Natural Killer Cells as Biomarkers of Hyperbaric Stress During a Dry Heliox Saturation Dive

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Introduction: Diving, hyperbaric oxygen, and decompression have been described as inducers of alterations in various components of the human immune system, such as the distribution of circulating lymphocytes. Hypothetically, the monitoring of specific lymphocyte subsets during hyperbaric exposure, including T- and NK-cell subsets, can serve as biomarkers of hyperbaric stress. ***Methods:*** Eight experienced saturation divers and eight reference subjects, naive to deep saturation diving, were examined. Peripheral blood mononuclear cells were isolated before and at different points during a 19.3-d dry heliox saturation dive to 2.64 MPa (254 msw). The NK cell cytotoxicity was estimated in a 4-h ^{51}Cr-release assay using the NK cell sensitive tumor cell-line K562 as target cells. The major lymphocyte subpopulations, with special emphasis on the NK cell subsets, were phenotypically delineated by the use of 4-color flow cytometry. ***Results:*** Although NK cell cytotoxicity increased significantly in the divers during the compression phase and the reference subjects who remained in normoxic conditions outside the chamber, the NK cell cytotoxicity was significantly higher in the divers. ***Discussion:*** This finding, together with augmentation in the absolute number of circulating NK cells in the divers due to a possible activation of specific parts of the innate cellular immune system during hyperbaric exposure, suggests the monitoring of specific immune functions can be useful as biomarkers of hyperbaric-induced inflammatory stress.

Keywords: NK cells, multicolor flow cytometry, cytotoxicity, hyperbaric, stress.

ALTERATIONS OF human immune responses in relation to diving and hyperbaric exposure, including hyperbaric oxygen (HBO) treatment and decompression, have been described previously (4). Still, 10 years later, the pathophysiological consequences of such alterations remain poorly understood, although the generation of inflammatory and bio-regulatory immunological mediators has been suggested as being involved in the pathogenesis of decompression sickness (DCS) following diving (7,29) or experimental hyperbaric exposure to animals (20,21,30). Briefly, during deep saturation diving, the human body is exposed to a number of physio- and psychological stress factors, e.g., elevated ambient pressure, breathing of HBO and inert gases, elevated temperature and humidity, and changes in sleep patterns and interpersonal conflicts, which could be considered additional elements of stress in the hyperbaric saturation environment.

During simulated deep saturation diving, alterations of specific components of the immune system were described; these included activation of the complement system (25), changes in the distribution of T- and natural killer (NK) cell subsets in peripheral blood (23), and expression of heat shock proteins (15). It was hypothesized that during the compression phase an unbalanced endocrine/immunological response is induced, providing changes in the circulating lymphocyte subsets. Thus changes in the circulating lymphocyte subsets have been proposed as a useful immunological marker of hyperbaric stress during the early phases of deep saturation diving (15). Interestingly, as emphasized by Montcalm-Smith et al. (17), the symptoms of severe DCS have many features in common with immunological reactions such as anaphylaxis and complement activation (18).

Briefly, NK cells represent a distinct lymphoid population comprising 10–15% of the circulating peripheral blood lymphocytes in healthy individuals and are considered important bridge-builders between the innate and the adaptive immune responses. NK cells are characterized phenotypically and functionally by their ability to destroy target cells without prior stimulation in vivo and in vitro. NK cells also provide an early source of immunoregulatory cytokines (5,28). These capabilities suggests that NK cells play a pivotal role in the cross-talk and editing of other cell types involved in the regulation of the quality and strength of immune responses encountered during inflammatory processes throughout the human body (19).

The key question in this paper asks how the immune system adapts to life in a hyperbaric milieu during a simulated dry helium oxygen (heliox) saturation dive. Subsequently, how might alterations of specific parts of an

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innate cellular immune system influence the shaping of an adequate immune response or lead to pathophysiological events, manifested as hyperbaric stress? Thus, the overall aim of this study was to investigate the functional and phenotypical changes in the circulating subpopulations of peripheral blood lymphocytes, with special emphasis on NK cells, intended as biomarkers of hyperbaric stress. Furthermore, the effect of decompression per se to the blood samples taken during the dive was evaluated.

METHODS

In October 2002, a simulated dry heliox saturation dive to 2.64 MPa (254 msw) was carried out in the hyperbaric chamber complex of Norwegian Underwater Intervention AS (NUI) in Bergen. The main scientific objective was to verify revised saturation diving procedures with respect to the incidence of venous gas microemboli and possible induced health effects. The Regional Committee for Medical Research Ethics, Bergen, Norway, approved the present study on the condition it was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Participation in the study was based on written informed consent.

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Subjects

There were 19 male subjects who participated in the study. Of those subjects, seven were commercial divers certified for saturation diving and one was a medical doctor and qualified scientist with experience in scuba and saturation diving. The divers were divided into two teams, A and B respectively, according to the different tasks performed during the dive. All divers were asked to ingest fish oil once daily for the 2 wk leading to and including the dive in accordance with Bakken et al. (1). Working outside the chamber in a normobaric atmosphere during the dive, 11 healthy persons naïve to deep saturation diving served as reference persons to the divers. The groups were comparable in age and body mass index (BMI), with the divers having a mean age (SD) of 41 (6.5) yr and BMI (SD) of 27.9 (2.4), versus the reference subjects, who had a mean age (SD) of 42 (4.5) yr and a BMI (SD) of 27.5 (2.6).

Diving Profile

The dive lasted 19.3 d, the compression phase 20 h 35 min (0.86 d), the saturation phase 6.6 d and the decompression phase 11.9 d (22) (**Fig. 1**). During the saturation phase, the divers carried out four to six excursions to a maximum depth of 2.64 MPa (254 msw). The mean rate of saturation decompression was 20.1 msw (201 kPa) per day. The partial pressure of oxygen (Po_2) was ~35 kPa during the compression and saturation phases and 70 kPa during the excursions. During decompression Po_2 was ~50 kPa, except for 8 h around each of the first 10 6-h night-stops, when Po_2 was reduced to ~35 kPa.

During the saturation phase, four and six excursions were performed by teams A and B, respectively. Team A conducted a thermal emergency test on days 4 and 5, which explains the discrepancy in the numbers of excursions. Team A exceeded the planned maximal depth of 2.6 MPa (250 msw) at the first excursion, achieving a maximal excursion depth of 2.64 MPa (254 msw). During the dive, the time-averaged Po_2 was 44.5 kPa for team A and 44.3 kPa for team B, respectively. The divers all conducted the same kind of moderate exercise in relation to a number of physiological tests performed during the dive.

Blood Sampling and Preparation

For this study, 16 ml of venous blood was drawn into tubes containing lithium heparin (Greiner BioOne GmbH, Austria) and 4 ml of venous blood were drawn into tubes containing EDTA (BD Bioscience, Erembodegem, Belgium) just prior to beginning the dive, at day 2 following the compression phase, at day 8 following the saturation phase, and, finally, at day 22 when the divers had terminated the decompression (Fig. 1A). The divers were instructed prior to the dive how to perform the blood sampling inside the pressurized chamber during the dive; blood samples from the reference persons in normobaric atmosphere were drawn by a physical chemist. All blood samples were drawn from an antebrachial vein between 7 and 9 a.m. to avoid circadian variation. The pressurized blood samples were placed without caps in a heliox atmosphere in a small service lock and decompressed using a staged profile (Fig. 1B) having a mean decompression rate of 28 kPa · min^{-1}, ensuring minimal alterations in temperature and minimal formation of bubbles and hemolyzation in the blood samples. Similarly, during the decompression of the blood sam-

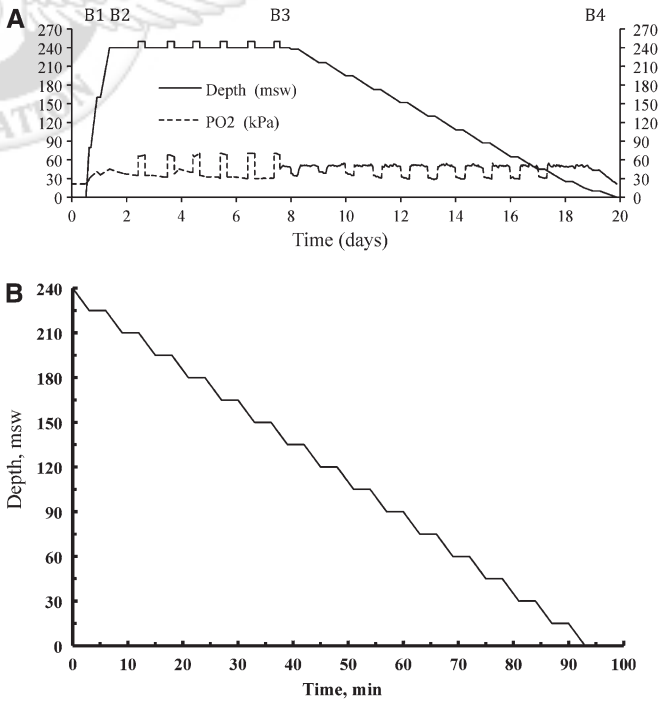

Fig. 1. A) The pressure profile of the dry simulated heliox dive to 240 msw (2.5 MPa). The dotted line indicates variations in Po_2 in relation to the activities at the depth during the dive. B1-B4: Time points for blood sampling. B) The decompression profile used for decompression of the blood samples in the medical lock.

ples obtained from the divers, the reference samples were placed in normobaric air at room temperature without caps. After decompression the PBMC from the heparinized blood samples were isolated using density gradient [Lymfoprep (1.077 g · ml^{-1}), Nycomed, Norway] centrifugation (3) and cryopreserved at −135°C until analyzed.

To evaluate whether the effect of the decompression procedure per se could induce any alterations on the investigated parameters, blood samples from four healthy reference persons were placed in the medical lock and compressed to 2.4 MPa and afterwards decompressed by the same profile (90 min) as the samples obtained from the divers. Likewise, blood samples from the same subjects were kept in normobaric air during the above-described procedure. No effect of decompression per se could be demonstrated on data relevant for this study (data not shown).

The ^{51}Cr-Release Assay

This assay was performed as previously described (12). Briefly, after the PBMC were thawed and washed once in RPMI 1640 (Gibco BRL, Eggenstein, Germany) that included 2% fetal calf serum (Gibco BRL), cell viability was tested by the trypan blue exclusion test (8).The effector cell suspension was adjusted to a final cell concentration of 2×10^6 viable PBMC · ml^{-1}. After the thawing procedure, the viability of the PBMC varied between 85 and 98%. There was, however, no relationship between viability and the NK cell cytotoxicity in this study (data not shown). The human erythroleukemic NK sensitive cell line K-562 (13) was used as target cells, kept in logarithmic growth, and labeled with 100 μCi $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Zaventem, Belgium) at 37° in 5% CO_2 in humidified air. Non-labeled K-562 cells were added to the spontaneous and maximum vials as surrogate cells to ensure that the concentration of cells in these two vials was comparable to that in the tests vials.

NK cytotoxicity was estimated in a conventional 4-h ^{51}Cr-release assay. Effector-to-target cell ratios tested were 5:1, 10:1, 20:1, and 40:1. The median values of counts per minute (cpm) in triplicates were used to calculate the NK cell activity by the formula:

$$\%\,\text{Cytotoxicity} = \frac{[\text{Experimental } {}^{51}\text{Cr release} - \text{Spontaneous } {}^{51}\text{Cr release}]}{[(0.8)\text{Max } {}^{51}\text{Cr release} - \text{Spontaneous } {}^{51}\text{Cr release}]} \times 100,$$

where maximum ^{51}Cr-release was obtained by harvesting both cells and supernatant. The ratio between the spontaneous and the maximal ^{51}Cr-release never exceeded 15%. For a daily internal control, cryopreserved PBMC from one healthy person with known NK cell cytotoxicity was included (data not shown).

Preparation of Stained Fixed Cells for Flow Cytometry

Antibody cocktails were prepared prior to staining using the concentration of monoclonal antibodies given by titration, defined as the optimal ratio between the positive and the negative mean channel fluorescence intensity of the gated lymphocyte population obtained by single color tube analysis. After the thawing procedure, the viability of the cryopreserved PBMC was tested using the trypan blue exclusion test (8). The PBMC concentration was adjusted to 5 x 10^6 viable PBMC · ml^{-1} in RPMI 1640 supplemented with streptomycin (5 g · ml^{-1}) and penicillin (1×10^6 IE · ml^{-1}), (Substratafdelingen, The Bartholin Building, University of Aarhus, Denmark) and 10% of fetal calf serum (Biochrome, Berlin, Germany). Fifty μl of PBMC in suspension were added to the tubes containing the respective antibody cocktails and incubated for 15 min at room temperature and in the dark. Following incubation the samples were gently mixed and 1 ml of phosphate buffered saline (PBS) at pH 7.4 containing 0.1% of sodium azide (Substratafdelingen) was added. To provide the best separation between signal and background, the samples were washed twice in PBS (200 × g in 5 min) and finally 500 μl of cold fixation buffer (PBS, pH 7.4, containing 1% of formaldehyde) were added during gentle whirl mixing.

Four-Color Flow Cytometry

The flow cytometry data were collected on a Cytomics™ FC500 dual laser instrument (Coulter Electronics Ltd., High Wycombe, UK). For optimization purposes, the instrument was adjusted daily using calibration beads (Epics Division, Coulter Corporation). The settings were: forward light scatter, 145 V, gain (g) 5.0; log side scatter, 356 V, g 1.0; log FL1 (FITC), 412 V, g 2.0; log FL2 (PE), 499 V, g 2.0; log FL4 (APC), 499 V, g 2.0; and log FL5 (PC7), 544 V, g 2.0, respectively. For each sample at least 10,000 viable lymphocytes, defined by the use of the viability marker 7AAD (BD Biosciences, Erembodegem, Belgium), were acquired in a forward light scatter vs. log side scatter histogram and a bitmap was placed around this lymphocyte population, defined as > 95% $CD14^-CD45^+$ cells. All gate-settings were established using the relevant isotype controls. Spectral overlap between flourochromes in the four-color flow cytometry protocol was compensated electronically using the Cytomics™ RXP compensation software (Beckman-Coulter, UK) and using the same compensation matrix for all analyses. All postacquisition analyses of the obtained list-mode data files were conducted in the FlowJo® software version 4.5.2 for MacIntosh (Tree Star Inc, Ashland, OR).

The flow cytometry data in **Fig. 2** are representative of the results generated using the following technique. In the four-color protocol, scatter plots were generated as a function of the expression of CD3 and CD56, respectively. From these plots the $CD3^-CD56^+$ population was divided into $CD56^{dim}$ and $CD56^{bright}$ subsets and, afterwards, the expression of the CD16 and CD8 markers was examined in these subsets. The gate-settings were predefined prior to analysis using single parameter histograms and were identical in all data units. The absolute number of cells in the cellular subsets defined by flow cytometry was calculated by multiplication of the gate-frequencies and the total number of lymphocytes

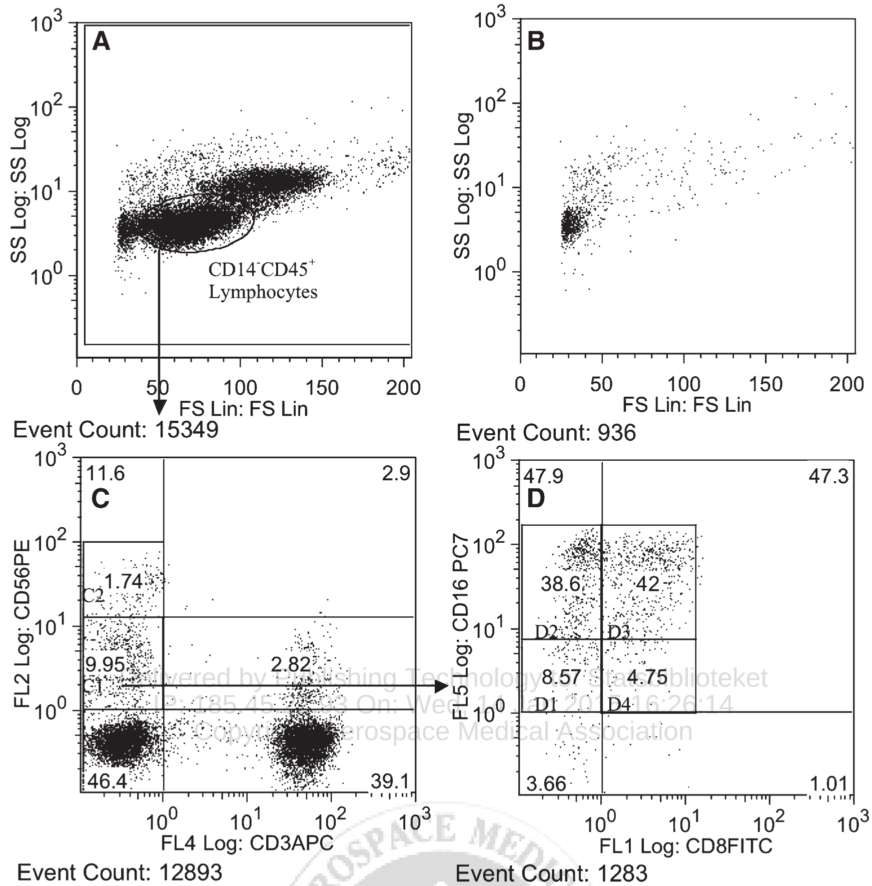

Fig. 2. Representative example of the four-color flow cytometry data. A) The lymphocyte population is defined as > 95% $CD14^-CD45^+$ leukocytes. B) The localization of dead lymphocytes. C) The NK cell population is identified by the expression of CD3 and CD56, respectively. The $CD3^-CD56^+$ population was divided into $CD56^{dim}$ (C1) and $CD56^{bright}$ (C2) NK cell subpopulations. D) The expression of the CD16 and CD8 markers are examined as demonstrated in D1-4.

counted by the Department of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway.

Statistical Methods

Statistical analyses were performed using the statistical package STATA (Stata 8.2, Stata Corporation, College Station, TX). The distribution of cytotoxicity was normalized by logarithmic transformation, which also ensured variance homogeneity between the time points and the groups of divers and control subjects. In a multiple linear regression model, with a random level for each individual describing the interindividual variation, the comparisons between groups were made using the Wald test (z-test). Furthermore, the linear regression model solved the multiple testing problems.

The comparisons between the data of day 0 and day 2, respectively, were made using a multivariate version of the Wald test (**Table I–III**). The covariation, i.e., the relation between the development of the two tested variables of interest, was tested using the above-mentioned multiple linear regression model with cytotoxicity as the dependent variable and the phenotypes or the leukocyte subpopulations, respectively, as the explaining variables (Table III). *P*-values and 95% confidence intervals are shown. *P*-values less than 0.05 are considered statistically significant.

RESULTS

Among the 11 reference subjects, 3 were excluded: 2 due to gastroenteritis during the test period and 1 due to a previous history of cancer. Furthermore, two reference subjects were absent at the scheduled blood sampling on day 2, which explains the lack of data at this point. Other lacking information is due to technical problems during the data processing.

Cytotoxicity

All cytotoxicity data presented were obtained at the effector-to-target ratio 10:1 and were considered the

TABLE I. DIFFERENCE IN MEAN PHENOTYPE LEVEL BETWEEN DIVERS AND REFERENCE SUBJECTS AT DAY 2 CORRECTED FOR THE PHENOTYPE LEVEL AT DAY 0 ($N_{DIVER} = 8$, $N_{REF} = 6$).

Phenotype	Reg. Coeff.	Mean Diff. [95% CI]	*P*-Value
Leukocytes	0.11	[−0.97 ; 1.19]	0.84
Neutrophils	−0.65	[−1.66 ; 0.36]	0.21
Lymphocytes	0.79	[0.39 ; 1.19]	< 0.001
Monocytes	−0.06	[−0.17 ; 0.04]	0.26
$CD14^-CD45^+$	0.79	[0.37 ; 1.22]	< 0.001
Viability (7AAD)	0.03	[−0.07 ; 0.14]	0.53

The mean differences in lymphocyte and NK cell subpopulations are estimated as absolute numbers (10^9 cells · L^{-1}). $CD14^-CD45^+$ and 7AAD data are based on multicolor flow cytometry.

TABLE II. DIFFERENCE IN MEAN LEVEL OF SPECIFIC LYMPHOCYTE SUBPOPULATIONS BETWEEN DIVERS AND REFERENCE SUBJECTS AT DAY 2 (N_{DIVER} = 8, N_{REF} = 6) CORRECTED FOR THE LEVEL OF LYMPHOCYTE SUBPOPULATIONS AT DAY 0 (N_{DIVER} = 8, N_{REF} = 8).

Lymphocyte Subpopulations	Reg. Coeff.	Mean Diff. [95% CI]	*P*-Value
$CD3^+$ (T cells)	0.33	[0.01 ; 0.64]	0.04
$CD19^+$ (B cells)	0.01	[−0.11 ; 0.11]	0.99
$CD3^-CD56^+$ (NK cells)	0.13	[0.03 ; 0.24]	0.01
NK Cell Subpopulations			
$CD3^-CD56^{dim}$	0.14	[0.04 ; 0.25]	0.01
$CD3^-CD16^{bright}CD56^{dim}$	0.12	[0.02 ; 0.22]	0.02
$CD3^-CD8^{dim}CD16^{bright}CD56^{dim}$	0.03	[−0.02 ; 0.08]	0.22
$CD3^-CD56^{bright}$	0.01	[−0.01 ; 0.02]	0.26

The mean differences in lymphocyte and NK cell subpopulations are estimated as absolute numbers (10^6 cells · L^{-1}) by multiplication of the multicolor flow cytometry data and the lymphocyte counts sampled during the dive.

physiologically correct ratio between cytotoxic NK cells and target cells during the in vitro conditions based on the kinetic in the ^{51}Cr-release assay (data not shown). During the dive, a significant increase in cytotoxicity was observed in both the divers and the reference subjects ($P < 0.001$ for both divers and reference subjects). The cytotoxicity in the group of divers increased relatively more compared to the group of reference subjects ($P < 0.001$) from day 0 until day 2 (**Fig. 3**). No difference in cytotoxicity between day 0 and day 22 was observed between the groups.

Leukocyte Counts

The number of circulating lymphocytes increased significantly in the group of divers from day 0 until day 2 ($P < 0.001$). There was no difference between the groups when the numbers of total leukocytes, neutrophils, or monocytes were compared (Table I) (**Fig. 4**).

NK Cell Phenotypes

The absolute number of $CD3^-CD56^+$ NK cells increased significantly ($P = 0.006$) from day 0 until day 2 in the divers. This increase was due to an increase in the $CD3^-CD56^{dim}$ and $CD3^-CD16^{bright}CD56^{dim}$ NK cell subsets in this group, whereas such increases could not be demonstrated in the reference subjects. No difference between the two groups was found either in the $CD3^-CD56^{bright}$ or the $CD3^-CD8^{dim}CD16^{bright}CD56^{dim}$ NK cell subsets (Table II) (Fig. 4).

Relationship Between Cytotoxicity, Lymphocyte Counts, and Phenotypes

A significant covariation between the increase in cytotoxicity and the increase in the numbers of circulating lymphocytes ($P < 0.001$) was observed in the divers, whereas this was not found in the reference subjects. The covariation between the increase in absolute number of PBMC expressing the CD56 marker and the increased cytotoxicity was also found to be significant ($P < 0.001$) in the divers, whereas this could not be demonstrated in the reference subjects ($P = 0.243$). This covariation was, however, independent of the level of expression of CD56 (Table III). No covariation between the cytotoxicity and the absolute numbers of $CD3^+$ T lymphopcytes ($p_{diver} = 0.284$ and $p_{reference} = 0.407$) or $CD19^+$ B lymphocytes ($p_{diver} = 0.181$ and $p_{reference} = 0.342$), respectively, could be demonstrated between the two study groups (Table III).

DISCUSSION

The main finding in this study is the pronounced increase in NK cell cytotoxicity during the compression phase accompanied by alterations in the cytotoxic NK cell subsets in the divers' peripheral blood. The influences

TABLE III. COVARIATION BETWEEN THE CYTOTOXICITY AND SPECIFIC LYMPHOCYTE SUBPOPULATIONS DURING THE DIVE.

	Divers			Reference Subjects		
Cell Type	**Reg. Coeff.**	**[95% CI]**	***P*–Value**	**Reg. Coeff.**	**[95% CI]**	***P*–Value**
$CD3^+$ (T cells)	0.3	[−0.3 ; 0.9]	0.28	−0.3	[−1.0 ; 0.4]	0.41
$CD19^+$ (B cells)	−1.1	[−2.7 ; 0.5]	0.18	−1.5	[−4.4 ; 1.5]	0.34
$CD3^-CD56^+$ (NK cells)	2.6	[1.4 ; 3.9]	< 0.001	1.5	[−0.9 ; 4.0]	0.24
NK cell subpopulations						
$CD3^-CD56^{dim}$	7.0	[1.9 ; 4.5]	0.01	4.8	[−4.9 ; 14.0]	0.30
$CD3^-CD16^{bright}CD56^{dim}$	3.0	[1.6 ; 4.3]	< 0.001	2.0	[−0.6 ; 4.7]	0.13
$CD3^-CD8^{dim}CD16^{bright}CD56^{dim}$	7.8	[4.4 ; 11.4]	< 0.001	5.6	[−1.2 ; 12.4]	0.11
$CD3^-CD56^{bright}$	275	[114 ; 467]	0.001	5.5	[−91 ; 102]	0.91

The covariation i.e., the relation between the development of the two tested variables of interest, were tested using a multiple linear regression model with a random level for each individual describing the interindividual variation, having the cytotoxicity as the dependent variable and the phenotypes or the leukocyte types, respectively, as the explaining variables.

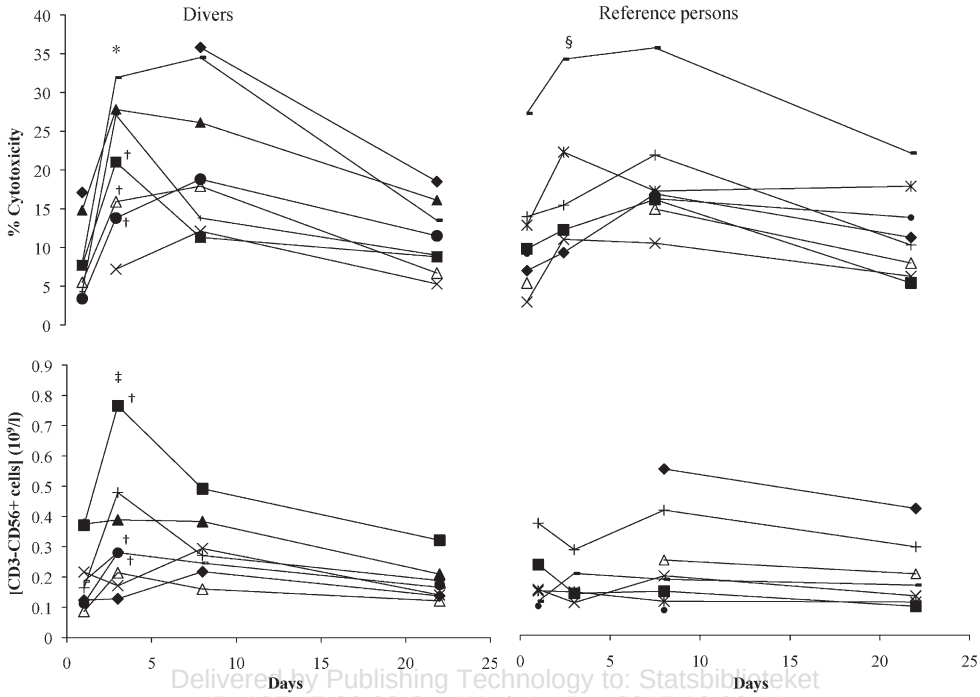

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Fig. 3. The individual NK-cell cytotoxicity of the divers and the reference subjects, respectively, plotted against time (days). A significant increase was observed in both the divers (*) and the reference persons (§) during the saturation phase (day 0–day 2). This increase was accompanied by a significant increase in the absolute numbers of circulating $CD3^-CD56^+$ NK-cells (10^9/l), which only could be demonstrated in the group of divers (‡). † indicates the two divers who had symptoms of high-pressure nervous syndrome during the dive and the one who had DCS after surfacing.

of compression on human NK cell cytotoxicity have, to our knowledge, never been studied before. A significant increase in the absolute numbers of circulating cytotoxic ($CD3^-CD16^{bright}CD56^{dim}$) NK cells in the divers accompanied the increase in cytotoxicity. Furthermore, the significant covariation between the estimated NK cell cytotoxicity and the absolute numbers of circulating NK cells, which could only be demonstrated in the group of divers, suggests that the divers' immune systems behaved differentially during the stay in the hyperbaric chamber, compared to the immune response observed in the group of reference persons living outside the chamber in the normobaric atmosphere.

The increased absolute number of circulating NK cells during the compression phase is in accordance with the findings in previous studies (15,23). Although it should be considered preliminary data, it is noteworthy that the dive produced symptoms of high-pressure nervous syndrome in two of the divers and one who expressed symptoms of DCS by the end of the dive (Fig. 3). Interestingly, two of the three divers seemed to have an increase in NK cell cytotoxicity, whereas the number of circulating NK cells did not increase during the dive to the same extent as in the asymptomatic divers. It should be stressed that it cannot be concluded, based on the limited observations in this study, that this "response" represents a link between the pathophysiological mechanisms and the observed symptoms of high-pressure nervous syndrome and DCS; however, this observation is intriguing.

It is evident that some of the hyperbaric environmental factors mentioned earlier had an impact on neuroendocrine homeostasis, influencing the immunological balance. Thus, a possible explanation for the findings in this study could be alterations induced by a neuroendocrine stress response induced by the release of stress hormones, such as catecholamines and cortisol. Interestingly, the cortisol level was elevated significantly in the divers during the dive compared to the reference subjects (A. Bårholm, Faculty of Psychology, University of Bergen, Norway. Dissertation; 2003). This response is well known as having a profound effect on the redistribution of circulating leukocytes in the peripheral blood, although cortisol generally dampens the immune system; on the other hand, the release of catecholamines augments immune functions, e.g., the cytotoxicity of circulating NK cells (16,27). Hypothetically, the increased NK cell cytotoxicity can reflect a state of arousal, including the release of catecholamines, in both the divers and reference subjects at the beginning of the dive, counterregulated by the release of cortisol. Although it is highly speculative, the reported difference in NK cell cytotoxicity and the numbers of circulating NK cells may represent an immunological reaction to the adaptation to the hyperbaric milieu in the pressurized chamber. Importantly in this relationship, hyperbaric exposure should be considered a multifactorial physiological stress (26), implying that a direct cause and effect relationship behind single environmental factors and suggestible immune modulating effects is difficult to identify. The unexpected changes observed in the group of reference persons can hypothetically reflect the physical and psychological stress levels outside the chamber during the initial phase of the dive.

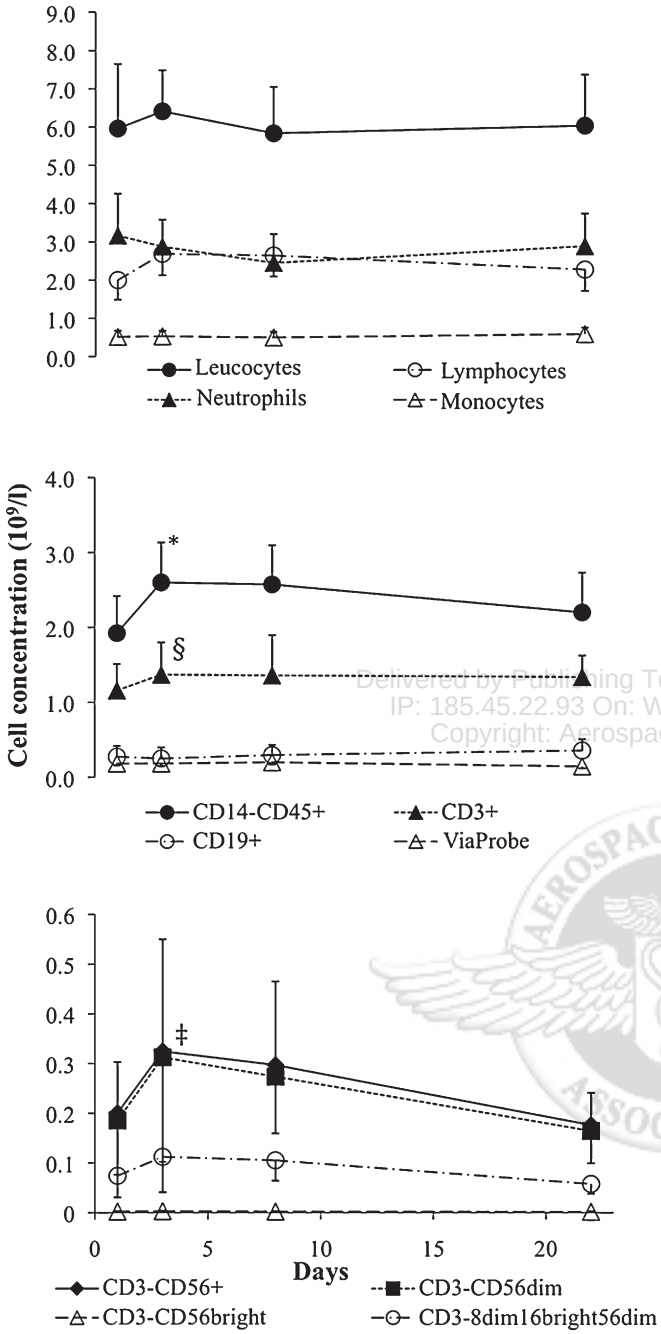

Fig. 4. The absolute number of leukocytes (10^9/L) plotted against time (days) (divers' data only). Mean and SD are shown. A significant increase in absolute number of the $CD14^-CD45^+$ lymphocytes (*) was observed during the saturation phase (day 0–day 2) due to an increase in the absolute numbers of circulating $CD3^+$ T-lymphocytes (§) and $CD3^-CD56^{dim}$ NK cells (‡).

The transition from normobaric air to the state of breathing heliox at the beginning of a saturation dive is abrupt, although the physiological impact of such changes in the composition of breathing gas is not known. Breathing helium instead of nitrogen reduces the breathing resistance (14), the narcotic effect of nitrogen at depth, and the time of decompression (9). The oxygen partial pressure in the diving chambers is moderately elevated to 30–50 kPa to prevent the risk of hypoxia during the dive. The oxygen partial pressure is tightly regulated to avoid pulmonary oxygen toxicity, which is known to induce, among other things, a state of interstitial pulmonary inflammation (24). As argued by Hofso et al. (10), no conventional signs of inflammation, such as C-reactive protein and leukocytosis, were associated with this dive. Intriguingly, in the same study, a significant increase in serum ferritin was found. This finding could support the theory that isolated inflammatory processes may be ongoing, despite lack of production of C-reactive protein, since ferritin can be released in response to a variety of cytokines and protects against the harmful effects of free oxygen radicals (11). Consequently the treatment of DCS could include the use of immune-modulating drugs, as suggested by Montcalm-Smith et al. (18).

The presence of oxidative stress during this dive was indicated by a reduction in glutathione levels in peripheral blood cells (6). It should be emphasized that the specific biological effects of compression and, concomitantly, the effects of increased partial pressure of the breathing gas on the NK cell system cannot readily be explained in this present study. However, it is noteworthy that redistribution of PBMC from the peripheral blood to the lungs, spleen, and lymph nodes has been demonstrated in rats during HBO studies. It has been proposed that some of the observed changes in the proportion of specific subsets of T-lymphocytes in the lungs could be related to the presence of stress proteins induced by HBO (2). The induction of stress proteins in the human lungs during hyperbaric exposure, and thereby activation of specific NK- and T-cell subsets, remains to be clarified. As such, the present study raises several issues that remain to be settled. It is still unknown whether the observed increase in cytotoxicity and changes in the phenotype reflects the in vivo conditions in the divers during the dive. Furthermore, it cannot be rejected that the innate immune systems of seasoned saturation divers may be somewhat different from that of the reference subjects due to repeated saturation diving. Such questions can be further delineated by investigations of the level of circulating soluble immune regulatory substances in the divers during the dive (e.g., cytokines, chemokines, NO, and complement factors). Another important consideration involves the fact that the investigated PBMC are cells isolated from the peripheral blood, whereas cells localized and/or redistributed to organs or sites of local inflammatory processes, such as the lungs and the lymph nodes, cannot be examined in the present set-up. The use of hyperbaric animal models will be preferable if such issues are studied.

It is important to note that, with regard to the significant increase in cytotoxicity in both groups of test subjects and the limited number of observations, we consider our data to be preliminary and interpret them with caution. The results of this study emphasize the need for further studies addressing the immunological aspects of conditions that affect human physiology in relation to hyperbaric exposure and saturation diving. In conclusion, the relative higher increase in the estimated NK

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cell cytotoxicity and absolute numbers of cytotoxic NK cells in the divers suggests that hyperbaric exposure during saturation diving can induce signals that activate specific parts of the cellular immune system.

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