu g*ml^{-1}$ streptomycin (both from Sigma–Aldrich) in a 5% CO_2-humified incubator (Thermo Fisher Scientific) at 37°C. The human NK cell line NK-92 was cultivated in RPMI 1640 supplemented with 10% FBS, 100 $U*ml^{-1}$ penicillin, 100 $\mu g*ml^{-1}$ streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine (both from Biochrom AG) and 200 $U*ml^{-1}$ human recombinant interleukin-2 (Novartis AG, Basel, Switzerland).

NK cell-mediated cytotoxicity assay

DELFIA® EuTDA Cytotoxicity Reagents (PerkinElmer, Waltham, USA) were used for NK cell-mediated cytotoxicity assay following the manufacturer's instructions. The target DLD-1 cells (1×10^6 cells per ml) were loaded with the fluorescence enhancing ligand (BATDA) for 15 min at 37°C and washed several times. NK-92 cells either remained un-stimulated or were stimulated with 10 and 100 $ng*ml^{-1}$ recombinant human leptin (R&D Systems) for 4 h.

Effector cells (2.5×10^6 cells per ml) were transferred into a V-bottom sterile plate, DLD-1 cells were added at effector:target ratio 3:1, centrifuged and incubated for 1 h at 37°C. Afterwards, plate was spun down and 20 µl supernatant from each well was transferred to DELFIA microtitation plate. After addition of 200 µl Europium Solution, plate was incubated for 15 min at room temperature and finally the time-resolved fluorescence was measured by photometer (Synergy Mx, BioTek Instruments, Winooski, USA). Cytotoxicity assay was performed in five individual experiments each with $n = 4$.

Statistical analysis

Values are presented as means ± SEM or as medians (minimum to maximum). Statistical analyses were performed using Student's *t* test (cell culture) or two-way ANOVA (factors: gender and time point; intervention and time point) with GraphPad Prism 5.01 software (GraphPad Software, La Jolla, USA). Statistical differences in the two-way ANOVA were assessed with Bonferroni post-hoc test and considered significant at $P < 0.05$.

Results

In the present human study, various data were collected at three time points: at the beginning (baseline), at the end of the study, and 3 months after study end. Baseline and "end of study" values are demonstrated for anthropometric, metabolic, and fitness measurements. Alterations of cellular and molecular parameters were firstly detected at the end of study, but significant differences could only be revealed 3 months after the end of the study.

Study population anthropometric and metabolic parameters

The experimental group was composed of 8 male and 10 female participants with a mean age of 48.8 ± 3.3 and 46.8 ± 3.4, respectively (Table 1). Male participants reduced their waist and hip circumferences significantly, whereas female participants showed no significant reduction of body circumferences (Table 1). Compared to baseline, the intervention had no significant impact on weight, BMI and lean mass in both gender groups. However, men significantly decreased their body fat mass (−10 kg) upon intervention. Fasting metabolic parameters revealed a significant reduction in glucose level in male participants from baseline to week 12 of the study. Plasma concentrations of cholesterol, its subsets, and triacylglycerols showed no alterations after intervention. In the control group, all parameters were also determined and remained unchanged within the study period (data not shown).

Increase of physical fitness

Male participants had a higher PWC (171.9 ± 14.5 W) than female participants (115.0 ± 7.6 W) at baseline and end of study (181.3 ± 14.8 vs. 127.5 ± 7.9 W), but no significant differences were achieved by the intervention (Figure 1A). $VO_{2\ max}$ was significantly increased both in male (23 ± 1.4 ml * kg^{-1} vs. 28.8 ± 2.1 ml * kg^{-1}) and female volunteers (16.8 ± 0.8 ml * kg^{-1} vs. 21.8 ± 1.1 ml * kg^{-1}) in response to the intervention (Figure 1B). $VO_{2\ max}$ also showed a significant gender impact because women had a significantly lower $VO_{2\ max}$, both at baseline and end of study, than men.

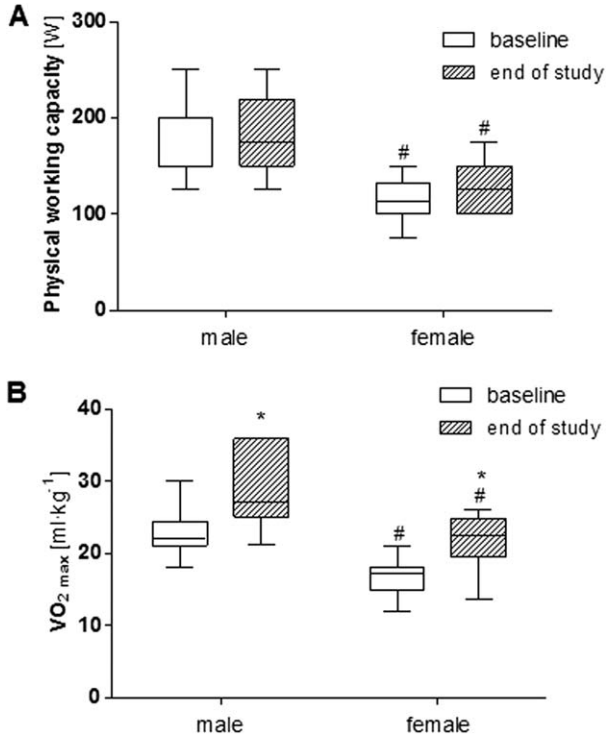

Figure 1 (**A**) Physical working capacity (PWC) and (**B**) related maximal oxygen uptake ($VO_{2\ max}$) of male and female experimental group at baseline and end of study time point. PWC ($n = 18$) and $VO_{2\ max}$ ($n = 17$) were significantly higher in male than female participants. $VO_{2\ max}$ was significantly increased in both genders after intervention. #$P < 0.01$ vs. male participants; *$P < 0.05$ vs. baseline.

Leukocyte subset-specific alterations in male subjects

Changes in monocyte and lymphocyte populations of male participants are presented in Figure 2 (A-F). Interestingly, the number of monocytes significantly decreased 3 months after the study end, whereas numbers of B lymphocytes did not change, and the T lymphocyte count significantly increased. In flow cytometric analyses T lymphocytes were divided into CD8 and CD4 positive cells (Figure 2F). Both cell populations were not influenced by the intervention (Figure 2D and E). Both the control groups and female subjects after the intervention showed no alterations (data not shown).

IFN-γ expression in CD56^{dim} NK cells increases after weight loss in male subjects

For the detection of NK cells, isolated PBMC were stained with CD3 and CD56 (Figure 3A-G). NK cells are composed of ∼10% CD56^{bright} and ∼90% CD56^{dim} NK cells. No differences in NK cell numbers in the male experimental group before and after study was detected. To investigate whether weight loss resulted in an increased activity of NK cells, we measured the TRAIL and IFN-γ expression of NK cells by means of flow cytometry. Figure 2C and F demonstrate TRAIL positive CD56^{bright} (2.5 ± 1.0%) and CD56^{dim}

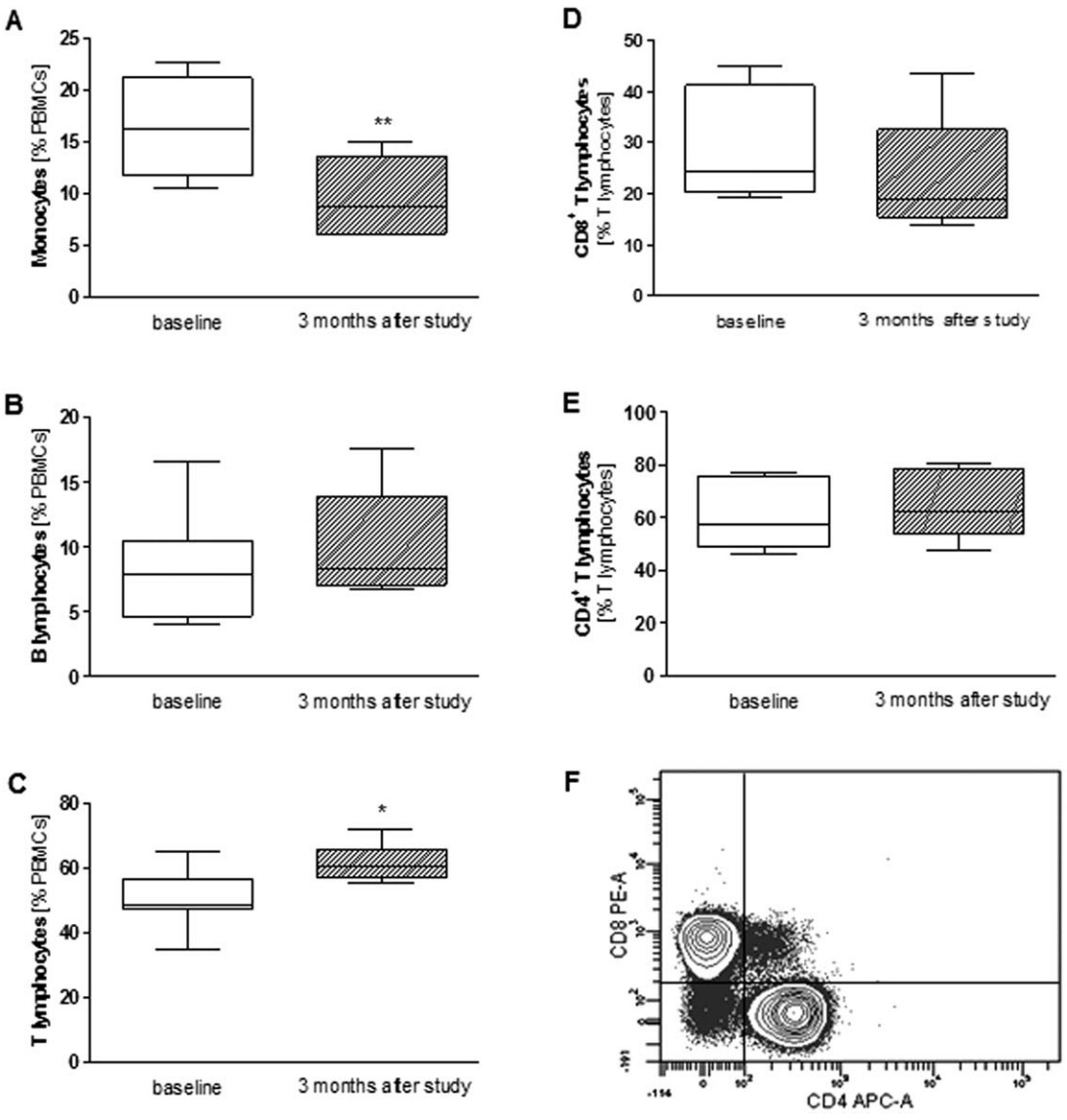

Figure 2 Different leukocyte subset populations from male participants of the experimental group at baseline and 3 months after study end. (**A**) Monocytes ($CD14^+$) as percentage of peripheral blood mononuclear cells (PBMC) with significant reduction after intervention. (**B**) B lymphocytes ($CD\ 20^+$) and (**C**) T lymphocytes ($CD3^+$) as percentage of PBMC with significant increase after study period. (**D**) $CD8^+$ T lymphocytes and (**E**) $CD4^+$ T lymphocytes as percentage of T lymphocytes before and 3 months after the study. (**F**) Illustration of T lymphocytes expressing CD8 conjugated with phycoerythrin (PE) and CD4 conjugated with allophycocyanin (APC) by flow cytometry. $^*P < 0.05$ vs. baseline; $^{**}P < 0.01$ vs. baseline. $N = 14$.

(1.1% ± 0.2%) NK cells. Comparable to the whole NK cell population, the intervention resulted in no significant changes in the NK cell subset numbers. Interestingly, a twofold increase of IFN-γ expression in $CD56^{dim}$ NK cells (35.9% ± 4.8% vs. 70.8% ± 5.7%) could be detected after the intervention.

Plasma leptin decreased in male participants after the intervention

Leptin levels significantly decreased in male subjects after weight loss (11.7 ± 1.6 ng * ml^{-1} vs. 5.0 ± 1.4 ng * ml^{-1}). Compared to men, women had more than twofold higher plasma leptin levels which, however, did not change during the study period (Figure 4A). Leptin receptor (Ob-R) positive NK cells were detected, but no effect of the intervention on the receptor expression could be determined (Figure 4B). Furthermore, no gender effect could be detected. Figure 4C shows the plasma resistin level in the experimental groups. Resistin, a predominantly macrophage-derived protein that seems to play a pro-inflammatory role in metabolic, coronary heart, and inflammatory disease (18), was not significantly influenced by weight loss or gender. MCP-1, a pro-inflammatory cytokine with higher levels in patients with type 2 diabetes and adults with obesity (19), also showed no significant modification after the intervention and by gender (Figure 4D).

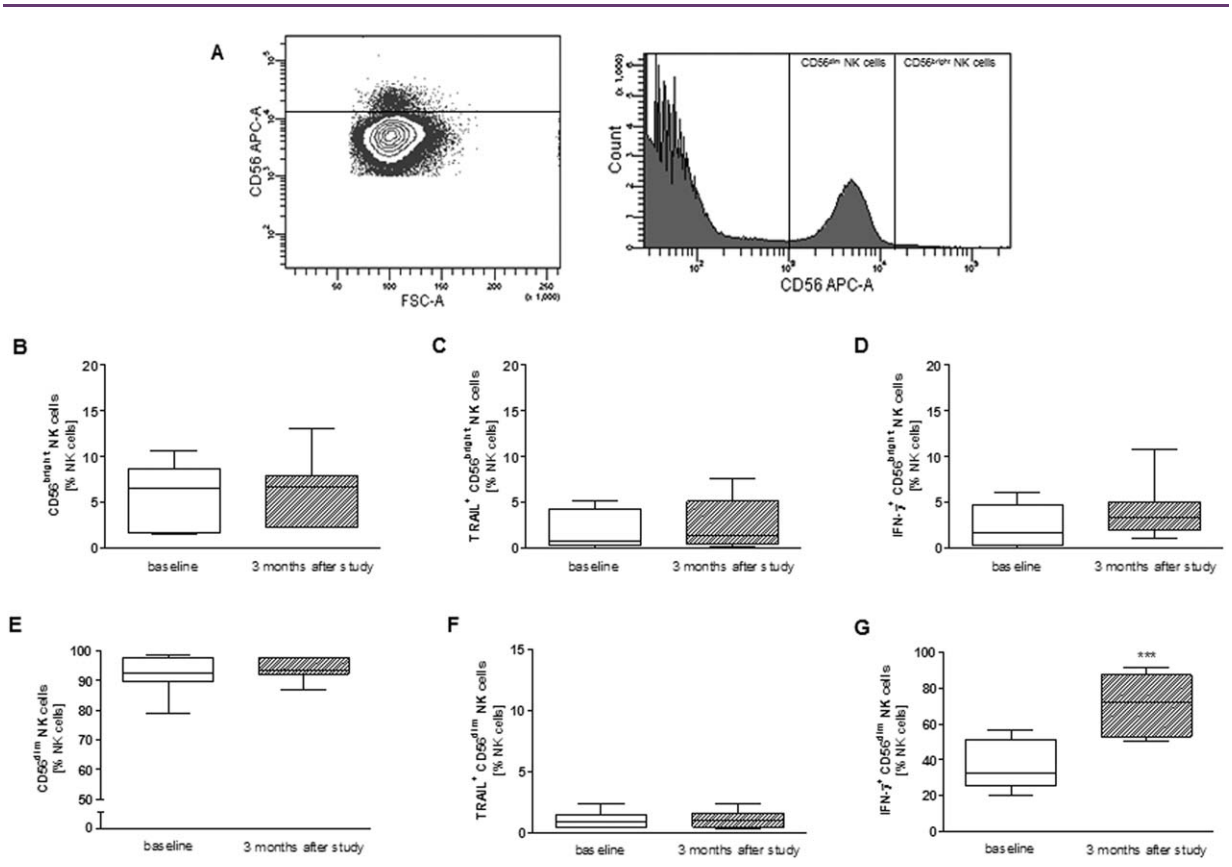

Figure 3 NK cell subsets CD56^{bright} and CD56^{dim} with expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and interferon gamma (IFN- γ) in the male experimental group at baseline and 3 months after study end. (**A**) After gating for CD3- cells, CD56^{+} NK cells were divided into CD56^{bright} and CD56^{dim} cells by flow cytometry; (**B**) CD56^{bright} NK cells as percentage of NK cells; (**C**) TRAIL-expressing CD56^{bright} NK cells as percentage of NK cells; (**D**) IFN-γ-expressing CD56^{bright} NK cells as percentage of total NK cells; (**E**) CD56^{dim} NK cells as percentage of total NK cells; (**F**) TRAIL-expressing CD56^{dim} NK cells as percentage of total NK cells; (**G**) IFN-γ-expressing CD56^{dim} NK cells as percentage of total NK cells. Three months after the study, IFN-γ expression in CD56^{dim} NK cells increased significantly. $^{***}P < 0.001$ vs. baseline. $n = 12$.

NK cell-mediated cytotoxicity is reduced after leptin stimulation

High elevated leptin levels, comparable to the pre-intervention leptin levels of the male participants of this study, are supposed to negatively influence NK cell mediated tumor cell lysis. Thus, an *in vitro* cytotoxicity test was performed with NK-92 cells, which are comparable with CD56^{bright} NK cells with high cytotoxic activity (20), to evaluate NK cell mediated lysis of tumor cells after stimulation with leptin with different dosages. Figure 5 illustrates a significant decrease of specific cell lysis after stimulation of NK-92 cells with 100 ng $*$ ml^{-1} leptin compared to unstimulated cells. Stimulation with 10 ng $*$ ml^{-1} leptin revealed no significant changes.

No effect on phosphorylation of STAT3 and ERK1/2 in PBMC after the intervention

STAT3 and ERK1/2 are two major phospho-kinases involved in leptin signaling and we hypothesized altered protein levels in participants with obesity after weight loss. For this experiment we decided to compare age and body weight matched controls versus experimental males 3 months after study end. Western blot analysis showed no significant effect of phosphorylation of STAT3 and ERK1/2 in PBMC, as well as amounts of STAT3 and ERK1/2 in relation to the protein standard (Figure 6A-D).

Discussion

Colorectal, renal, endometrial and postmenopausal breast cancer are only some cancer entities with increasing risks due to highly elevated body weight (21). Male volunteers of our study reduced their BMI to less than 30 kg $*$ m^{-2}. Furthermore, waist and hip circumferences as well as fat mass were significantly decreased. BMI is less accurate to determine body weight alterations because it includes only weight and height, but neither body composition nor fat distribution, two central parameters to assess obesity-related health risks. Although a correlation between waist circumference and BMI has been described, waist circumferences vary among patients and individuals with similar BMI values, and therefore, they have to be measured and considered as well (22). Although the relative $VO_{2\ max}$, a marker of physical performance, was significantly improved in both genders after the end of the study, it could not be increased to physiological levels (23).

In general, women have higher body fat content compared to men with matched BMI (24). Additionally, men have a visceral fat

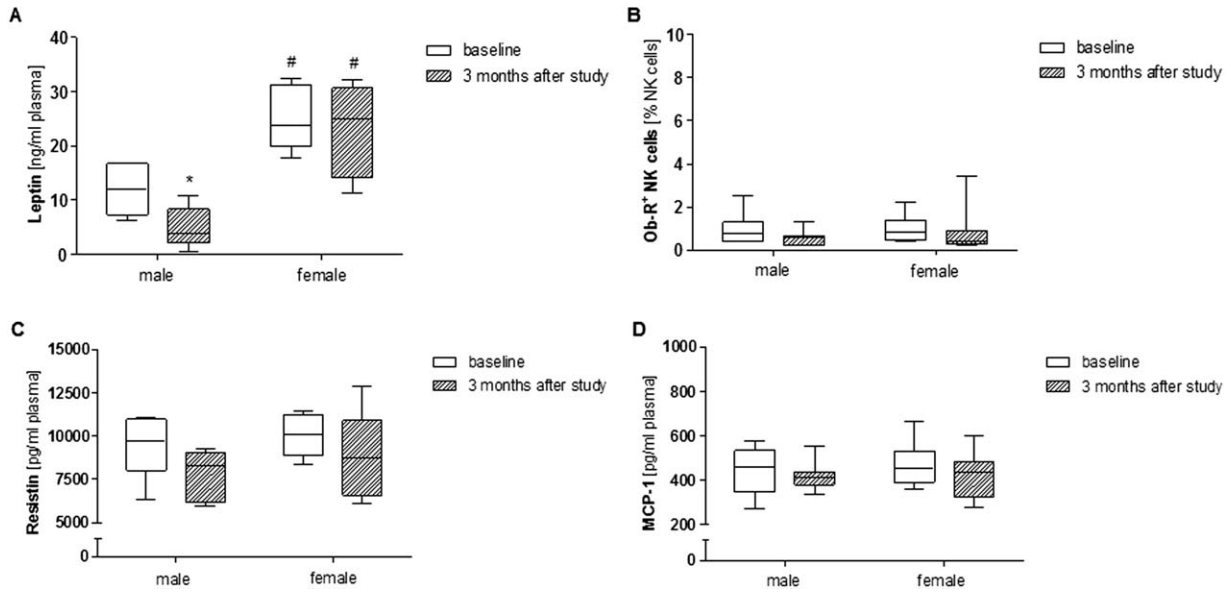

Figure 4 Leptin, leptin receptor (Ob-R), resistin, and monocyte chemoattractant protein-1 (MCP-1) concentration in male and female experimental group at baseline and 3 months after study end. (**A**) Plasma leptin concentration of male and female experimental group at baseline and 3 months after study. (**B**) Ob-R-expressing NK cells as percentage of NK cells. (**C**) Plasma resistin concentration. (**D**) Plasma MCP-1 concentration. #$P < 0.001$ vs. male participants; *$P < 0.05$ vs. baseline. $n = 15$.

distribution, whereas women mostly have a gynoid phenotype (25). The leptin amount and secretion varies by region and increases proportionally to body fat mass (26). The present study shows significantly decreased plasma leptin levels in male subjects after the intervention. Concerning leptin metabolism in individuals with obesity, several studies suggest a leptin resistance mechanism: chronically elevated circulating leptin levels in obesity lead to a central and peripheral receptor resistance and, therefore, a reduction of biological effectiveness of leptin, e.g. in brain, and immune system (10,27). Thus, as a consequence of the reduction of fat mass the male participants in the present study significantly reduced peripheral leptin levels to levels of normal weight men (28) and herewith reduced the chronic activation of Ob-Rs.

Stimulation of NK-92 cells with high dosages of leptin significantly decreased lysis of tumor target cells. These findings are in line with a previous study of our group (10) demonstrating an inhibitory effect of long-term leptin exposure on NK cell functions. However, we did not observe alterations of leptin signaling components in PBMC, including STAT3 and ERK1/2, two major intracellular signal transducer acting as downstream effectors from Ob-R binding (29).

We could show a significant decrease in percentage of monocytes and an increase in percentage of T lymphocytes in isolated PBMC after the intervention. Fantuzzi and Faggioni (30) found decreased T lymphocyte numbers and an activated monocyte/macrophage system in *ob*/*ob* mice with leptin deficiency, while an administration of leptin induced the proliferation of T lymphocytes and inhibited the monocyte/macrophage system.

We observed higher levels of IFN-γ expression in CD56^{dim} NK cells as compared to CD56^{bright} NK cells at the baseline time point. After the intervention, a twofold increase in IFN-γ-expressing CD56^{dim} NK cells could be seen in the male experimental group. In former studies of our group, we showed a significant increase of IFN-γ

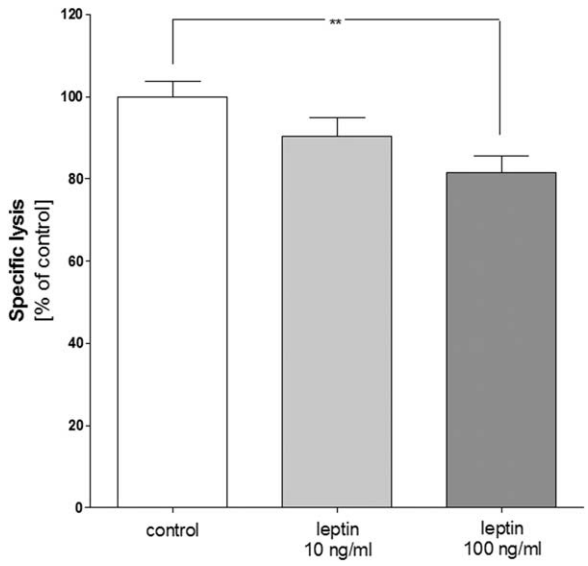

Figure 5 Effect of leptin stimulation on cytotoxicity of human NK cells. Human NK-92 cells were stimulated with different leptin concentrations for 4 h and incubated with the colon adenocarcinoma cell line DLD-1. Cytotoxicity was measured with the DELFIA® EuTDA Cytotoxicity Reagents. As controls served unstimulated NK-92 cells likewise co-incubated with tumor target cells. The measured fluorescence signal correlates directly with specific cell lysis which was calculated. In comparison to unstimulated NK cells, 100 ng*ml^{-1} leptin stimulation led to a significant decrease of specific cell lysis. Cytotoxicity assay was performed in five individual experiments each with $n = 4$. **$P < 0.01$ vs. control.

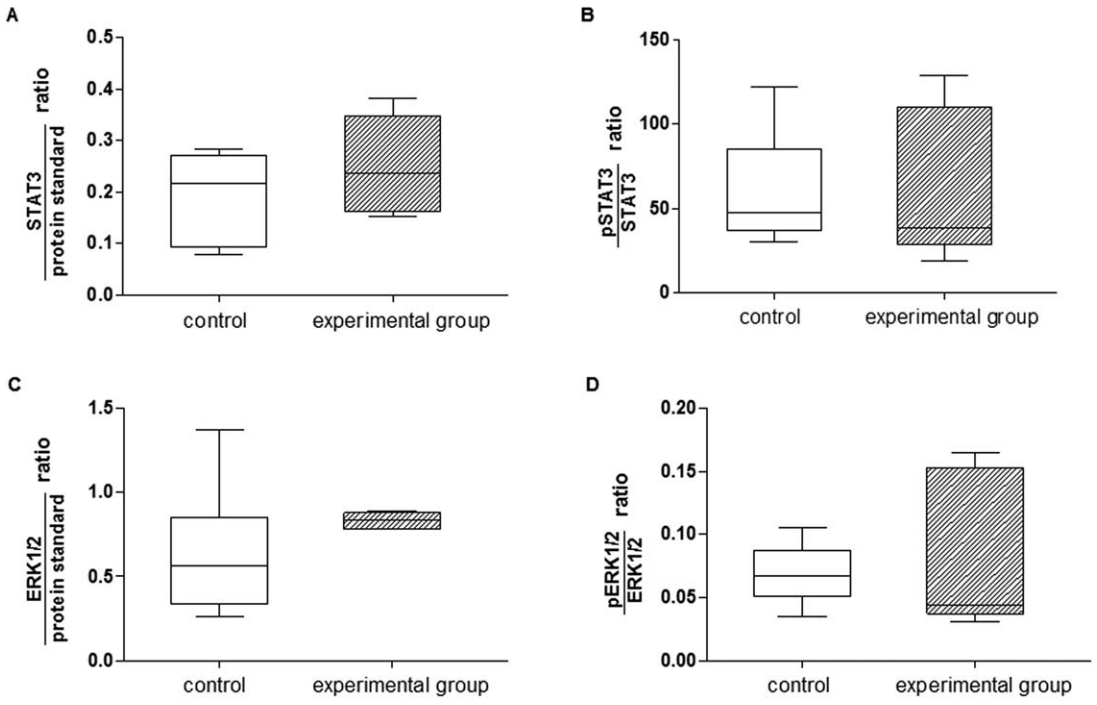

Figure 6 Relative protein amount of STAT3, ERK1/2, and their phosphorylation levels in peripheral blood mononuclear cells (PBMC). (**A**) Amount of STAT3 in relation to protein standard (SK Hep-1 cell line) and (**B**) relative phosphorylation of STAT3 in male control and experimental groups 3 months after the end of the study. (**C**) Amount of ERK1/2 in relation to protein standard (SK Hep-1 cell line) and (**D**) relative phosphorylation of ERK1/2 in male control and experimental groups 3 months after the end of the study. To correct loading differences, detective signals were adjusted in relation to β-actin signal in the same blot, or respectively, calculated as the ratio of phosphorylated to unphosphorylated protein form. A total of six persons (analyzed twice) were included.

secretion after short-term leptin exposure of human NK cells (10,31). De Maria et al. (32) also described a rapid and significantly enhanced expression of IFN-γ in $CD56^{dim}$ NK cells after stimulation for few hours as an early function of defense. IFN-γ regulates the function and differentiation of many immune cells, e.g. monocytes, macrophages, T cells and NK cells (33). An animal study showed increased regression of tumor nodules and survival of animals after treatment with IFN-γ (34).

Several studies (35,36) have shown that a combination of exercise and dietary program is more effective to reduce body weight than either alone. The design of the present study combined exercise and dietary programs and herewith caused both a significant reduction in body fat mass in male participants and a significant increase of physical fitness (by means of enhanced $VO_{2\ max}$) in male and female participants. The absence of a weight (fat mass) loss in the female participants was due to a lower compliance.

Exercise training increases NK cell activity (NKCA) (37) but high training intensity is followed by a period of immunosuppression (38). Besides plasma leptin reduction, loss of body weight leads to enhanced NKCA and IFN-γ production after bariatric surgery (17). The positive effect on the IFN-γ production after the exercise training in the male participants can be compared to effects of a surgical intervention described by Moulin et al. (17).

NK cells are essential for an appropriate cancer defense. It is known that especially moderate exercise decreases the risk of recurrence or progression of malignancy in cancer survivors (39). Potential mechanisms for reduction of cancer risks are decreases of circulating adipokines, sex hormones, inflammatory cytokines, as well as psychological benefits with an increase of happiness and life quality (40).

For the first time, the present study demonstrates a reactivation of NK cell functionality after fat mass reduction as a consequence of a combined exercise and dietary program in adults with obesity. In future studies, further NK cell functions after a longer study period have to be addressed.

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