Monocytes inhibit NK activity *via* TGF-β in patients with obstructive sleep apnoea

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ABSTRACT Obstructive sleep apnoea (OSA) is associated with cancer incidence and mortality. The contribution of the immune system appears to be crucial; however, the potential role of monocytes and natural killer (NK) cells remains unclear.

Quantitative reverse transcriptase PCR, flow cytometry and *in vitro* assays were used to analyse the phenotype and immune response activity in 92 patients with OSA (60 recently diagnosed untreated patients and 32 patients after 6 months of treatment with continuous positive airway pressure (CPAP)) and 29 healthy volunteers (HV).

We determined that monocytes in patients with OSA exhibit an immunosuppressive phenotype, including surface expression of glycoprotein-A repetitions predominant protein (GARP) and transforming growth factor-β (TGF-β), in contrast to those from the HV and CPAP groups. High levels of TGF-β were detected in OSA sera. TGF-β release by GARP+ monocytes impaired NK cytotoxicity and maturation. This altered phenotype correlated with the hypoxic severity clinical score (CT90). Reoxygenation eventually restored the altered phenotypes and cytotoxicity.

This study demonstrates that GARP+ monocytes from untreated patients with OSA have an NK-suppressing role through their release of TGF-β. Our findings show that monocyte plasticity immunomodulates NK activity in this pathology, suggesting a potential role in cancer incidence.

Introduction

The intermittent hypoxia that characterises obstructive sleep apnoea (OSA) has been identified as an important determinant of cancer incidence and mortality in humans [1–6] and in animal models [7–10]. The immune system could play a crucial role in the association between OSA and cancer [7, 11–13]. Regulatory T-cell lymphocytes (Tregs) and alternatively activated macrophages are critically important players in this immunosuppressive status [14]. In fact, macrophages from mice exposed *in vivo* to intermittent hypoxia (IH) exhibit a patent polarisation towards an immunosuppressive phenotype in the surrounding tissue that favours tumour aggressiveness [7, 15].

Although the effect of hypoxia on tumour immune escape remains poorly understood, substantial evidence suggests that hypoxia might affect the phenotype and activity of monocytes and natural killer (NK) cells [16], which are crucial for driving a potent immune response. In turn, oxygen plays a critical role in NK cell development [17], modulating important transcription factors (RAR-related orphan receptor C (RORC), T-Box protein 21 (Tbet) and eomesodermin (EOMES)) involved in the late stages of NK cell maturation [18]. It has also been reported that transforming growth factor β (TGF-β) modulates NK differentiation and cytotoxicity *via* the SMAD3 pathway [19, 20]. Activation of this pathway is mediated by the release of TGF-β from its inactive form, TGF-β-latency-associated-peptide (TGF-β-LAP); in this context, glycoprotein-A repetitions predominant protein (GARP) plays an important role in regulating bioavailability and provides a cell surface platform for TGF-β activation [21]. Interestingly, we have previously reported that hypoxia-inducible factor-1α (HIF-1α) regulates the expression of TGF-β in human monocytes, enhancing the immunosuppression phenotype [22].

We therefore hypothesised that patients with OSA would have an altered phenotype and altered activity of circulating monocytes and NK cells, promoting the immune escape that might eventually favour an increased incidence of cancer.

Herein, we report that monocytes isolated from patients with OSA assume the Treg role, downregulating the NK cytotoxic subset in a TGF-β-dependent manner. We have identified the correlation between hypoxic severity and impaired immune response in this pathology, as well as determining recovery of the appropriate phenotype and immune activity after reoxygenation.

Materials and methods

For in-depth details on the materials and methods, please refer to the supplementary material.

Study participants

Patients with OSA were prospectively recruited from the sleep unit of La Paz-Cantoblanco University Hospital, Madrid, Spain. Patients aged 40–65 years with an apnoea–hypopnoea index (AHI) ⩾30 were included in the study.

According to previous treatment with continuous positive airway pressure (CPAP), patients with OSA were classified as being CPAP-naïve (the untreated group, OSA) or having been treated for at least 6 months (the treated group, CPAP) with a mean daily use of more than 4.5 h per day, as measured with a run-time counter. In the CPAP group, optimal CPAP pressure was titrated by an auto CPAP device (REMstar Pro M Series with C-Flex, Philips Respironics, Pittsburgh, PA, USA) and verified by repeated respiratory polygraphy at the time of inclusion in the study. As a control group, healthy volunteers (HV), homogeneous in sex, age, smoking habits and body mass index, were selected. None of these volunteers were being treated with any type of medication, and the presence of OSA was ruled out by respiratory polygraphy.

The study was approved by the local ethics committee (PI-1857), and informed consent was obtained from all the participants.

Cell culture

Monocytes and NK cells from the HV group were cultured either under normoxic conditions (21% O_2, 5% CO_2, 37°C) or using an *in vitro* model of IH. In the IH protocol, cells were exposed in a hypoxic chamber (3% O_2, 5% CO_2, 37°C) for 12 cycles and changed from the preconditioned hypoxic medium (5 min) to a normoxic medium (10 min), as previously described by RYAN *et al.* [23]. The use of a preconditioned medium was necessary to allow instantaneous exposure to hypoxia. Intracellular oxygen levels were determined by the ability of oxygen to quench the excited state of the oxygen-sensitive probe from the intracellular oxygen concentration assay (Ab197245; Abcam, Cambridge, UK). The fluorescence lifetime of the oxygen-sensitive probe diminished after exposure to the hypoxic medium and increased under normoxic conditions.

To assess the effect of restoring adequate oxygenation to the innate immune system, peripheral blood mononuclear cells (PBMCs) from the more hypoxemic untreated patients with OSA were cultured for 16 h in standard cell culture conditions (normoxia, incubated at 37°C in a humidified tissue culture incubator, 5% CO_2).

Biomarker expression by quantitative PCR

Total RNA was purified from PBMCs and monocytes were isolated using the High Pure RNA Isolation Kit from Roche Diagnostics (Basel, Switzerland). Gene expression levels were analysed by reverse transcription of 1 mg RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster, CA, USA), then quantitative PCR (qPCR; Light-Cycler, Roche Diagnostics), using specific primers. In the CD14$^+$ monocytes, HIF-1α, TGF-β1, vascular endothelial growth factor (VEGF) and interleukin (IL)-10 were used as M2 markers, whereas IL-12p40 served as the M1 marker [24]. The relative expression was based on the levels of target genes compared with the housekeeping gene (β-Actin).

Flow cytometry

The PBMCs were stained after isolation with the indicated antibodies (CD3, CD14, TGF-β-LAP, CD56, CD16, CD4, CD25, CD8, CD28, P30, P44, P46, p-SMAD2/3 and CD117) from BD Bioscience (Grenoble, France). For the intracellular staining, the cells were labelled with forkhead box P3 (FOXP3), Tbet, RORC, EOMES and perforin (all from BD Bioscience), following a standard protocol using the Transcription Factor Buffer Set (BD Bioscience). Appropriate isotype controls were used for each experiment. The

TABLE 1 Baseline characteristics of the study participants

	OSA group	CPAP group (before treatment)[#]	Healthy volunteers	p-value[¶]
Subjects n	60	32	29	
Age years	54±8	55±7	55±6	0.865
Male n (%)	50 (83.3)	26 (81.2)	24 (82.5)	0.969
Body mass index kg·m^{-2}	28.5±4.6	27.4±4.8	27.4±5.0	0.463
Smoking habit n (%)				0.987
Current smoker	21 (35.0)	12 (37.5)	10 (34.5)	
Former smoker	21 (35.0)	12 (37.5)	10 (34.5)	
Never smoker	18 (30.0)	8 (25.0)	9 (31.0)	
ESS	10.8±4.5	10.7±4.4	4.1±3.6	<0.001
Daytime sleepiness (ESS ⩾10)	33 (55.0)	20 (62.5)	1 (3.4)	<0.001
AHI events·h^{-1}	55.1±18.2	54.3±17.9	2.7±1.2	<0.001
Oxygen desaturation index events·h^{-1}	49.6±22.3	49.5±19.9	1.9±1.1	<0.001
Sleep time with S_{pO_2} <90% %	33.1±29.0	31.8±29.1	2.3±2.8	0.004
Sleep time with S_{pO_2} <80% %	5.6±9.6	6.1±8.5	0	0.117
Low nocturnal S_{pO_2} %	77.6±6.9	77.2±7.0	87.6±2.3	<0.001
Mean nocturnal S_{pO_2} %	89.9±4.0	89.3±4.3	92.3±1.5	0.102
Obstructive events %	88.7±6.6	87.2±6.4	86.0±6.4	0.343
Systolic blood pressure mmHg	121.6±10.5	123.0±10.2	122.8±11.5	0.807
Diastolic blood pressure mmHg	74.6±6.4	75.6±6.5	75.7±6.5	0.693
White cell count mm^{-3}				
Total	7.60±1.27	7.63±1.25	7.77±1.33	0.834
Neutrophils	5.02±0.95	4.89±0.91	5.03±1.04	0.785
Lymphocytes	1.93±0.56	2.10±0.63	2.09±0.61	0.314
Monocytes	0.50±0.14	0.51±0.13	0.48±0.09	0.690
Haemoglobin g·dL^{-1}	13.6±1.2	13.7±1.3	14.0±1.2	0.392
Cholesterol total mg·dL^{-1}	186.6±41.9	182.6±52.7	183.9±40.4	0.938
HDL-cholesterol mg·dL^{-1}	52.5±7.0	50.6±5.8	49.0±4.4	0.089
LDL-cholesterol mg·dL^{-1}	122.7±36.9	121.5±47.9	122.6±35.7	0.992
Triglycerides mg·dL^{-1}	142.9±40.8	138.4±46.2	136.1±18.4	0.789

Data are presented as mean±SD, unless otherwise stated. OSA: obstructive sleep apnoea; CPAP: continuous positive airway pressure; ESS: Epworth Sleepiness Scale; AHI: apnoea-hypopnea index; S_{pO_2}: arterial oxygen saturation measured by pulse oximetry; HDL: high-density lipoprotein; LDL: low-density lipoprotein. [#]: baseline values of the treated patients with OSA refer to the diagnostic time, before starting treatment with CPAP; [¶]: comparisons between groups were performed by an ANOVA or Chi-squared test.

antibodies' details are included in supplementary table E1. After staining, the cells were acquired using a BD FACSCalibur flow cytometer, and the collected data were analysed using FlowJo v10.

Statistical analysis

Unless otherwise indicated, data are presented as mean±SEM. Comparisons between groups were performed using analysis of variance with the Kruskal–Wallis test, with Dunn's multiple comparison for the non-normally distributed variables. For the *in vivo* and *in vitro* studies, statistical significance was calculated using the Wilcoxon test or a paired t-test where appropriate. The correlations were assessed with Spearman's rank correlation for non-normally distributed data. The differences were considered significant at $p < 0.05$ and the analyses were conducted using Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Patient demographics

In total, 92 patients with severe OSA were included, 60 who had been recently diagnosed and were CPAP-naïve (OSA group) and 32 who had been treated with CPAP for >6 months (CPAP group); 29 HVs were also included as controls. Table 1 shows the baseline anthropometric and clinical data of the three groups. At the time of their diagnoses, patients in the OSA and CPAP groups showed similar sleep characteristics. However, at the time of study inclusion, i.e. after the CPAP group had undergone >6 months of treatment, patients in this group showed recovered sleep characteristics (supplementary table E2).

Obstructive sleep apnoea monocytes are polarised towards an immunosuppressive phenotype expressing GARP and TGF-β

Analysis of biomarker expression in isolated CD14$^+$ monocytes from the three study groups showed that OSA monocytes are polarised towards an immunosuppressive phenotype. We found high mRNA levels of HIF-1α, TGF-β1, VEGF and IL-10, and decreased levels of IL-12p40 in the OSA group compared with the HV or the CPAP group (figure 1a).

In addition, our analysis revealed that TGF-β1 protein levels were significantly elevated in the OSA sera compared with that from the HV and CPAP groups; this expression correlated with a night-time arterial oxygen saturation measured by pulse oximetry (S_{pO_2}) of <90% (CT90) (figure 1b, r=0.7109). Although TGF-β is an inducer of the Treg population [25], patients with OSA exhibited a significant reduction in their Treg subset (supplementary figure E1a). We studied the expression of the TGF-β-related proteins, GARP and TGF-β-LAP, which play an important role in the release of active TGF-β. Both GARP and TGF-β-LAP were detected on monocytes from the patients with OSA, but not on monocytes from the HV and the CPAP groups (figure 1c, d); however, TGF-β-LAP was not detected on Treg cell surfaces (supplementary figure E1b).

Obstructive sleep apnoea NK cells exhibit an immature subset and impaired cytotoxic activity

The immunosuppressive role of TGF-β in NK cells is well known [26–30]. Although no differences were detected in the total number of NK cells (CD3$^-$CD56$^+$) among the three study groups (supplementary figure E2a), an analysis of the NK phenotype indicated a patent reduction in the cytotoxic subset (CD3$^-$CD56^{dim}CD16$^+$) in the OSA group compared with the HV and the CPAP group (figure 2a, b). Curiously, we observed a new NK subset (CD3$^-$CD56^{dim}CD16$^-$) only in the patients with OSA (see OSA dot blot, figure 2a, b). Moreover, we found significantly increased levels of p-SMAD2/3 (figure 2c), indicating activation of the TGF-β pathway in this new NK subset. NK cytotoxic ability was patently impaired in the patients with OSA compared with the CPAP-treated patients and the HV (figure 2d). NK cytotoxicity correlated with the surface TGF-β-LAP on monocytes (figure 2e, r = −0.7757), indicating their immunosuppressive role. The expression of natural cytotoxic receptors, including p30, p44 and p46, was significantly decreased in the CD3$^-$CD56^{dim}CD16$^-$ subset compared with the cytotoxic (CD3$^-$CD56^{dim}CD16$^+$) NK subset (figure 3a–c). Reduced perforin expression was also observed in the CD3$^-$CD56^{dim}CD16$^-$ subset (figure 3d).

The phenotype of the NK cells was analysed using the cell surface marker CD117, which is implicated in the intermediate stage of differentiation of NK cells [18], and a set of transcription factors (RORC, Tbet and EOMES) related to NK differentiation. The surface expression of CD117 was significantly decreased in the CD3$^-$CD56^{dim}CD16$^-$ NK subset (figure 3e). Whereas Tbet and EOMES are associated with a mature NK phenotype, RORC defines an immature NK status [31]. The CD3$^-$CD56^{dim}CD16$^-$ subset exhibited an immature phenotype supported by the upregulation of RORC and the inhibition of both Tbet and EOMES (figure 3f–h). Other T-cell subsets with reported cytotoxic activity were also analysed, including CD3$^+$CD56$^+$, CD3$^+$CD8$^+$, CD3$^+$CD28$^+$ and CD3$^+$γδ$^+$ (supplementary figure E2b–e). Only the CD3$^+$γδ$^+$ subpopulation exhibited a significant increase in patients with OSA. The rate of each cell subset obtained
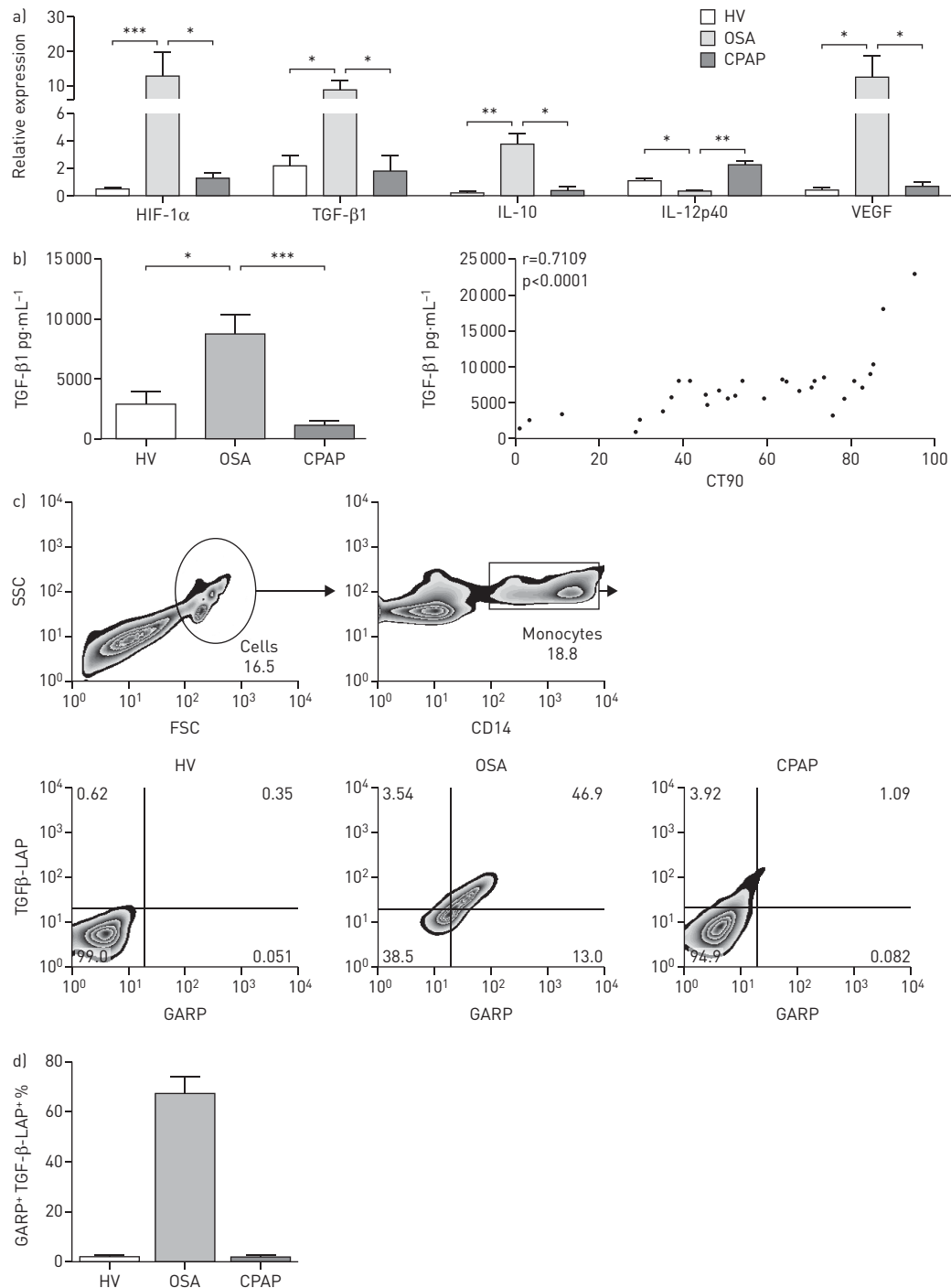
FIGURE 1 Monocytes from patients with obstructive sleep apnoea (OSA) exhibit an immunosuppressive phenotype, expressing TGF-β and GARP. CD14^{+} monocytes were isolated from healthy volunteers (HV) (n=20 randomly selected), patients with OSA (n=20 randomly selected) and patients treated with continuous positive airway pressure (CPAP) (n=20 randomly selected). a) Expression analysis by quantitative PCR of HIF-1α, TGF-β1, IL-10, IL-12p40 and VEGF mRNA in CD14^{+} monocytes. Relative expression is shown. b) TGF-β1 protein levels in sera were analysed from HV (n=29), patients with OSA (n=30 randomly selected) and patients treated with CPAP (n=32). TGF-β1 protein levels in sera from patients with OSA correlated with CT90. Spearman correlation coefficients (r and p-value) are shown. c) Gating strategy for the detection of monocytes. Numbers adjacent to outlined areas indicate the percentage of cells and monocytes. Representative dot blots of TGF-β-LAP and GARP expression on CD14^{+} cells are shown. Peripheral blood mononuclear cells from HV (n=29), patients with OSA (n=20 randomly selected) and patients treated with CPAP (n=20 randomly selected) were analysed by flow cytometry. d) The percentage distribution of GARP^{+}TGF-β-LAP^{+} on total gated CD14^{+} cells is shown. *: p<0.05; **: p<0.01; ***: p<0.001 for OSA using the Kruskal–Wallis test with Dunn's multiple comparison.
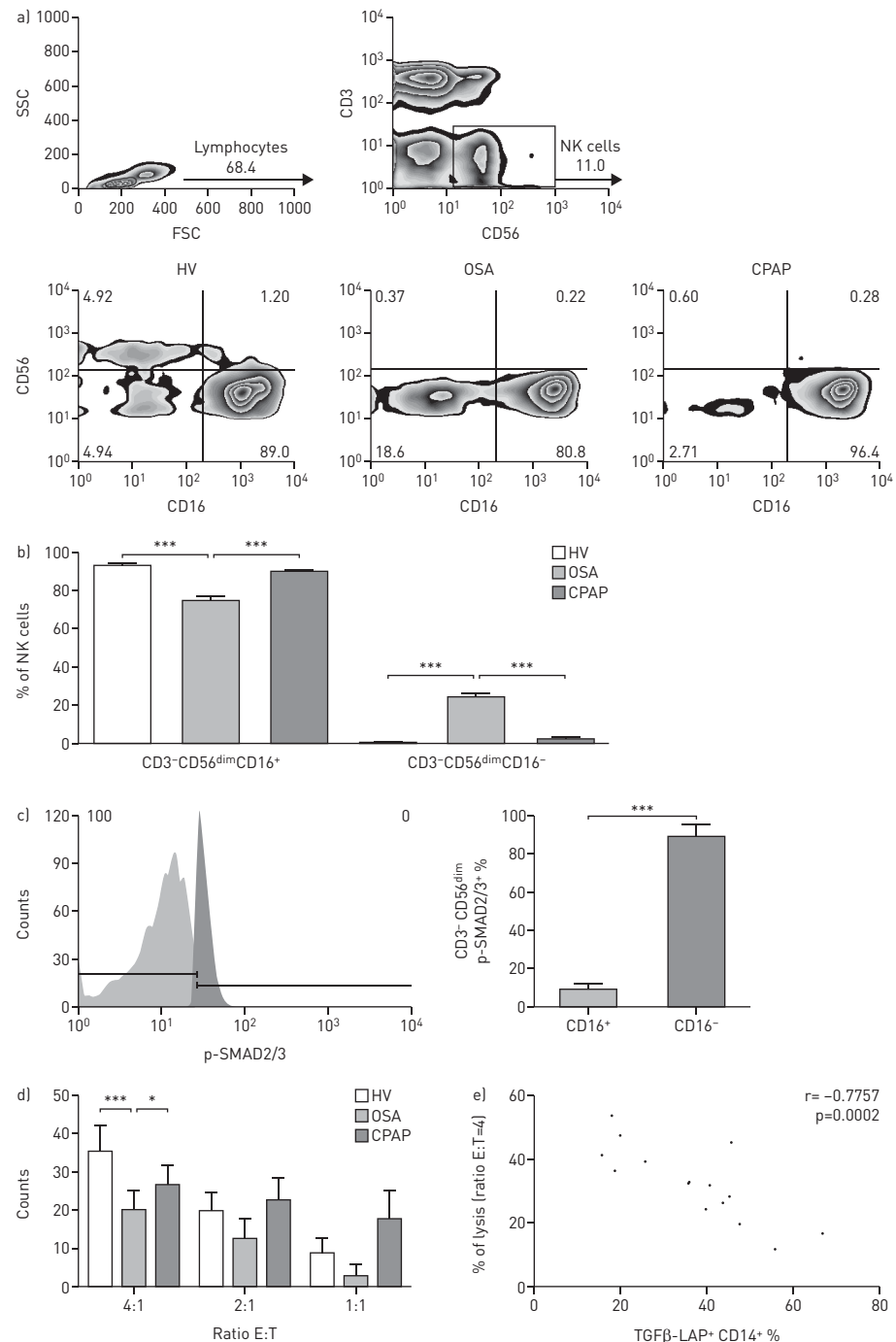
FIGURE 2 The NK subset from patients with obstructive sleep apnoea (OSA) shows that CD16 is downregulated, p-SMAD2/3 upregulated and that impaired cytotoxicity correlates with TGF-β-LAP on monocytes. Peripheral blood mononuclear cells from healthy volunteers (HV) (n=29), patients with OSA (n=20 randomly selected) and patients treated with continuous positive airway pressure (CPAP) (n=20 randomly selected) were analysed by flow cytometry. a) Gating strategy for the detection of NK cells. Numbers adjacent to outlined areas indicate the percentage of lymphocytes and NK cells. Representative dot blots of CD56 and CD16 expression on NK cells are shown. b) The percentage distribution of CD3⁻CD56^{dim}CD16⁺ and CD3⁻CD56^{dim}CD16⁻ on total gated NK cells is shown. ***: p<0.001, using the Kruskal–Wallis test with Dunn's multiple comparison. c) The percentage distribution of CD3⁻CD56^{dim}p-SMAD2/3⁺ on CD3⁻CD56^{dim}CD16⁺ (light grey bars) and CD3⁻CD56^{dim}CD16⁻ (dark grey bars) from patients with OSA (n=20 randomly selected). A standard histogram is shown. ***: p<0.001, using a paired t-test. d) Cytotoxic activity by europium–TDA release assay after 2 h of incubation of NK (E) with K562 as target cells (T) at different ratios. Percentages of lysis are shown. *: p<0.05; ***: p<0.001, using the Kruskal–Wallis test with Dunn's multiple comparison. e) Isolated peripheral blood mononuclear cells from patients with OSA (n=16 randomly selected) were analysed by flow cytometry. NK cytotoxic activity (% of lysis, ratio E:T=4) correlated with mean intensity of fluorescence of TGF-β-LAP on total gated CD14⁺ cells. Spearman's correlation coefficient (r and p-value) is shown.
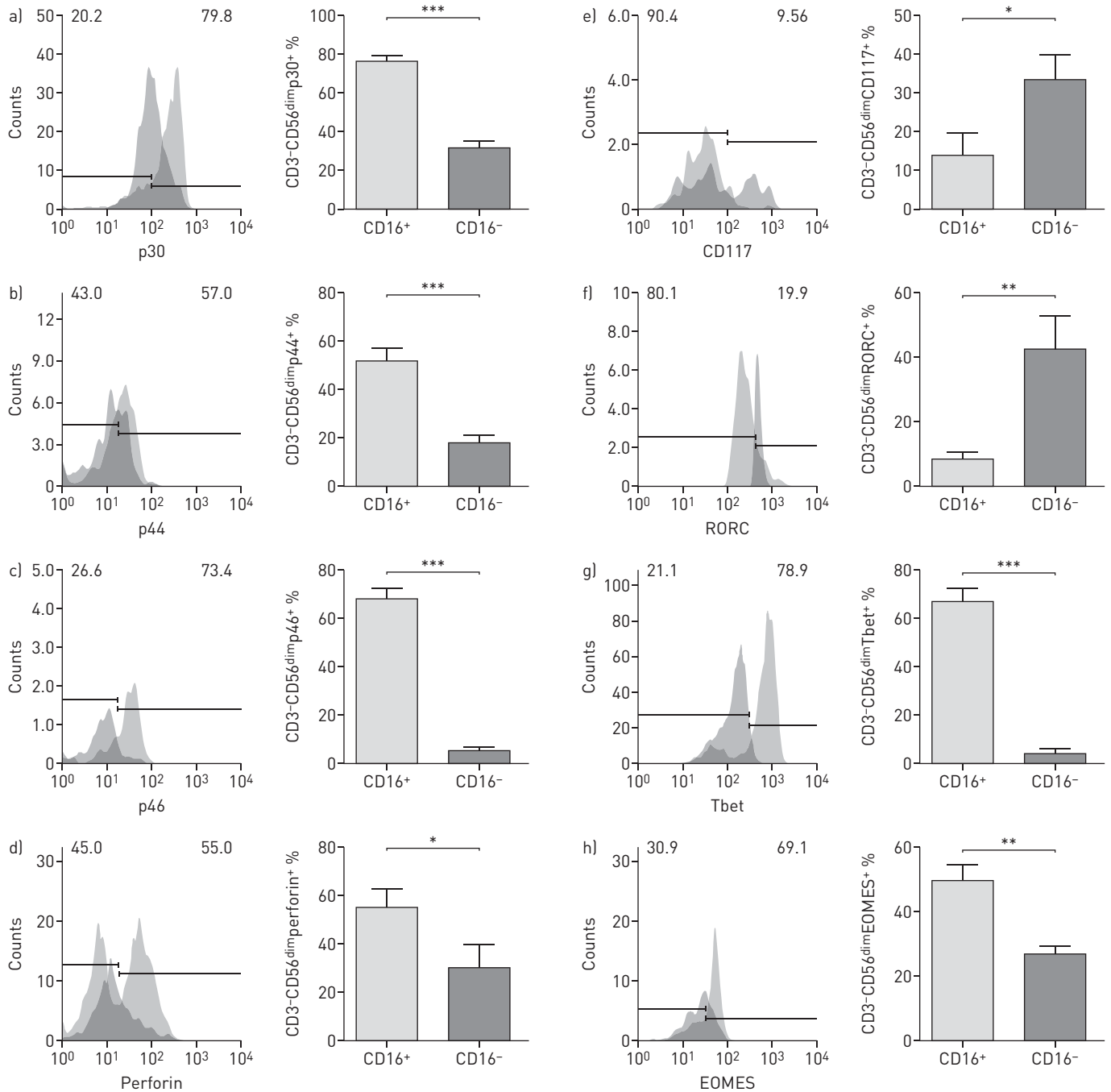
FIGURE 3 The CD3⁻CD56^dim CD16⁻ NK subset exhibits an immature state. CD3⁻CD56^dimCD16⁺ (light grey bars) and CD3⁻CD56^dimCD16⁻ (dark grey bars) from patients with obstructive sleep apnoea (OSA) (n=20 randomly selected) were analysed by flow cytometry. For each, the percentage distribution and a standard histogram are shown. a) CD3⁻CD56^dimp30⁺; b) CD3⁻CD56^dimp44⁺; c) CD3⁻CD56^dimp46⁺; d) CD3⁻CD56^dimPerforin; e) CD3⁻CD56^dimCD117⁺; f) CD3⁻CD56^dimRORC⁺; g) CD3⁻CD56^dimTbet⁺; h) CD3⁻CD56^dimEOMES⁺. *: p<0.05, **: p<0.01; ***: p<0.001, using a paired t-test.

in the HV and in the CPAP-treated patients matched the reported value for their age range distribution [32, 33].

GARP⁺ monocytes regulate the NK cytotoxicity subset

In order to evaluate the implication of the GARP⁺ monocyte in NK cytotoxicity, we performed an *in vitro* model of IH by culturing isolated CD14⁺ monocytes from HV in preconditioned hypoxic medium followed by preconditioned normoxic medium. This model resulted in cyclic fluctuations of intracellular oxygen, mimicking the IH episodes (supplementary figure E3a). The analysis of HIF-1α, TGF-β1, VEGF, IL-10 and IL-12p40 corroborated the switch to an immunosuppressive phenotype (supplementary figure
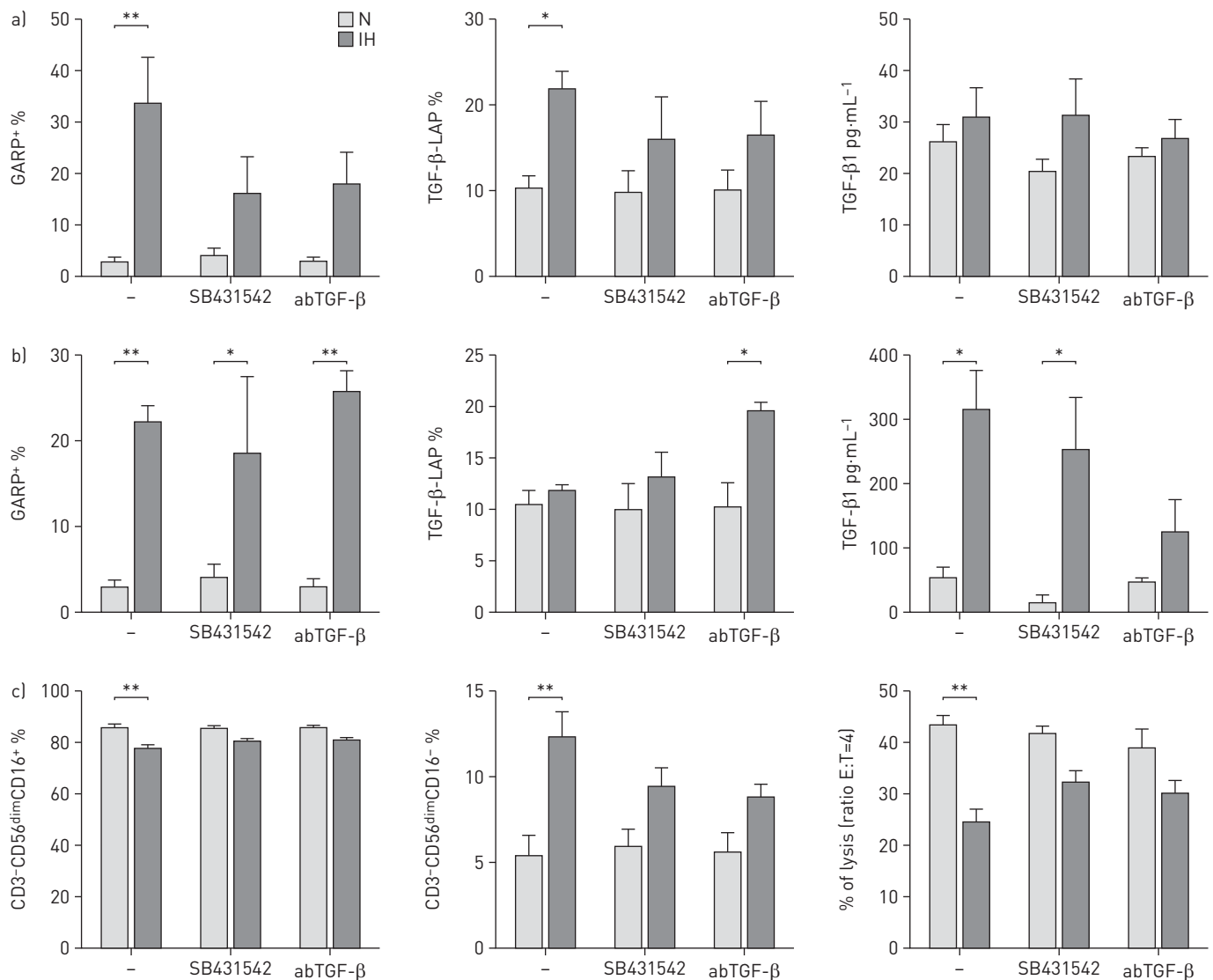
FIGURE 4 GARP+ monocytes release TGF-β1, modulating the CD3−CD56dimCD16+ NK subset. CD14+ monocytes isolated from healthy volunteers were cultured under normoxic conditions (N, n=7) or intermittent hypoxia (IH, n=7) conditions. The media were supplemented with a TGF-β receptor inhibitor (SB431542, 10 μM) or TGF-β1 antibody (abTGF-β, 50 ng·mL−1). a) Percentage distributions of GARP+ and TGF-β-LAP+ on CD14+ cells are shown; TGF-β1 protein levels from cultured media were analysed after 2 h. b) Isolated homologous normoxic NK cells were then added at a ratio of 1:1 for 2 h of incubation. Percentage distributions of GARP+ and TGF-β-LAP on total gated CD14+ cells are shown; TGF-β1 protein levels from cultured media were analysed after 2 h. c) Percentage distributions of CD3−CD56dimCD16+ and CD3−CD56dimCD16− on total gated NK cells are shown. Cytotoxic activity by europium-TDA release assay after 2 h of incubation with K562 as target cells. Percentage of lysis at ratio E:T=4 is shown. *: p<0.05; **: p<0.01; using a paired t-test.

E3b). Remarkably, the HIF-1α knockdown reverted the observed phenotype under IH (supplementary figure E3c). The percentage of GARP+ monocytes increased after IH (figure 4a, left panel); however, this effect was slightly mitigated by the TGF-β receptor inhibitor (SB431542) and the TGF-β1 antibody (abTGF-β1). The surface expression of TGF-β-LAP on monocytes showed a similar pattern (figure 4a, central panel). Interestingly, we did not observe TGF-β1 active protein in the cell supernatant after 2 h (figure 4a, right panel). To elucidate the interaction between monocytes and NK cells, we co-cultured isolated CD14+ HV monocytes under IH conditions with isolated homologous normoxic NK cells at a ratio of 1:1 for 2 h. We observed that the distribution of GARP+ monocytes increased under IH in the presence or not of SB431542 and abTGF-β1 (figure 4b, left panel). The surface expression of TGF-β-LAP on monocytes under IH diminished in the co-culture, except in abTGF-β1 conditions (figure 4b, central panel). The soluble TGF-β1 active form was detected only in the presence of both IH monocytes and normoxic NK cells, except in abTGF-β1 conditions (compare figure 4a, right panel, with figure 4b, right panel). Furthermore, a significant reduction in the CD3−CD56dimCD16+ NK subset and an increase in the
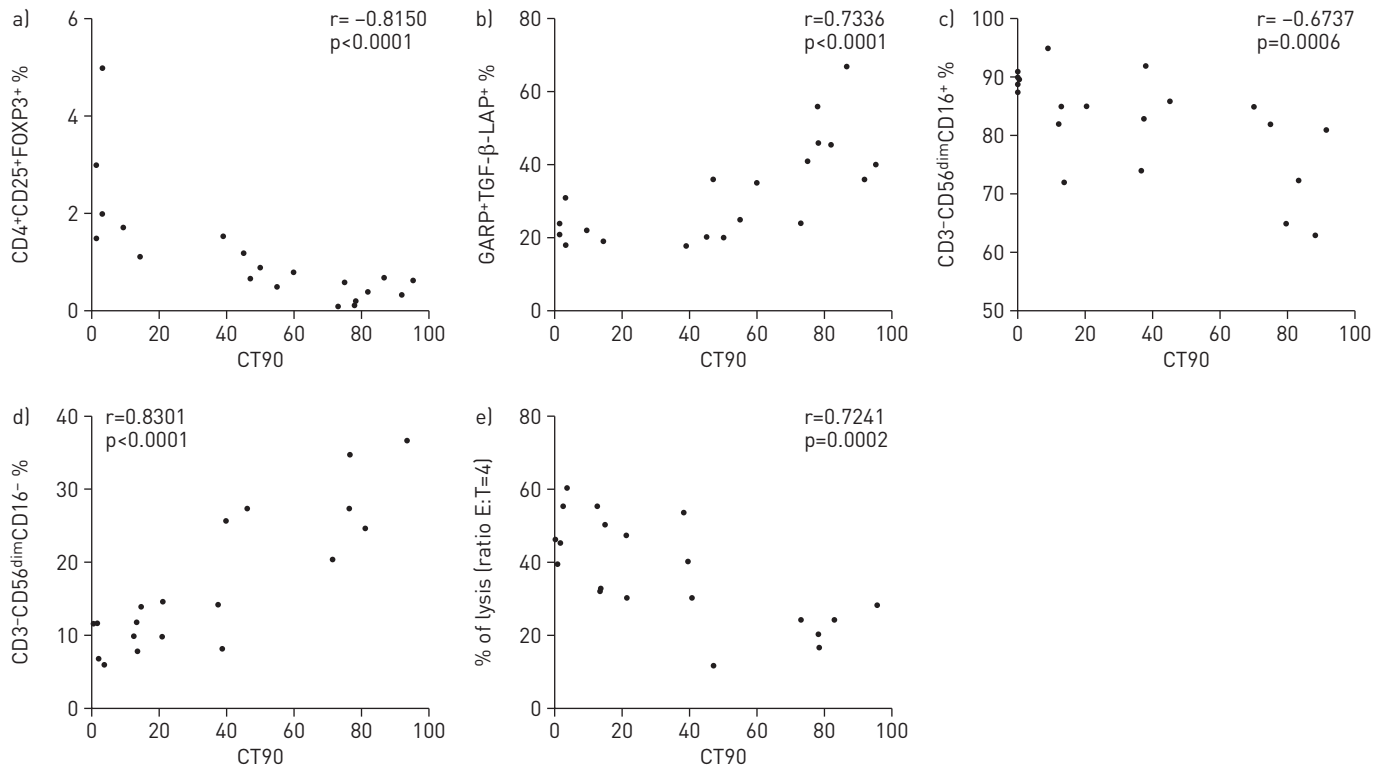
FIGURE 5 Night-time arterial oxygen saturation measured by pulse oximetry (S_{pO_2}) <90% (CT90) correlates with Treg distribution, monocytes, NK phenotypes and cytotoxicity. Isolated peripheral blood mononuclear cells from patients with obstructive sleep apnoea (n=20 randomly selected) were analysed by flow cytometry. Correlations with CT90 of a) percentage distribution of CD4⁺CD25⁺FOXP3⁺ on total gated T-lymphocytes; b) percentage distribution of GARP⁺ and TGF-β-LAP⁺ on CD14⁺ cells; c) percentage distribution of CD3⁻CD56^dimCD16⁺ on gated NK cells; d) percentage distribution of CD3⁻CD56^dimCD16⁻ on gated NK; and e) NK cytotoxic activity (% of lysis, ratio E:T=4). Spearman's correlation coefficients (r and p-value) are shown.

CD3⁻CD56^dimCD16⁻ NK subset were observed (figure 4c, left and central panels). These data are in agreement with the reduced lysis activity observed (figure 4c, right panel). This immunosuppressive effect was mitigated by SB431542 and abTGF-β1 conditions (figure 4c).

Hypoxic severity correlates with the altered obstructive sleep apnoea phenotype and NK cytotoxicity, whereas oxygenation restores the immunosuppressive phenotype

The altered distribution and phenotype of Tregs, monocytes and NK subsets in patients with OSA correlated with hypoxic severity as assessed by night-time S_{pO_2}<90% (CT90) (figure 5). In addition, isolated cells from the most hypoxemic patients with OSA recovered a healthy phenotype and cytotoxic ability after 16 h in normoxic conditions (figure 6). In line with these findings, supplementary figure E4 shows the phenotypes from five patients with OSA analysed before and after 6 months of CPAP treatment, suggesting that CPAP treatment restores the impaired immune phenotype.

DISCUSSION

Our findings demonstrate that patients with OSA have altered immune cell phenotypes, including monocytes and NK cells. These impaired phenotypes were not observed in patients with OSA who had received >6 months of CPAP treatment, indicating the role of oxygenation in immune plasticity. According to our findings, OSA modulates the phenotype of circulating monocytes towards an immunosuppressive state (high levels of HIF-1α, TGF-β, IL-10 and VEGF and decreased levels of IL-12p40). We have previously reported that HIF-1α plays a crucial role in immunosuppressive reprogramming, increasing the expression of TGF-β and VEGF [22]. Moreover, the upregulation of IL-10 and the attenuation of IL-12p40 are crucial markers to classify the alternative phenotype in monocytes [24]. It has been reported that several cytokines and metabolites directly reprogramme the immune system, compromising cytotoxic ability [27, 34]. Among these, the cytokine TGF-β plays a major role in suppressing the immune response [35]. Curiously, sera from untreated OSA showed that increased levels of TGF-β correlated with the hypoxemic index CT90, supporting the association between hypoxic severity and immunosuppression. We note that hypoxia, inflammation and TGF-β are primary components of the
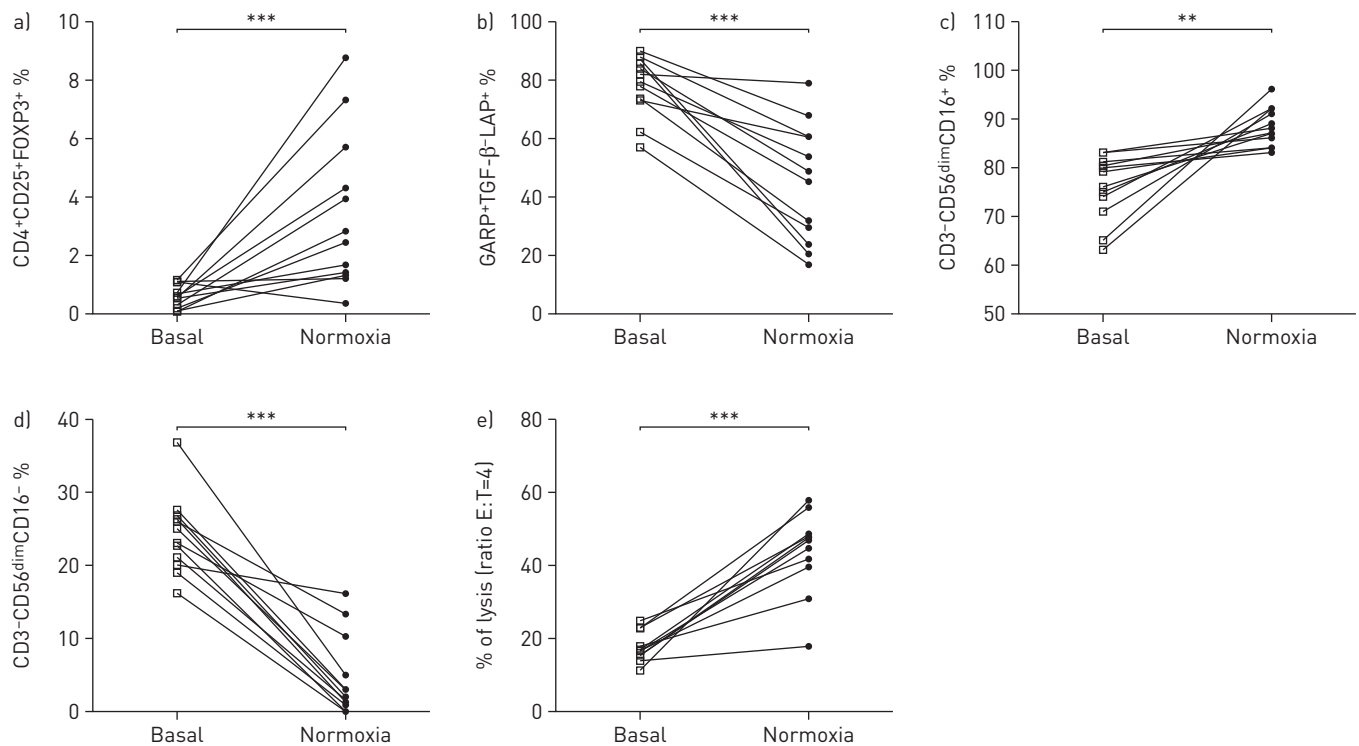
FIGURE 6 Normoxia reverts altered phenotypes in patients with obstructive sleep apnoea (OSA). Peripheral blood mononuclear cells isolated from more hypoxemic patients with OSA (CT90 >60%, n=12) were analysed in baseline conditions (open squares) and after culture for 16 h in normoxic conditions (dark spots). Shown are the percentage distributions of a) Tregs (CD4+CD25+FOXP3+) on total gated T-lymphocytes; b) GARP+TGF-β-LAP+ on total gated CD14+ cells; and c) CD3−CD56dimCD16+ and d) CD3−CD56dimCD16− on total gated NK cells. e) Cytotoxic activity by europium-TDA release assay after 2 h of incubation with K562 as target cells; percentages of lysis (ratio E:T=4) are shown. **: p<0.01; ***: p<0.001, using Wilcoxon test.

tumour microenvironment [36–38]. In this context, it is well accepted that patients with OSA have basal systemic inflammation and intermittent hypoxia; we have also reported high levels of TGF-β in circulation. Therefore, we propose a polarisation towards an immunosuppressive phenotype of OSA monocytes due to this particular environment.

Importantly, TGF-β diminishes the maturation, proliferation and functional activities of NK cells [39]. Interestingly, our patients with OSA exhibited a new NK subset (CD3−CD56dimCD16−) with increased levels of p-SMAD2/3, indicating activation of the TGF-β pathway [26]. The observed new NK subset was characterised by decreased natural cytotoxic receptors and maturation markers. NK functionality also showed reduced perforin levels with decreased cytotoxicity.

Other studies have demonstrated that Tregs reduce the cytotoxic NK subset *via* TGF-β, favouring the appearance of an immature phenotype [40]. However, our results and previously reported data [12] show that Tregs are significantly reduced in patients with OSA, probably due to the fact that hypoxia attenuates Treg development by binding FOXP3 and targeting it for proteosomal degradation [41]. We therefore studied the expression of TGF-β-related proteins (GARP and TGF-β-LAP) in other immune cells. In our patients with OSA, TGF-β-LAP was not found on Treg cell surfaces, whereas both GARP and TGF-β-LAP were detected on monocytes. GARP regulates the bioavailability of TGF-β, joining the LAP and releasing the TGF-β active form [21, 42]. Other authors have reported that GARP on the Treg surface captures TGF-β-LAP from its surroundings, resulting in local suppression of the anti-tumour immune response [42]. In line with these findings, we propose a novel central role for monocytes in the impairment of anti-tumour immunosurveillance in patients with OSA (figure 7). Our findings suggest that immunosuppressive monocytes, but not Tregs, express inactive TGF-β-LAP and promote the release of active TGF-β1, which regulates the NK phenotype and its activity. TGF-β has been reported to inhibit the anti-tumour activity of NK cells by repressing the mTOR pathway [26].

The immunosuppressive changes described for the Treg phenotype, monocytes and NK cells as well as the NK cytotoxicity in patients with OSA were correlated with CT90. These findings are consistent with previous studies which reported that CT90 was the only sleep parameter independently associated with increasing cancer incidence [4] and mortality [5] in patients with OSA. Remarkably, in these studies,
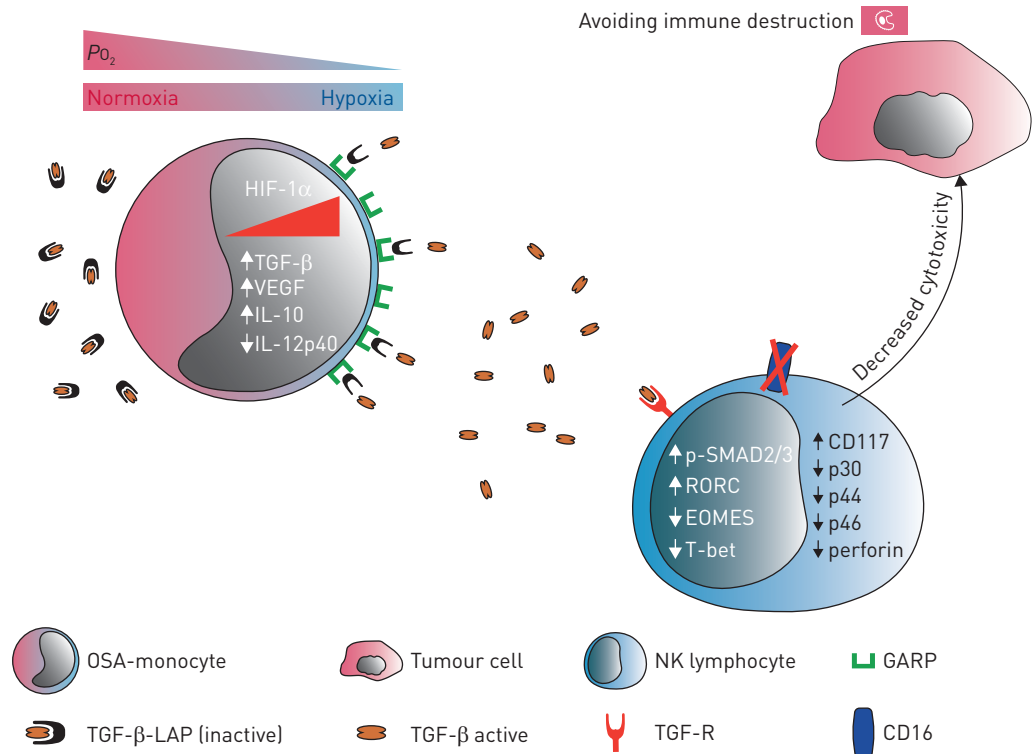
FIGURE 7 Schematic representation of the crosstalk between monocytes and NK cells in patients with obstructive sleep apnoea (OSA). The intermittent hypoxic environment in patients with OSA provokes a monocyte polarisation towards an immunosuppressive phenotype. In addition, OSA monocytes showed GARP and TGF-β-LAP surface expression, modulating the NK cytotoxicity thorough TGF-β activation. This NK modulation shows a reversion from the mature to the immature stage, affecting the cytotoxicity phenotype and lysis activity. Collectively, OSA conditions induce an immune suppression that might explain cancer incidence in patients with OSA. P_{O_2}: oxygen tension.

CT90 was a stronger predictor of cancer incidence or mortality than the AHI or the desaturation index [4, 5]. Thus, because patients with respiratory failure or those treated with long-term oxygen therapy were excluded from the present study to minimise the impact of chronic sustained hypoxia, our results appear to indicate that the effect of IH on the innate immune system depends more on the magnitude of the hypoxia than on the number of oxygenation-desaturation sequences.

Our study confirmed that the restoration of adequate oxygenation reverses the immune response impairment found in the context of OSA. These data are concordant with previous observations that suppression of hypoxia in a tumoural microenvironment reverses the immunosuppressive state, decreasing molecules such as TGF-β [43]. These effects appear to be Treg- and NK cell-dependent, and they extend previous observations of the critical importance of NK cells in enabling anti-tumour immune activity under normal oxygen conditions [44]. In a longitudinal study of HVs, individuals harbouring NK cells with low natural cytotoxicity presented a significantly higher incidence of cancer after 11 years [45].

Taking into account the remarkable differences between human and mouse immune cell subsets, particularly NK cells [46], the main strength of our study is the analysis of the innate immune response in a well-characterised cohort of patients with OSA, characterising the molecular mechanisms of IH-dependent immune escape.

Nevertheless, the present study has several limitations. We only selected patients with severe OSA; consequently, it is not possible to know whether the changes identified would also be present in milder forms of the disease. Although groups of untreated and treated patients with OSA were homogeneous at baseline, a pre–post treatment design was not established (only a small percentage of patients were included in the two groups). No random assignment of patients with OSA to treatment with CPAP or control was performed. The group of patients with OSA treated with CPAP was included to evaluate the plasticity of the innate immune system and to strengthen the association between sleep apnoea and the alterations in the immunosuppression phenotypes, showing their recovery when apnoea was suppressed. The clinical significance of our results must be confirmed; our findings do not allow us to establish clinical

recommendations for CPAP management in patients with OSA. To date, there is no clinical evidence that treatment with CPAP reduces the incidence or progression of cancer in patients with OSA. Several studies that have analysed the association between OSA and cancer mortality have excluded patients with good adherence to CPAP treatment [4, 5]. Our *in vitro* model does not allow an assessment of the contribution of other components of OSA, such as sympathetic tone or sleep fragmentation, which also appear to have some effect on the innate immune system [47].

In summary, the present study shows that OSA induces changes in the functionality of circulating monocytes and NK cells, which could favour tumour immune escape. Moreover, our data provide a new mechanism by which monocytes under intermittent hypoxic conditions impair the maturation and cytotoxic capacity of NK cells. Overall, these findings provide biological plausibility to recent epidemiological data suggesting that OSA is associated with increased cancer incidence. Further studies should focus on the link between our findings and other reported OSA comorbidities, such as cardiovascular diseases, hypertension and obesity [48–50]. Nevertheless, the previously mentioned OSA-related pathologies have been associated with high levels of TGF-β, which we have shown in this study.

References

1   Campos-Rodriguez F, Martinez-Garcia MA, Martinez M, *et al.* Association between obstructive sleep apnea and cancer incidence in a large multicenter Spanish cohort. *Am J Respir Crit Care Med* 2013; 187: 99–105.

2   Marshall NS, Wong KK, Cullen SR, *et al.* Sleep apnea and 20-year follow-up for all-cause mortality, stroke, and cancer incidence and mortality in the Busselton Health Study cohort. *J Clin Sleep Med* 2014; 10: 355–362.

3   Martínez-García MA, Martorell-Calatayud A, Nagore E, *et al.* Association between sleep disordered breathing and aggressiveness markers of malignant cutaneous melanoma. *Eur Respir J* 2014; 43: 1661–1668.

4   Nieto FJ, Peppard PE, Young T, *et al.* Sleep-disordered breathing and cancer mortality: results from the Wisconsin Sleep Cohort Study. *Am J Respir Crit Care Med* 2012; 186: 190–194.

5   Martínez-García MA, Campos-Rodriguez F, Durán-Cantolla J, *et al.* Obstructive sleep apnea is associated with cancer mortality in younger patients. *Sleep Med* 2014; 15: 742–748.

6   Martínez-García MA, Campos-Rodriguez F, Barbé F. Cancer and OSA: current evidence from human studies. *Chest* 2016; 150: 451–463.

7   Almendros I, Wang Y, Becker L, *et al.* Intermittent hypoxia-induced changes in tumor-associated macrophages and tumor malignancy in a mouse model of sleep apnea. *Am J Respir Crit Care Med* 2014; 189: 593–601.

8   Almendros I, Montserrat JM, Torres M, *et al.* Intermittent hypoxia increases melanoma metastasis to the lung in a mouse model of sleep apnea. *Respir Physiol Neurobiol* 2013; 186: 303–307.

9   Almendros I, Montserrat JM, Ramirez J, *et al.* Intermittent hypoxia enhances cancer progression in a mouse model of sleep apnoea. *Eur Respir J* 2012; 39: 215–217.

10   Almendros I, Montserrat JM, Torres M, *et al.* Obesity and intermittent hypoxia increase tumor growth in a mouse model of sleep apnea. *Sleep Med* 2012; 13: 1254–1260.

11   Gaoatswe G, Kent BD, Corrigan MA, *et al.* Invariant natural killer T cell deficiency and functional impairment in sleep apnea: links to cancer comorbidity. *Sleep* 2015; 38: 1629–1634.

12   Ye J, Liu H, Zhang G, *et al.* The treg/th17 imbalance in patients with obstructive sleep apnoea syndrome. *Mediators Inflamm* 2012; 2012: 815308.

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   Ruffell B, Affara NI, Coussens LM. Differential macrophage programming in the tumor microenvironment. *Trends Immunol* 2012; 33: 119–126.

15   Almendros I, Gileles-Hillel A, Khalyfa A, *et al.* Adipose tissue macrophage polarization by intermittent hypoxia in a mouse model of OSA: effect of tumor microenvironment. *Cancer Lett* 2015; 361: 233–239.

16   Barsoum IB, Koti M, Siemens DR, *et al.* Mechanisms of hypoxia-mediated immune escape in cancer. *Cancer Res* 2014; 74: 7185–7190.

17   Yun S, Lee SH, Yoon SR, *et al.* Oxygen tension regulates NK cells differentiation from hematopoietic stem cells in vitro. *Immunol Lett* 2011; 137: 70–77.

18   Freud AG, Yokohama A, Becknell B, *et al.* Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med* 2006; 203: 1033–1043.

19   Allan DS, Rybalov B, Awong G, *et al.* TGF-beta affects development and differentiation of human natural killer cell subsets. *Eur J Immunol* 2010; 40: 2289–2295.

20   Trotta R, Dal Col J, Yu J, *et al.* TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. *J Immunol* 2008; 181: 3784–3792.

21   Wang R, Zhu J, Dong X, *et al.* GARP regulates the bioavailability and activation of TGFβ. *Mol Biol Cell* 2012; 23: 1129–1139.

22   Shalova IN, Lim JY, Chittezhath M, *et al.* Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1α. *Immunity* 2015; 42: 484–498.

23   Ryan S, Taylor CT, McNicholas WT. Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. *Circulation* 2005; 112: 2660–2667.

24   Mantovani A, Sozzani S, Locati M, *et al.* Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; 23: 549–555.

25 Chen W, Jin W, Hardegen N, *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; 198: 1875–1886.

26 Viel S, Marçais A, Guimaraes FS, *et al.* TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci Signal* 2016; 9: ra19.

27 Cerdeira AS, Rajakumar A, Royle CM, *et al.* Conversion of peripheral blood NK cells to a decidual NK-like phenotype by a cocktail of defined factors. *J Immunol* 2013; 190: 3939–3948.

28 Vacca P, Cantoni C, Vitale M, *et al.* Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci U S A* 2010; 107: 11918–11923.

29 Zhou H, Chen L, You Y, *et al.* Foxp3-transduced polyclonal regulatory T cells suppress NK cell functions in a TGF-beta dependent manner. *Autoimmunity* 2010; 43: 299–307.

30 Bierie B, Moses HL. Transforming growth factor beta (TGF-beta) and inflammation in cancer. *Cytokine Growth Factor Rev* 2010; 21: 49–59.

31 Hughes T, Briercheck EL, Freud AG, *et al.* The transcription Factor AHR prevents the differentiation of a stage 3 innate lymphoid cell subset to natural killer cells. *Cell Rep* 2014; 8: 150–162.

32 Chidrawar SM, Khan N, Chan YL, *et al.* Ageing is associated with a decline in peripheral blood CD56bright NK cells. *Immun Ageing* 2006; 3: 10.

33 Roden AC, Morice WG, Hanson CA. Immunophenotypic attributes of benign peripheral blood gammadelta T cells and conditions associated with their increase. *Arch Pathol Lab Med* 2008; 132: 1774–1780.

34 Artis D, Spits H. The biology of innate lymphoid cells. *Nature* 2015; 517: 293–301.

35 Massagué J. TGFbeta in cancer. *Cell* 2008; 134: 215–230.

36 Mantovani A, Allavena P, Sica A, *et al.* Cancer-related inflammation. *Nature* 2008; 454: 436–444.

37 Loomans HA, Andl CD. Intertwining of activin A and TGFβ signaling: dual roles in cancer progression and cancer cell invasion. *Cancers* 2014; 7: 70–91.

38 Carmeliet P, Dor Y, Herbert JM, *et al.* Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 1998; 394: 485–490.

39 Sun JC, Lanier LL. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nat Rev Immunol* 2011; 11: 645–657.

40 Keskin DB, Allan DS, Rybalov B, *et al.* TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16- NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci U S A* 2007; 104: 3378–3383.

41 Dang EV, Barbi J, Yang HY, *et al.* Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* 2011; 146: 772–784.

42 Tran DQ, Andersson J, Wang R, *et al.* GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2009; 106: 13445–13450.

43 Hatfield SM, Kjaergaard J, Lukashev D, *et al.* Immunological mechanisms of the antitumor effects of supplemental oxygenation. *Sci Transl Med* 2015; 7: 277ra230.

44 Raulet DH, Gasser S, Gowen BG, *et al.* Regulation of ligands for the NKG2D activating receptor. *Annu Rev Immunol* 2013; 31: 413–441.

45 Imai K, Matsuyama S, Miyake S, *et al.* Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* 2000; 356: 1795–1799.

46 Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. *Nat Immunol* 2016; 17: 1025–1036.

47 Hakim F, Wang Y, Zhang SX, *et al.* Fragmented sleep accelerates tumor growth and progression through recruitment of tumor-associated macrophages and TLR4 signaling. *Cancer Res* 2014; 74: 1329–1337.

48 Ikonomidis I, Stamatelopoulos K, Lekakis J, *et al.* Inflammatory and non-invasive vascular markers: the multimarker approach for risk stratification in coronary artery disease. *Atherosclerosis* 2008; 199: 3–11.

49 Ikonomidis I, Tzortzis S, Triantafyllidi H, *et al.* Association of impaired left ventricular twisting-untwisting with vascular dysfunction, neurohumoral activation and impaired exercise capacity in hypertensive heart disease. *Eur J Heart Fail* 2015; 17: 1240–1251.

50 Ale A, Zhang Y, Han C, *et al.* Obesity-associated extracellular mtDNA activates central TGFβ pathway to cause blood pressure increase. *Am J Physiol Endocrinol Metab* 2016; 312: E161–E174.