Eur Respir J 2005; 25: 820–828
DOI: 10.1183/09031936.05.00103204

Activated CD8+ T-lymphocytes in obstructive sleep apnoea

L. Dyugovskaya*, P. Lavie*, M. Hirsh# and L. Lavie*

ABSTRACT: T-lymphocytes are implicated in the development of atherosclerosis. The aim of this study was to assess whether the CD8+ T-lymphocytes of obstructive sleep apnoea (OSA) patients undergo phenotypic and functional changes that may exaggerate atherogenic sequelae in OSA.

A total of 36 OSA patients, 17 controls and 15 single-night-treated OSA patients were studied. Phenotype and cytotoxicity against K562 target cells were analysed by flow cytometry. Cytotoxicity against human umbilical vein endothelial cells (HUVECs) was assessed by ^{51}Cr release assay.

The cytotoxicity of the CD8+ T-lymphocytes of OSA patients against K562 and HUVECs was significantly greater than controls. This increased cytotoxicity directly depended on the presence of perforin and natural killer receptors (CD56, CD16), which were significantly increased in OSA CD8+ T-lymphocytes. Also the percentage of the CD56bright subset, which mediates initial interactions with vascular endothelium, significantly increased in OSA. Nasal continuous positive airway pressure treatment significantly decreased CD8+ T-cell cytotoxicity and CD56 expression, and was positively correlated with natural killer inhibitory NKB1 receptor expression either after a single-night treatment or after a prolonged treatment.

In conclusion, the CD8+ T-lymphocytes of obstructive sleep apnoea patients undergo phenotypic and functional changes, rendering them cytotoxic to target cells *via* increased CD56+/perforin+ expression, which can be ameliorated by nasal continuous positive airway pressure treatment. These results are compatible with the current authors' hypothesis of atherogenic sequelae in obstructive sleep apnoea.

KEYWORDS: Atherosclerosis, CD8+ T-lymphocytes, cytotoxicity, nasal continuous positive airway pressure treatment, obstructive sleep apnoea, perforin

Obstructive sleep apnoea (OSA) syndrome is characterised by intermittent and recurrent pauses in respiration during sleep, leading to a fall in oxygen saturation and sleep fragmentation. It constitutes a major public health problem because of its association with cardiovascular morbidity and mortality, and its pervasive impact on patients' quality of life [1–4].

So far, the exact nature of the underlying pathophysiology of cardiovascular morbidity in OSA has not been fully elucidated. However, attempts have focused on the potential influence of two of the syndrome's major features: intermittent hypoxia and sleep fragmentation. Both could affect the inflammatory and immune responses that are considered to be fundamental mechanisms underlying atherosclerosis and its major complications, such as ischaemic heart disease and stroke [5–9]. It has been recently established that OSA is associated with increased oxidative stress and a state of inflammatory cell activation [10–12]. Specifically, it was demonstrated that circulating monocytes and γδ T-lymphocytes of OSA patients were activated and cytotoxic to endothelial cells [12, 13]. Hence, it became increasingly evident that cytotoxic CD8+ T-lymphocytes are also potential contributors to atherosclerosis, since they exhibit cytotoxicity against vascular endothelial and smooth muscle cells [5, 7].

Among the CD8+ T-lymphocytes, having two subpopulations CD8dim and CD8bright, the greatest cytotoxicity was found in subsets that also express the natural killer (NK) receptors CD56 and CD16 (also termed NK T-cells) [14–18]. These CD8+/CD56+ T-lymphocytes generally contain high amounts of perforin, which explains their greater cytotoxic capacity [18]. Moreover, among CD56+ cells, the CD56bright subset mediates initial interactions with vascular endothelium.

For editorial comments see page 780.

AFFILIATIONS
*The Lloyd Rigler Sleep Apnea Research Laboratory, Unit of Anatomy and Cell Biology, and
#The Laboratory for Shock and Trauma Research, The Ruth and Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel.

CORRESPONDENCE
L. Lavie
Unit of Anatomy and Cell Biology
The Ruth and Bruce Rappaport
Faculty of Medicine
Technion
POB 9649, 31096
Haifa
Israel
Fax: 972 48295403/972 48343934
E-mail: lenal@techunix.technion.ac.il

Received:
September 02 2004
Accepted after revision:
January 10 2005

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

In the current study, the expression of stimulatory (CD56, CD16) and inhibitory (NKB1) NK receptors and the expression of perforin, a cytotoxicity marker, in CD8+ T-lymphocytes of OSA patients and controls were characterised. In addition, the cytotoxicity of CD8+ T-lymphocytes against two types of target cells was investigated. In order to ensure that the observed changes were due to the sleep apnoea and not to other concurrent factors, the expression of these markers, after a single night of treatment with nasal continuous positive airway pressure (nCPAP) and after prolonged nCPAP use, was also investigated.

MATERIALS AND METHODS

Patients and controls

A diagnosis of OSA was based on an in-laboratory polysomnographic recording, which included monitoring of electroencephalography, respiration using chest and abdomen respiratory belts and oronasal temperature sensors, or pressure canulas, as substitute measurements of respiratory effort and flow, and arterial oxygen saturation (S_{a,O_2}) by a finger oximeter. Apnoea–hypopnoea index (AHI) was calculated as the total number of apnoeas plus hypopnoeas divided by hours of sleep, and the percentage of sleep time spent with oxygen saturation <90% was determined. Height and weight were recorded, and the body mass index (BMI) was calculated ($kg \cdot m^{-2}$). Participants for this study were recruited from the patient population of the Technion Sleep Medicine Center in Haifa, Israel. Consecutive patients were recruited for the study (n=36). The inclusion criteria were an AHI >15 and not being sick during the last week before the study. Six out of the 36 untreated patients were re-investigated after 4.8±2.7 months of treatment with nCPAP. Individuals who were referred for diagnostic sleep recordings, mostly because of snoring and fatigue, but who were found to have an AHI <15 comprised the control group, provided that they mostly had hypopnoeas without arterial oxygen desaturation (n=17). A separate group of 15 OSA patients treated for one night with nCPAP was also studied simultaneously with the untreated patients and the controls. This group, which was similar to the untreated patients with respect to the pre-treatment severity of OSA, differed from untreated patients only with respect to AHI and percentage of time spent with oxygen saturation <90% after the treatment. A diagnosis of hypertension was based on either blood pressure measurements >140/90 mmHg or the usage of antihypertensive medication. A diagnosis of ischaemic heart disease was based on a history of myocardial infarction or angiographic findings. Table 1 provides clinical and demographic data.

Due to the fact that phenotyping and cytotoxicity assays are tedious, time consuming, strategically difficult to perform, require large quantities of blood, and that lymphocyte preparation from blood and preparation of human umbilical vein endothelial cells (HUVECs) in culture do not always coincide, the tests could not be performed simultaneously on all subjects. Thus, the design was based on comparing the cellular functions of subgroups of OSA patients, single-night-treated OSA patients and controls at the same time for a given assay. The assignment of subjects to the different experiments was based on similarity in age and BMI. The number of subjects participating in each experiment is shown in table 2. The protocol was approved by the local human rights committee, and all participants signed an informed consent.

Isolation of CD8+ T-lymphocytes

Blood samples were withdrawn in the morning after overnight fasting. Enriched fractions of CD8+ T-lymphocytes were prepared using magnetic microbeads conjugated with anti-fluorescein isothiocyanate (FITC) and a magnetic field system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) [13]. FITC-conjugated mouse monoclonal antibody (mAb) anti-human CD8 (Serotec Ltd, Oxford, UK) was used. In addition, enriched CD8+/CD56-/CD16- T-lymphocytes were prepared by RosetteSep antibody cocktail (StemCell Technologies Inc., Vancouver, Canada).

TABLE 1 Demographic, sleep and clinical data of obstructive sleep apnoea (OSA) patients, with and without single-night nasal continuous positive airway pressure (nCPAP) treatment, and control subjects participating in the different experiments

	OSA	OSA nCPAP	Controls	3-way p-value	Controls *versus* OSA p-value	Controls *versus* nCPAP p-value	OSA *versus* nCPAP p-value
Subjects n	36	15	17				
Age yrs	52.8±12.0	53.4±10.8	46.4±10.7	NS	0.06	0.10	NS
Males %	81	93	82	NS	NS	NS	NS
BMI $kg \cdot m^{-2}$	29.6±5.0	27.4±3.3	26.4±3.1	0.04	0.01	NS	NS
AHI events$\cdot h^{-1}$	31.7±14.4	11.3±4.9	9.7±3.8			NS	
Time spent with S_{a,O_2} <90% %	11.2±16.0	0.3±1.2	1.0±2.2	0.0001	0.001	0.10	0.0001
HTN %	39	20	19	NS	NS	NS	NS
IHD %	3	13	0	NS	NS	NS	NS
DM %	22	0	6	0.07	NS	NS	0.09
Current smokers %	25.0	20.0	29.4	NS	0.06	NS	NS
Never-smokers %	38.9	53.3	64.7	NS	0.06	NS	NS

Data are presented as n, mean±SD and %, unless otherwise stated. BMI: body mass index; AHI: apnoea–hypopnoea index (total number of hypopnoeas plus hypopnoeas divided by hours of sleep); S_{a,O_2}: arterial oxygen saturation; HTN: hypertension; IHD: ischaemic heart disease; DM: diabetes mellitus; NS: nonsignificant.

TABLE 2 Lymphocyte markers on peripheral blood lymphocytes in controls, obstructive sleep apnoea (OSA) patients without treatment and OSA patients after one night of nasal continuous positive airway pressure (nCPAP) treatment

Markers	Controls	OSA	OSA nCPAP	3-way p-value	Controls *versus* OSA p-value	Controls *versus* CPAP p-value	OSA *versus* CPAP p-value
CD8#	29.5±6.6 (16)	28.0±7.6 (31)	28.1±6.0 (13)	NS	NS	NS	NS
CD16#	13.6±5.1 (12)	17.2±5.4 (14)	15.0±5.1 (3)	NS	0.11	NS	NS
CD56#	18.1±5.2 (12)	20.2±6.8 (30)	24.5±11 (13)	0.2	NS	0.1	NS
CD56bright¶	3.6±1.2 (7)	6.0±1.3 (12)	3.1±1.9 (9)	0.002	0.003	NS	0.004
CD8+/CD56++	35.2±11.0 (15)	50.0±13.9 (30)	40.7±15.2 (13)	0.003	0.0007	NS	0.06
CD8+/CD16++	19.1±7.6 (12)	36.1±13.7 (12)			0.004		
CD8+/NKB1++	5.4±4.2 (11)	5.3±4.0 (15)	10.5±4.5 (9)	0.03	NS	0.05	0.01

Data are presented as %±SD (n), unless otherwise stated. NKB1: natural killer inhibitory receptor. #: % of labelled lymphocytes; ¶: % CD56bright (mean fluorescence intensity 800–1200) among CD56+ T-lymphocytes; +: % of double-stained cells among CD8+ T-lymphocytes.

All procedures were performed according to the manufacturer's instructions. Flow cytometry revealed 85–90% purity and ≈90% viability by trypan-blue exclusion.

Flow cytometry

The cell-surface phenotype was determined in whole blood using mouse immunoglobulin G mAb reacting with the following human molecules: CD8 (clone LT8), NKB1 (clone DX9), CD56 (clone B159) and CD16 (clone 3G8; PharMingen, San Diego, CA, USA). Cell samples were analysed by flow cytometry (FACS Calibur; Becton Dickinson, Lincoln Park, NJ, USA), using a single or a dual-staining protocol. Lymphoid cells were gated using forward and side light scatter. In parallel, for each subject, controls for autofluorescence (unstained cells) and isotypic controls were analysed. The percentage of fluorescent cells and mean fluorescence intensity (MFI) were determined in each case.

Intracellular perforin determination in CD8+ T-lymphocytes

Ficoll-separated peripheral blood mononuclear cells were incubated with FITC-conjugated CD8 mAb. After two washes, the cells were fixed with 4% paraformaldehyde PBS, and were washed twice and permeabilised with 0.1% saponin (Sigma-Aldrich Ltd, Rehovot, Israel). Thereafter, cells were incubated with R-phycoerythrin (PE)-labelled anti-perforin antibody (clone δG9; Ancell Corporation, Bayport, MN, USA) for 20 min at room temperature in the dark, washed twice with PBS/0.1% saponin, resuspended in PBS and analysed by flow cytometry. The results were expressed as percentages of CD8+/perforin+ containing cells.

Cytotoxicity assay

The cytotoxicity of CD8+ T-lymphocytes was determined by utilising two different target cells: erythroleukaemic K562 cells, normally utilised for NK-induced cytotoxicity, and HUVECs. Cytotoxicity against K562 cells was measured using flow cytometry [19]. Briefly, suspended CD8+ T-lymphocytes ($2 \times 10^5 \cdot 100$ μL^{-1}) and washed K562 were mixed in effector/target ratios of 5:1, 10:1 and 20:1 in 200 μL of culture medium, incubated for 1 h at 37℃ and 5% CO_2, centrifuged, resuspended in buffered formaldehyde and analysed. A total of 20,000 cells was collected for each sample. The cytotoxicity index was calculated [19].

HUVECs were kindly provided by N. Lanir (Rambam Medical Center, Haifa, Israel) and treated as previously described [13]. After detachment with trypsin ethylenediamine tetraacetic acid, HUVECs were seeded onto fibronectin-pretreated (50 $\mu L \cdot well^{-1}$ at 10 $\mu g \cdot mL^{-1}$) 96-well microplates and loaded overnight with ^{51}Cr (1 $\mu Ci \cdot mL^{-1}$). Following washing, purified CD8+ T-lymphocytes were added to the ^{51}Cr-labelled HUVECs (effector/target 10:1). After 16 h of co-culture, counts per min radioactivity was determined and calculated [13].

Statistical analysis

Univariate analysis was used to compare the demographic and clinical data of the three groups: OSA, nCPAP-treated OSA and controls. Categorical data were analysed by Chi-squared tests or Fisher's exact probability tests. Continuous variables were analysed by the Kruskal-Wallis test. To account for the differences between OSA patients and controls in age and BMI, in each of the comparisons, data were adjusted to both variables, as well as to sex, by analysis of the covariance. Spearman rank order correlations were used to determine the relationship between the variables.

RESULTS

Immunophenotyping of peripheral blood lymphocytes in OSA patients and controls

Flow cytometry analysis by single-staining protocol was used to determine the distribution of the main lymphocyte markers in whole blood by two parameters: percentage of cells expressing the given receptor and its intensity of expression as attested by MFI. No differences were found in the percentage of T-lymphocytes expressing CD8 and CD16 receptors in whole blood (table 2), nor in MFI among the groups investigated (data not shown). Also the percentage of T-lymphocytes expressing CD56 molecules did not differ significantly among the groups (table 2). However, when CD56+ T-lymphocytes were subdivided according to the intensity of expression into two subpopulations [20–22] of CD56dim (MFI 150–300) and CD56bright (MFI 800–1200), the percentage of the CD56bright subset among the CD56+ T-lymphocytes was significantly greater in OSA patients (table 2). Three examples for each study group are given in figure 1.

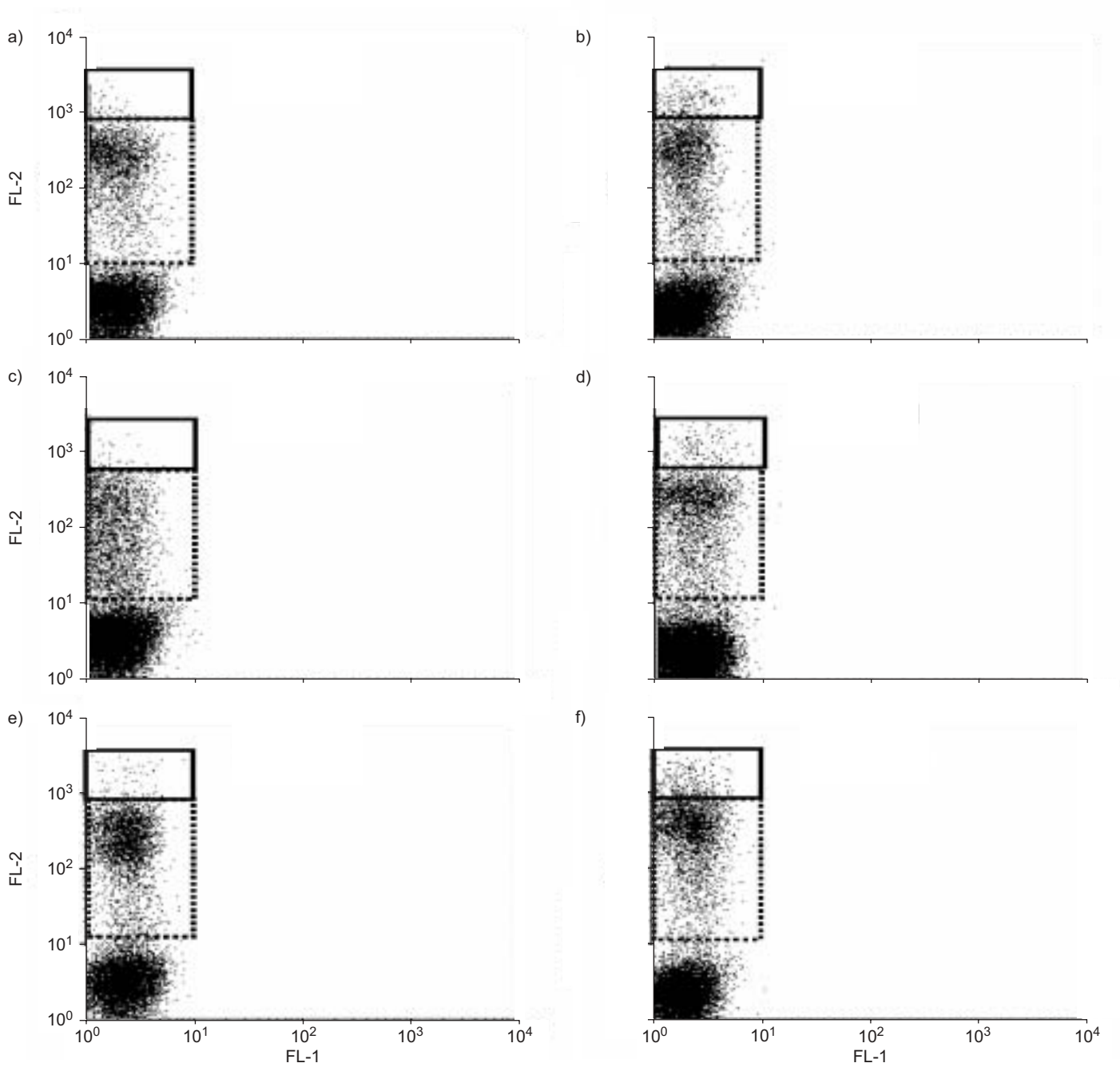

FIGURE 1. The expression of CD56bright in the lymphocytes of controls (a, c, e) and obstructive sleep apnoea (b, d, f) patients, illustrated by the dot plots of flow cytometric analysis of lymphocytes labelled with R-phycoerythrin-conjugated CD56 monoclonal antibody (mAb). Whole peripheral blood was stained with CD56 mAb and cell samples were analysed by flow cytometry using a single-staining protocol in lymphocyte gates. CD56+ T-lymphocytes were subdivided according to the intensity of expression into CD56dim (mean fluorescence intensity (MFI) 150–300; dotted rectangle) and CD56bright (MFI 800–1200; solid rectangle) subsets. The percentages of the CD56bright subset among CD56+ T-lymphocytes were: 2.1% (a), 8.4% (b), 1.9% (c), 5.3% (d), 3.1% (e) and 6.6% (f). Three typical subjects are presented for each group. FL-1: fluorescence detected in channel 1; FL-2: fluorescence detected in channel 2.

NK expression on CD8+ T-lymphocytes of OSA patients and controls

Since NK receptors are useful markers for the identification and monitoring of CD8+ T-lymphocytes in different clinical settings, NK-receptor expression (PE staining) on peripheral blood CD8+ T-lymphocytes (FITC staining) was analysed using double staining. The percentage of CD8+ T-lymphocytes that also expressed CD56 and/or CD16 molecules was significantly higher in OSA (table 2). Specifically, the differences in CD56 expression were detected in the CD8bright subset (fig. 2a), whereas CD16 expression was mainly detected in the CD8dim subpopulation (fig. 2b). By contrast, no differences were found between the OSA patients and controls in the percentage of CD8+ T-lymphocytes expressing NKB1 inhibitory receptors

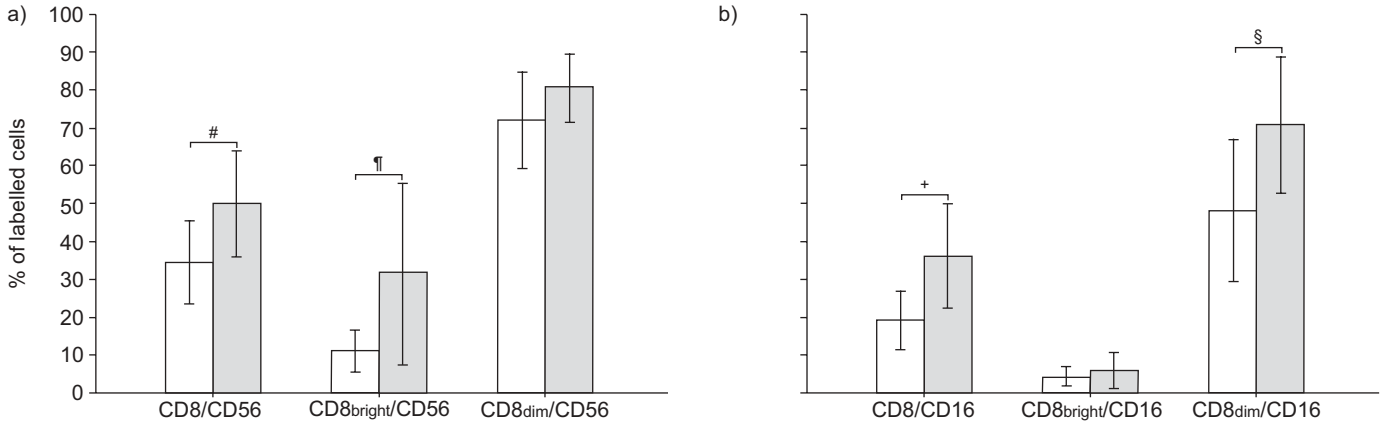

FIGURE 2. CD56 and CD16 expression among CD8+ T-lymphocytes in obstructive sleep apnoea (OSA) patients (■) and controls (□). a) The percentage expression of CD56+ among CD8+ T-lymphocytes, subdivided according to $CD8_{bright}$ and $CD8_{dim}$ expression. CD8/CD56 denotes the percentage of CD56-bearing cells among CD8-bearing T-lymphocytes. $CD8_{bright}$/CD56 denotes the percentage of CD56-expressing cells among the $CD8_{bright}$ subset, and $CD8_{dim}$/CD56 denotes the percentage of CD56-expressing cells among the $CD8_{dim}$ subset (OSA n=30; controls n=15). b) The percentage expression of CD16+ among CD8+ T-lymphocytes, subdivided according to $CD8_{bright}$ and $CD8_{dim}$ expression. CD8/CD16 denotes the percentage of CD16-bearing cells among CD8-bearing T-lymphocytes. $CD8_{bright}$/CD16 denotes the percentage of CD16-expressing cells among the $CD8_{bright}$ subset, and $CD8_{dim}$/CD16 denotes the percentage of CD16-expressing cells among the $CD8_{dim}$ subset (OSA n=13; controls n=11). Data were analysed by flow cytometry in whole peripheral blood using dual-staining protocols, and presented as mean±SD. #: p=0.001; ¶: p=0.008; +: p=0.003; §: p=0.004.

(table 2). Also, similar values were reported for normal individuals [23].

The correlation between the presence/absence of hypertension and NK-receptor expression on CD8+ T-lymphocytes was also studied. No differences (p=0.83) were found in the percentage of CD8+ T-lymphocytes expressing CD56 between normotensive (n=22; AHI 29.0±12.3; CD8/CD56 51.2±15.8%) and hypertensive (n=11; AHI 37.3±18.4; CD8/CD56 47.8±9.4%) OSA patients. Also, no differences were found between the groups in $CD56_{bright}$ expression (p=0.6)

Cytotoxicity against K562 erythroleukaemic cells: involvement of NK receptors and perforin

The cytotoxicity of CD8+ T-lymphocytes against K562 erythroleukaemic target cells was ≈2-fold higher in patients with OSA compared with controls at the three different effector/target ratios investigated (fig. 3). Since the cytotoxicity of CD8+ T-lymphocytes depends on the presence of NK receptors, lymphocytes with and without NK receptors were used in parallel experiments from the same donors (CD8+/CD56+/CD16+ and CD8+/CD56-/CD16- T-lymphocytes, respectively). As illustrated in figure 3, the cytotoxicity of CD8+ T-lymphocytes depended on the presence of CD56+/CD16+ receptors, since by depleting these CD8+ T-lymphocytes that also express CD56+/CD16+ receptors, the ability of K562 killing was dramatically decreased.

The cytotoxicity of CD8+/CD56+ T-lymphocytes is largely attributed to the presence of perforin. Perforin molecules are enclosed within cytoplasmic granules and, when released, injure cellular membranes in their vicinity. Thus, CD8+/CD56+ T-lymphocytes of healthy donors generally contain high amounts of perforin and, therefore, have a greater cytotoxic capacity than T-lymphocytes lacking NK receptors [18]. A positive correlation was found between the percentage of CD8+ lymphocytes expressing CD56/CD16 molecules (CD8+/CD56+/CD16+) and the percentage of CD8+ T-lymphocytes containing perforin (CD8+/perforin+) in OSA patients (fig. 4a). Figure 4b further illustrates this association by demonstrating the low amounts of perforin that are present in the CD8+/CD56-/CD16- subpopulation compared with the CD8+/CD56+/CD16+ subpopulation, rendering the latter more cytotoxic.

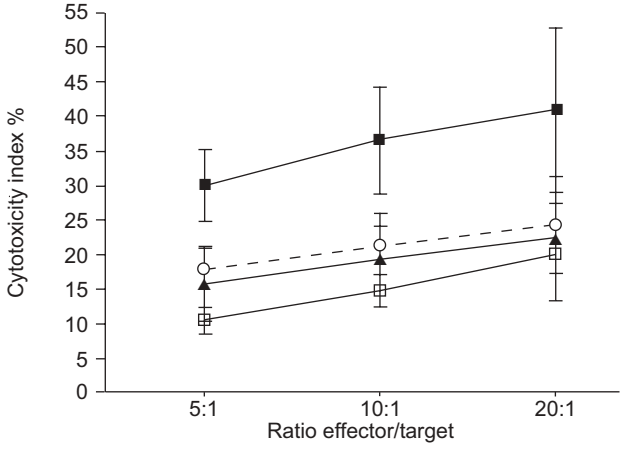

FIGURE 3. Flow cytometry analysis of CD8+ T-lymphocyte cytotoxicity against K562 target cells. CD8+ T-lymphocyte cytotoxicity was compared between controls (○; n=5), obstructive sleep apnoea (OSA) patients (■; n=7), single-night nasal continuous positive airway pressure-treated OSA patients (▲; n=6) and OSA CD8+ T-lymphocytes depleted from CD56+/CD16+ cells (□; n=6). All comparisons were statistically significant as compared with OSA patients (p<0.001, p<0.005, p<0.05 for 5:1, 10:1, and 20:1, respectively). Data are presented as mean±SD.

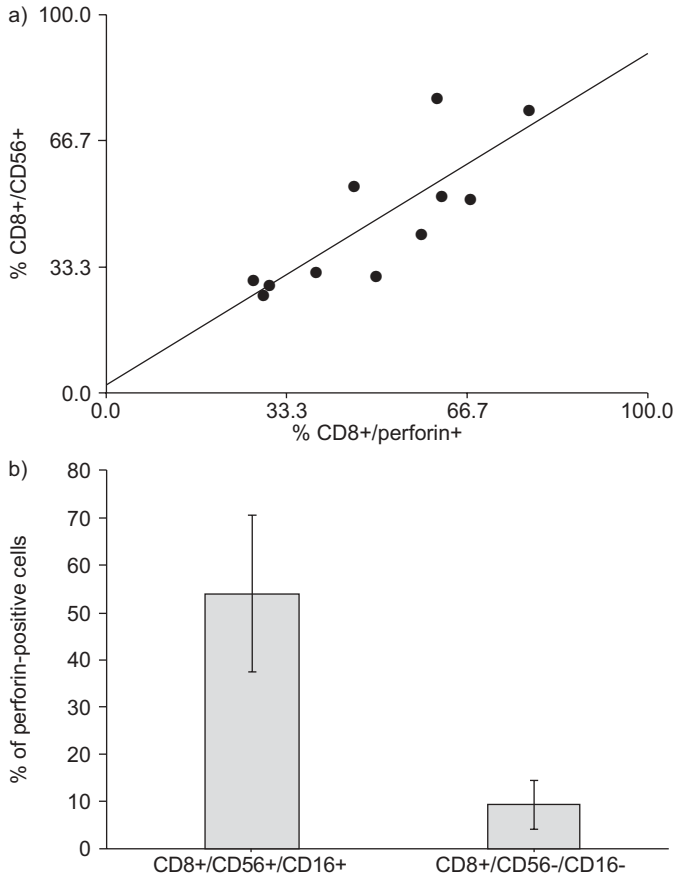

FIGURE 4. Perforin expression in obstructive sleep apnoea (OSA) CD8+ T-lymphocytes. a) Correlation between the percentage of CD56 expression *versus* the percentage of perforin expression in CD8+ T-lymphocytes of OSA patients. Data are presented for each of the patients individually (R=0.8076; p=0.0026). b) The percentage of perforin+ lymphocytes among OSA CD8+/CD56+/CD16+ and CD8+/CD56-/CD16- T-lymphocytes from the same patients (p=0.002). Purified, magnet-separated CD8+ T-lymphocytes were used as a source of CD8+/CD56+/CD16+ T-lymphocytes, and CD8+/CD56-/CD16- T-lymphocytes were prepared using RosetteSep antibody cocktail (StemCell Technologies Inc., Vancouver, Canada). Data are presented as mean±SD.

Cytotoxicity against human umbilical vein endothelial cells

Since cytotoxicity against endothelial cells is of great relevance to atherosclerosis, HUVECs were additionally utilised as target cells. The cytotoxicity of CD8+ T-lymphocytes was assessed by co-culturing with HUVECs at an effector/target ratio of 10:1. In contrast to cytotoxicity against K562 (data not shown), the lysis of HUVECs was positively significantly correlated with the severity of the syndrome, as attested by AHI (r=0.73; p<0.0017; fig. 5). The cytotoxicity was also positively correlated with percentage time spent with S_{a,O_2} <90% (r=0.62; p=0.02) and was negatively correlated with minimum oxygen saturation (r=-0.66; p=0.01). Yet, BMI had no effect on the cytotoxicity against HUVECs (fig. 5), as no significant correlation was found between cytotoxicity *versus* BMI (r=0.29; p=0.3). Similar to the cytotoxicity against K562 cells, HUVECs killing was significantly lowered to 5.3±4.0% (p=0.0001) after depletion of the cytotoxic CD16+/CD56+ lymphocytes from the CD8+ population.

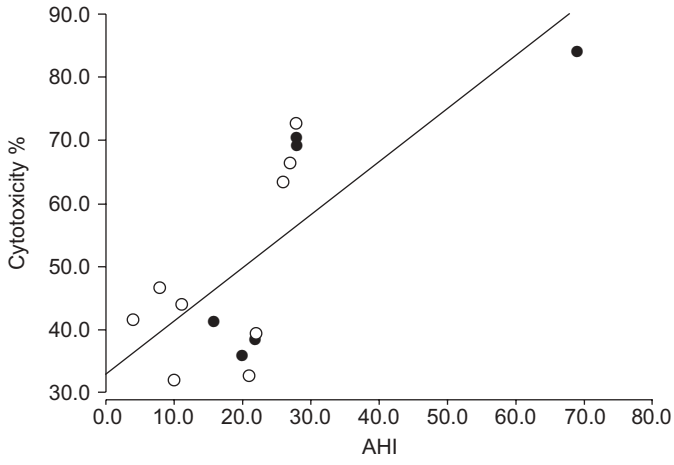

FIGURE 5. Correlation between the cytotoxicity of CD8+ T-lymphocytes against human umbilical vein endothelial cells and the apnoea–hypopnoea index (AHI). Cytotoxicity was detected by ^{51}Cr release assay as described in the *Materials and methods* section. Data are presented individually for each subject (R=0.73; p=0.0017). ○: individuals with body mass index (BMI) <30; ●: individuals with BMI >30.

Effects of a single-night nCPAP treatment on phenotype and function of CD8+ T-lymphocytes

To further demonstrate that sleep apnoea affected lymphocyte function, and not any other associated variables, a group of OSA patients after their first night of nCPAP treatment was compared simultaneously with OSA patients and controls. This group was very similar to the OSA group with respect to age, BMI and comorbidities, but their breathing in sleep was normalised by the treatment, as was evident by the decreased AHI (from 27.5±8.0 to 11.3±4.9 events·h^{-1}) and the percentage time spent with S_{a,O_2} <90% (from 8.5±6.5 to 0.3±1.2).

No significant changes were found in the total numbers of peripheral blood CD8+ or CD56+ T-lymphocytes (table 2) between OSA patients, controls or single-night nCPAP-treated patients. However, the percentage of CD8+/CD56+ T-lymphocytes was on borderline statistical significance, and the percentage of CD56^{bright} subsets among CD56 T-lymphocytes was significantly lower in the nCPAP-treated OSA patients compared with the OSA patients. Moreover, these values were found to be similar to the controls (table 2). Interestingly, the expression of NKB1 inhibitory receptors on CD8+ T-lymphocytes of nCPAP-treated patients was elevated compared with either OSA patients or controls (table 2). This increased NKB1 expression was noted in both CD8+ T-lymphocyte subpopulations, *i.e.* the CD8^{dim} and the CD8^{bright} (fig. 6). Also, the cytotoxicity of CD8+ T-lymphocytes of the nCPAP-treated group against K562 target cells was only 20–25% of that of the OSA group and was identical to control values (fig. 3).

Comparison of patients before and after prolonged nCPAP treatment

An additional group comprising six of the 36 untreated OSA patients were re-studied after 4.8±2.7 months of nCPAP treatment. A marked reduction in AHI (25.5±2.23 *versus* 3.0±1.6; p<0.00003) was noted. Similar to the single-night

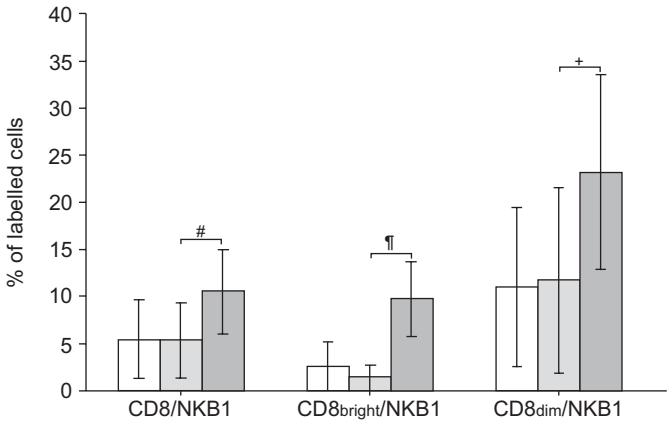

FIGURE 6. Natural killer inhibitory NKB1 receptor expression on CD8+ T-lymphocytes of controls (□; n=11), untreated obstructive sleep apnoea patients (■; n=15) and single-night nasal continuous positive airway pressure-treated patients (■; n=9). Frequencies of CD8+/NKB1+ among CD8bright and CD8dim T-lymphocytes were determined by flow cytometry in whole blood using dual-staining protocols. CD8/NKB1 denotes the percentage of CD8+ T-lymphocytes also expressing NKB1. CD8bright/NKB1 denotes the percentage of cells expressing NKB1 among the CD8bright subset, and CD8dim/NKB1 denotes the percentage of cells expressing NKB1 among CD8dim subsets. Data are presented as mean±SD. #: p=0.01; ¶: p=0.005; +: p=0.05.

nCPAP treatment, prolonged treatment also lowered the expression of both CD56 (53.5±11.3 *versus* 36.6±16.2%; $p<0.004$) and perforin (56.2±8.3 *versus* 36.6±11.6%; $p<0.006$) of CD8+ T-lymphocytes, whereas NKB1 receptor expression was increased (4.4±2.7 *versus* 8.3±4.5%; $p<0.06$).

DISCUSSION

In the current study, the authors demonstrated, for the first time, altered CD8+ T-lymphocyte phenotype and function in patients with OSA. In summary, the following findings were made. 1) The expression of two NK receptors, CD56 and CD16, was increased in the CD8+ T-lymphocytes of OSA patients. 2) The cytotoxicity of OSA CD8+ T-lymphocytes against K562 target cells and HUVECs was greater than in controls and was AHI-severity dependent against HUVECs. 3) The expression of NK receptors and perforin correlated with the increased cytotoxicity of CD8+ T-lymphocytes against both target cells. 4) Single-night or prolonged nCPAP treatment dramatically affected CD8 receptor expression; it decreased CD56 stimulating receptor and increased NKB1 inhibitory receptor. Single-night nCPAP use also inhibited cytotoxicity to control values, and decreased CD56bright subsets among CD56+ T-lymphocytes.

Intermittent hypoxia, the hallmark of OSA, comprises of multiple hypoxia/reoxygenation cycles [11]. Several studies indicate that hypoxia/reoxygenation directly, or *via* inflammatory cytokines, activates a variety of cells, including endothelial cells and leukocytes, thus propagating inflammatory/immune responses [11]. Currently, available data have shown that immunological activation is an early step in inflammatory/immunological mechanisms that govern atherogenesis [5–7]. It has already been established that the monocytes and γδ T-lymphocytes of patients with OSA expressed an activated phenotype, which led to increased adhesion, free radical formation and cytotoxicity towards endothelial cells [12, 13]. In the present study, these earlier findings were further expanded upon by demonstrating that the CD8+ T-lymphocytes of OSA patients also express an activated phenotype and increased cytotoxicity against various target cells.

Interestingly, analysis of the surface expression of CD8, CD16 and CD56 receptors in whole blood revealed no differences in the percentage of expression between the groups. However, when analysing lymphocytes according to the density of CD56 expression [20–22], increased CD56bright activity was noted in OSA T-lymphocytes. CD56bright cells correspond to a subset of activated circulating lymphocytes, which produce higher levels of cytokines in response to stimulation [20, 22], express higher levels of adhesion molecules (CD2, CD11c, CD44, CD62L) [21, 24, 25] and exhibit highly efficient adhesion to endothelial cells [25]. Importantly, a single night of nCPAP treatment (table 2) significantly decreased the percentage expression of CD56bright subsets compared with untreated patients and was similar to controls.

Hence, the higher percentages of CD56+/CD16+ cells that were found among CD8+ T-lymphocytes of OSA patients were highly cytotoxic and expressed higher levels of perforin, compared with CD8+/CD56-/CD16- lymphocytes. The increase in CD8+/CD56+ and CD8+/CD16+ T-lymphocytes suggests activation, particularly in severe OSA cases, where HUVECs lysis was higher.

Surprisingly, a two-fold increase in the percentage of CD8+/NKB1+ T-lymphocytes was found in OSA patients treated with nCPAP as compared with untreated OSA or controls. This was evident in either a single or a prolonged nCPAP treatment. Since the cytotoxic function is a result of a balance between activating and inhibiting signals, which are delivered by the corresponding receptors [26], nCPAP treatment could have decreased the cytotoxicity of CD8+ T-lymphocytes either by increasing inhibitory receptor expression and/or by decreasing the expression of stimulating receptors. Moreover, triggering NKB1 molecules can induce apoptosis of these cells and, *via* that, limit tissue destruction caused by activated CD8+ T-lymphocytes [27].

The necessary intracellular signals to elicit T-lymphocyte cytotoxicity are poorly understood. In OSA, however, increased cytotoxicity could result from changes in the surrounding oxygen tension. This possibility is strongly supported by the increase in lytic CD8+ T-lymphocytes during hypoxia *in vitro* [28]. The current data from the nCPAP-treated group further support this notion. Due to single-night nCPAP use, the hypoxaemia and apnoea severity were ameliorated concomitantly with a significant decrease in CD8+ T-lymphocyte cytotoxicity. Furthermore, the decreased cytotoxicity in nCPAP-treated patients also correlated with decreased CD56bright and increased NKB1 receptor expression. Since these changes were observed after a single night of nCPAP treatment, this attests to a rapidly altered cellular state induced by changes in S_{a,O_2}.

Another possibility that cannot be ignored is that inflammatory/immune activation is modulated by increased sympathetic activation and catecholamine release observed in OSA

patients [29]. Sympathetic activation can affect lymphocyte traffic, circulation, proliferation and modulate functional activities [30]. Generally, although data regarding the specific effects of catecholamines on CD8+ T-lymphocyte cytotoxicity are scarce, they are mostly described as potent inhibitors of NK activity [31]. In addition, investigating the immune system in various models of insomnia revealed suppressed immune activity in sleep disturbances associated with sympathetic activation [32, 33]. Thus, only a controlled study that will differentially manipulate hypoxia and sympathetic activation could determine the relative contribution of each of them to the cytotoxicity of OSA T-lymphocytes.

The results of the comparison between OSA patients and the group of patients examined after their first nCPAP titration night added strength to the current findings. The two groups that were very similar to each other, with respect to the history of sleep apnoea and its severity and in clinical and demographic variables, differed by a single night of treatment that normalised breathing in sleep. This was sufficient to bring T-lymphocyte functions to levels comparable with those of the control group. The results of the single-night treatment group were identical to those of patients after prolonged treatment. In both groups, normalising breathing by nCPAP was associated with increased NKB1 inhibitory and decreased CD56 stimulatory receptors.

In summary, this study provides direct evidence for the generation of cytotoxic CD8+/CD56+/perforin+ T-lymphocytes in patients with obstructive sleep apnoea. Thus, it is clearly evident that, together with monocytes [12] and γδ T-lymphocytes [13], CD8+ T-lymphocytes also participate in inflammatory/immune mechanisms in the setting of obstructive sleep apnoea. These may exaggerate atherogenesis and, thus, may provide a partial explanation of the high prevalence of cardiovascular morbidity in obstructive sleep apnoea patients. Nasal continuous positive airway pressure treatment, which downregulates some immune functions and activation in various leukocyte subpopulations, may attenuate atherogenic sequelae in obstructive sleep apnoea.

ACKNOWLEDGMENTS

The authors would like to thank F. Barbara, P. Herer and the staff of the Sleep Medicine Center, Rambam Hospital, Haifa, Israel, for their invaluable help.

REFERENCES

1 Lavie P, Herer P, Hoffstein V. Obstructive sleep apnea syndrome as a risk factor for hypertension: population study. *BMJ* 2000; 320: 479–482.
2 Shahar E, Whitney CW, Redline S, *et al.* Sleep-disordered breathing and cardiovascular disease cross-sectional results of the Sleep Heart Health Study. *Am J Respir Crit Care Med* 2001; 163: 19–25.
3 Nachtmann A, Stang A, Wang Y-M, Wondzinski E, Thilmann A. Association of obstructive sleep apnea and stenotic artery disease in ischemic stroke patients. *Atherosclerosis* 2003; 169: 301–307.
4 Malhotra A, White DP. Obstructive sleep apnoea. *Lancet* 2002; 360: 237–245.
5 Hansson GK. Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001; 21: 1876–1890.
6 Ludewig B, Zinkernagel RM, Hengartner H. Arterial inflammation and atherosclerosis. *Trends Cardiovasc Med* 2002; 12: 154–159.
7 Song L, Leung C, Schindler C. Lymphocytes are important in early atherosclerosis. *J Clin Invest* 2001; 108: 251–259.
8 Zhou X, Hansson GK. Immunomodulation and vaccination for atherosclerosis. *Expert Opin Biol Ther* 2004; 4: 599–612.
9 Sherer Y, Shoenfeld Y. Immunomodulation for treatment and prevention of atherosclerosis. *Autoimmun Rev* 2002; 1: 21–27.
10 Lavie L, Vishnevsky A, Lavie P. Evidence for lipid peroxidation in obstructive sleep apnea. *Sleep* 2004; 27: 123–128.
11 Lavie L. Obstructive sleep apnoea syndrome: an oxidative stress disorder. *Sleep Med Rev* 2003; 7: 35–51.
12 Dyugovskaya L, Lavie P, Lavie L. Increased adhesion molecules expression and production of reactive oxygen species in leukocytes of sleep apnea patients. *Am J Respir Crit Care Med* 2002; 165: 934–939.
13 Dyugovskaya L, Lavie P, Lavie L. Phenotypic and functional characterization of blood gammadelta T cells in sleep apnea. *Am J Respir Crit Care Med* 2003; 168: 242–249.
14 Mingari MC, Ponte M, Vitale C, Bellomo R, Moretta L. Expression of HLA class I-specific inhibitory receptors in human cytolytic T lymphocytes: a regulated mechanism that controls T-cell activation and function. *Hum Immunol* 2000; 61: 44–50.
15 Moretta L, Romagnani C, Pietra G, Moretta A, Mingari MC. NK-CTLs, a novel HLA-E-restricted T-cell subset. *Trends Immunol* 2003; 24: 136–143.
16 Satoh M, Seki S, Hashimoto W, *et al.* Cytotoxic gamma/delta or alpha/beta T-cells with a natural killer cell marker, CD56, induced from human peripheral blood lymphocytes by a combination of IL-12 and IL-2. *J Immunol* 1996; 157: 3886–3892.
17 Werwitzke S, Tiede A, Drescher BE, Schmidt RE, Witte T. CD8beta/CD28 expression defines functionally distinct populations of peripheral blood T lymphocytes. *Clin Exp Immunol* 2003; 133: 334–343.
18 Pittet NJ, Speiser DE, Valmori D, Cerottini JC, Romero P. Cutting edge: cytolytic effector function in human circulating CD8+ T-cells closely correlates with CD56 surface expression. *J Immunol* 2000; 164: 1148–1152.
19 Zamai L, Mariani AR, Zauli G, *et al.* Kinetics of *in vitro* natural killer activity against K562 cells as detected by flow cytometry. *Cytometry* 1998; 32: 280–285.
20 Jacobs R, Hintzen G, Kemper A, *et al.* CD56bright cells differ in their KIR receptoire and cytotoxic features from CD56bright NK cells. *Eur J Immunol* 2001; 31: 3221–3227.
21 Lima M, Teixeira MA, Queiros ML, *et al.* Immunophenotypic characterization of normal blood CD56+lo *versus* CD56hi NK-cell subsets and its impact on the understanding of their tissue distribution and functional properties. *Blood Cells Mol Dis* 2001; 27: 731–743.

22 Cooper MA, Fehniger TA, Turner SC, *et al.* Human natural killer cells: a unique innate immunoregulatory role for the CD56 (bright) subset. *Blood* 2001; 97: 3146–3151.

23 Tayebi H, Kuttler F, Saas P, *et al.* Effect of granulocyte colony-stimulating factor mobilization on phenotypical and functional properties of immune cells. *Exp Hematol* 2001; 29: 458–470.

24 Campbell JJ, Qin S, Unutmaz D, *et al.* Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* 2001; 166: 6477–6482.

25 Frey M, Packianathan NB, Fehniger TA, *et al.* Differential expression and function of L-selectin on CD56bright and CD56dim natural killer subsets. *J Immunol* 1998; 161: 400–408.

26 Solana R, Mariani E. NK and NK/T cells in human senescence. *Vaccine* 2000; 18: 1613–1620.

27 Nakajima H, Moore YI, Takiguchi M. Functional expression of the NKB1 killer cell inhibitory receptors on a gamma/delta CTL clones. *Immunogenetics* 1997; 45: 226–228.

28 Caldwell CC, Kojima H, Lukashev D, *et al.* Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol* 2001; 167: 6140–6149.

29 Peled N, Greenberg A, Pillar G, Zinder O, Levi N, Lavie P. Contributions of hypoxia and respiratory disturbance index to sympathetic activation and blood pressure in obstructive sleep apnea syndrome. *Am J Hypertens* 1998; 11: 1284–1289.

30 Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve: an integrative interference between two supersystems: the brain and the immune system. *Pharmacol Rev* 2000; 52: 595–638.

31 Hellstrand K, Hermodsson S. An immunopharmacological analysis of adrenaline-induced suppression of human natural killer cytotoxicity. *Int Arch Allergy Appl Immunol* 1989; 89: 334–341.

32 Irwin M. Effects of sleep loss on immunity. *Brain Behav Immun* 2002; 16: 503–512.

33 Irwin M, Clark C, Kennedy B, Gillin GC, Ziegler M. Nocturnal catecholamines and immune function in insomniacs, depressed patients and control subjects. *Brain Behav Immun* 2003; 17: 365–372.