Hypoxia-induced PD-L1/PD-1 crosstalk impairs T-cell function in sleep apnoea

Carolina Cubillos-Zapata[1,2], Jose Avendaño-Ortiz[3], Enrique Hernandez-Jimenez[1,3], Victor Toledano[1,3], Jose Casas-Martin[3], Anibal Varela-Serrano[3], Marta Torres[1,4], Isaac Almendros[1,5], Raquel Casitas[1,2], Isabel Fernández-Navarro[2], Aldara Garcia-Sanchez[2], Luis A. Aguirre[3], Ramón Farre[1,5], Eduardo López-Collazo[1,3,7] and Francisco García-Rio[1,2,6,7]

Affiliations: [1]Biomedical Research Networking Center on Respiratory Diseases (CIBERES), Madrid, Spain. [2]Respiratory Diseases Group, Respiratory Service, La Paz University Hospital, IdiPAZ, Madrid, Spain. [3]The Innate Immune Response Group, La Paz University Hospital, IdiPAZ, Madrid, Spain. [4]Sleep Laboratory, Pneumology Service, Hospital Clinic, University of Barcelona, Barcelona, Spain. [5]Biophysics and Bioengineering Unit, Faculty of Medicine, University of Barcelona-IDIBAPS, Barcelona, Spain. [6]Dept of Medicine, Autonomous University of Madrid, Madrid, Spain. [7]Joint principal investigators.

Correspondence: Francisco García Rio, Hospital Universitario La Paz, IdiPAZ; Paseo de La Castellana 261, 28046 Madrid, Spain. E-mail: fgr01m@gmail.com

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ABSTRACT Obstructive sleep apnoea (OSA) is associated with higher cancer incidence, tumour aggressiveness and cancer mortality, as well as greater severity of infections, which have been attributed to an immune deregulation. We studied the expression of programmed cell death (PD)-1 receptor and its ligand (PD-L1) on immune cells from patients with OSA, and its consequences on immune-suppressing activity. We report that PD-L1 was overexpressed on monocytes and PD-1 was overexpressed on $CD8^+$ T-cells in a severity-dependent manner. PD-L1 and PD-1 overexpression were induced in both the human *in vitro* and murine models of intermittent hypoxia, as well as by hypoxia-inducible factor-1α transfection. PD-L1/PD-1 crosstalk suppressed T-cell proliferation and activation of autologous T-lymphocytes and impaired the cytotoxic activity of $CD8^+$ T-cells. In addition, monocytes from patients with OSA exhibited high levels of retinoic acid related orphan receptor, which might explain the differentiation of myeloid-derived suppressor cells. Intermittent hypoxia upregulated the PD-L1/PD-1 crosstalk in patients with OSA, resulting in a reduction in $CD8^+$ T-cell activation and cytotoxicity, providing biological plausibility to the increased incidence and aggressiveness of cancer and the higher risk of infections described in these patients.

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Introduction

Obstructive sleep apnoea (OSA) is a highly prevalent disorder characterised by recurrent episodes of total or partial obstruction of the upper airway during sleep which are associated with intermittent hypoxaemia, increased inspiratory effort and sleep disruption [1]. Experimental and clinical data suggest that OSA could be associated with a higher incidence of cancer, tumour aggressiveness and cancer mortality [2–4]. In addition, OSA has been related to the severity of community-acquired pneumonia [5]. It has been proposed that hypoxia-induced immune deregulation might be responsible for many of these alterations [6].

Immune response is a complex phenomenon based on a critical balance between activator and inhibitor pathways modulating T-cell activity. This balance is destabilised in certain pathological processes in which the inhibition of the immune response will cooperate in the progression of the disorder. One key inhibitor pathway is the receptor of programmed cell death (PD)-1 and its ligand PD-L1, which plays a decisive role in the maintained quiescence of autoreactive T-cells [7].

The engagement of PD-1 on the T-cell surface with its ligand PD-L1 inhibits T-cell proliferation and effector functions and enhances myeloid-derived suppressor cell (MDSC) proliferation [8–11]. MDSCs are a heterogeneous population composed of myeloid cells at several stages of differentiation; they accumulate in the blood and tumour sites of patients and experimental animals with cancer, and they are capable of inhibiting both innate and adaptive immune response [12]. Moreover, retinoic acid related orphan receptor (RORC)1 protein expression promotes MDSC expansion in myeloid cells [13, 14].

Whereas upregulation of PD-1 is a natural consequence of T-cell activation and is necessary for the resolution of the immune response, a number of transcriptional factors have been shown to be involved in PD-L1 expression [11]. Interestingly, the hypoxia-inducible factor (HIF)-1 regulates PD-L1 through binding to a hypoxia response element of the PD-L1 promoter to activate PD-L1 transcription [15]. Therefore, we hypothesise that in patients with OSA, intermittent hypoxia might increase PD-L1 expression, enhancing the checkpoint inhibitor PD-1 receptor. The imbalanced activation of this immune checkpoint causes a downregulation of T-cell function, which might provide biological plausibility to the higher risk of developing cancer or chronic infections in OSA.

To explore the effect of OSA on the PD-L1/PD-1 co-inhibitory pathway, we assessed PD-L1 and PD-1 expression on monocytes and T-cells, respectively, of patients with OSA and healthy volunteers. To identify the role of intermittent hypoxia on this immune checkpoint, we used an *in vivo* murine model and a human *in vitro* intermittent hypoxia model. Finally, the consequences of PD-L1 overexpression on monocytes on their antigen-presenting capacity, T-cell cytotoxicity and suppressive effect were assessed.

Methods

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

Study participants

Recently diagnosed patients with OSA were consecutively recruited from the sleep unit of La Paz University Hospital-Cantoblanco (Madrid, Spain). Patients aged 40–65 years with an apnoea/hypopnoea index (AHI) of $\geqslant$5 events·h^{-1} were included in the study. The diagnosis of OSA was established using respiratory polygraphy. Mild–moderate OSA was defined as the presence of an AHI of 5–30 events·h^{-1}, whereas severe OSA was defined as AHI $\geqslant$30 events·h^{-1}. As a control group, healthy volunteers were selected who were homogeneous in sex, age, smoking habits and body mass index (BMI). None of these volunteers were being treated with any type of medication, and the diagnosis of OSA was ruled out using respiratory polygraphy. The study was approved by the local ethics committee (PI-1857), and informed consent was obtained from all the participants. Additional details are provided in the online supplementary material.

Isolation of monocytes and lymphocytes from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with OSA and healthy volunteers using Ficoll-Plus gradient (GE Healthcare Bio-Sciences, Little Chalfont, UK).

Intermittent hypoxia mouse model

The study was approved by the ethics committee for animal research at the University of Barcelona and was performed on 6-week-old pathogen-free C57BL/6 male mice (Charles River Laboratories, Lyon, France). A total of 28 mice were randomly assigned to intermittent hypoxia exposure (n=14 mice) or normoxia (n=14 mice) for 6 weeks, as previously reported [16].

Reagents

The carboxyfluorescein succinimidyl ester (CFSE) for the proliferation assays was purchased from Thermofisher (Darmstadt, Germany). The pokeweed (PWD) used was from Sigma-Aldrich (St Louis,

MO, USA). To inhibit PD-L1/PD-1 crosstalk, the anti-PD-1 antibody used was from Bristol-Myers Squibb (Bristol, UK). The 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamid (PCTA1) for the nuclear factor-κB (NF-κB) inhibitor assay was obtained from Tocris (Bristol, UK).

Flow cytometry analysis and cell culture

PBMCs were isolated and stained with surface or intracellular specific antibodies. Isolated splenocytes from the mice were labelled using surface-specific antibodies. In the intermittent hypoxia model, the cells were exposed to a hypoxic chamber (3% oxygen, 5% carbon dioxide, 37°C) for 12 cycles and changed from preconditioned-hypoxic medium to a normoxic medium, as previously described by Ryan *et al.* [17].

HIF1α overexpression studies

The HIF1α overexpression assay was performed by transfecting monocytes with a human HIF1α expression plasmid or a control plasmid, as we have previously described [18], using an Amaxa Nucleofector (Amaxa Biosystems, La Ferté-Macé, France).

T-cell proliferation and PD-L1/PD-1 blocking assays

T-cell proliferation, with or without PWD as stimulus, was analysed after 4 days. Isolated lymphocytes were either pretreated or not with anti-PD-1 antibody for 1 h. We then added the monocytes to the autologous CFSE-labelled lymphocytes (1:10) and stimulated them with PWD for 1 h. The cells were cultured for 4 days and were acquired by flow cytometry (BD-FACSCalibur; BD Biosciences, Ghent, Belgium).

RNA isolation and quantification

Total RNA was purified from $CD14^+$ isolated cells using the High Pure RNA Isolation Kit (Roche Diagnostics, Almere, the Netherlands). The mRNA quantitation gene expression levels were analysed using real-time quantitative (q)PCR using the LightCycler system (Roche Diagnostics). The real-time qPCRs were performed using the QuantiMix Easy SYG kit from Biotools (Madrid, Spain) and specific primers.

Statistical analysis

Data are presented as mean±SEM, unless otherwise stated. Comparisons between groups were performed using analysis of variance with a Dunnett *post hoc* test for the normal distributed variables and 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 Mann–Whitney U-test. The correlations were assessed with Spearman's rank correlation. For all the analyses, a p-value <0.05 was considered statistically significant. The analyses were conducted using Prism 5.0 (GraphPad, La Jolla, CA, USA) and SPSS 15.0 software (IBM, Armonk, NY, USA).

Results

Characteristics of the participants

46 patients with OSA were prospectively recruited: 32 with mild–moderate OSA and 14 with severe OSA. In addition, 29 healthy volunteers were included as the control group. The demographic, sleep and biochemical characteristics of the three groups are shown in table 1. Mean age, sex, BMI and smoking habits were similar across the three groups.

PD-L1 is overexpressed in monocytes from patients with OSA

PD-L1 expression on monocytes from the patients with severe OSA was higher than in the healthy volunteers or in the patients with mild–moderate OSA (figure 1a and b). Interestingly, several measures of OSA severity, such as AHI and the oxygen desaturation index (ODI), correlated positively with the percentage of monocytes that expressed PD-L1 (r=0.816, p<0.001 and r=0.715, p<0.001, respectively) (figure 1c). In contrast, the percentage of monocytes that expressed PD-L1 was not related with the recording time with arterial oxygen saturation measured by pulse oximetry (S_{pO_2}) <90% (r=0.228, p=0.362). In addition to PD-L1 surface protein expression, we analysed PD-L1 mRNA expression on isolated monocytes, corroborating that it was also significantly increased in the patients with severe OSA compared with the healthy volunteers (online supplementary figure S1).

PD-L1 expression is induced by intermittent hypoxia

To study whether intermittent hypoxia regulates PD-L1 expression, monocytes from healthy volunteers were cultured using a preconditioned hypoxic medium followed by preconditioned normoxic medium, inducing cyclic fluctuations of intracellular oxygen, mimicking OSA conditions (online supplementary figure S2). After exposure to this *in vitro* intermittent hypoxia model, the monocytes exhibited enhanced PD-L1 expression compared with the cells under normoxic conditions (figure 2a). In addition, we overexpressed HIF1α by plasmid transfection on isolated healthy monocytes and found an increase in the

TABLE 1 Demographic, respiratory and metabolic characteristics of the three study groups

	Severe OSA patients	Mild-moderate OSA patients	Healthy volunteers	p-value
Subjects n	14	32	29	
Male	10 (71)	23 (72)	24 (83)	0.553
Age years	58±9	56±10	55±6	0.599
Body mass index kg·m^{-2}	29.0±4.1	28.3±3.8	27.4±5.0	0.627
Smoking habit n (%)				0.994
Current smoker	5 (36)	10 (31)	10 (35)	
Former smoker	4 (29)	11 (34)	10 (35)	
Never-smoker	5 (36)	11 (34)	9 (31)	
Epworth sleepiness scale	12.9±3.6$^{\#,\P}$	7.8±4.8$^{\#}$	2.0±0.8	<0.001
AHI events·h^{-1}	62.6±35.4$^{\#,\P}$	18.1±6.7^{+}	2.7±1.2	<0.001
Oxygen desaturation index, events·h^{-1}	55.3±25.5$^{\#,\P}$	17.4±7.1^{+}	1.9±1.1	<0.001
Recording time with S_{pO_2} <90% %	29.0±19.2$^{\#,\S}$	11.4±15.9^{+}	2.3±2.1	<0.001
Mean nocturnal S_{pO_2} %	90.8±2.0$^{+,f}$	92.6±1.8	93.3±1.5	0.031
Blood pressure mmHg				
Systolic	123±12	122±10	123±11	0.968
Diastolic	74±8	76±7	76±6	0.685
White cell count cells·mm^{-3}				
Total	7.60±1.72	7.42±1.39	7.77±1.33	0.645
Neutrophils	4.70±1.11	4.90±1.11	5.03±1.04	0.638
Lymphocytes	2.24±0.73	1.81±0.50	2.09±0.61	0.287
Monocytes	0.50±0.17	0.47±0.15	0.48±0.09	0.777
Haemoglobin g·dL^{-1}	13.9±1.3	13.8±1.4	14.0±1.2	0.727
Cholesterol mg·dL^{-1}	203±38	196±49	184±40	0.536
HDL-cholesterol mg·dL^{-1}	132±32	130±41	123±36	0.777
LDL-cholesterol mg·dL^{-1}	56±7	55±10	49±4	0.045
Triglycerides mg·dL^{-1}	126±53	141±40	136±18	0.636

Data are presented as n, n (%) or mean. OSA: obstructive sleep apnoea; AHI: apnoea–hypopnea index; S_{pO_2}: arterial oxygen saturation measured by pulse oximetry; HDL: high-density lipoprotein; LDL: low-density lipoprotein. Comparisons between groups were performed by ANOVA or the Chi-squared test. $^{\#}$: p<0.001 *versus* control group; ¶: p<0.001 *versus* mild–moderate OSA patients; $^{+}$: p<0.05 *versus* control group; §: p<0.01 *versus* mild–moderate OSA patients; ^{f}: p<0.05 *versus* mild–moderate OSA patients.

percentage of monocytes expressing PD-L1 compared with the control group, which suggests that the overexpression of PD-L1 might be triggered by hypoxia (figure 2b). Moreover, a NF-κB blocking assay suggests the crucial role of intermittent hypoxia in this model (online supplementary figure S3). Finally, PD-L1 expression was assessed in the murine model of intermittent hypoxia. After 6 weeks, isolated F4/80 splenocytes from mice subjected to intermittent hypoxia showed higher PD-L1 expression than the control group (normoxia) (figure 2c). Collectively, these observations corroborate that intermittent hypoxia plays a role in PD-L1 regulation.

PD-1 expression on T-cells from patients with OSA

Although nonsignificant differences were found in PD-1 expression on CD4^{+} T-cells among the three study groups (figure 3a), PD-1 expression on CD8^{+} T-cells was significantly enhanced in the patients with severe and mild–moderate OSA compared with the healthy volunteers (figure 3b). PD-1 expression on CD4^{+} T-cells did not correlate with sleep characteristics (data not shown). In contrast, the percentage of CD8^{+} T-cells that expressed PD-1 was related to the AHI (r=0.477, p<0.0038) and, although not reaching statistical significance, to the ODI (r=0.332, p<0.0826) (figure 3c). No significant relationhsip was found between the percentage of CD8^{+} T-cells that expressed PD-1 and the recording time with S_{pO_2} <90% (r=0.1211, p=0.6214). Collectively, these data suggest that the activity of the PD-L1/PD-1 immune checkpoint is related to OSA severity.

PD-1 overexpression on CD8^{+} T-cells is induced under intermittent hypoxia

To assess whether intermittent hypoxia conditions could modulate PD-1 expression on T-cells, we analysed the T-cells from healthy volunteers submitted to the *in vitro* model of intermittent hypoxia. Whereas no differences in PD-1 expression on CD4^{+} T-cells between intermittent hypoxia and normoxia conditions were observed (figure 4a), we found a significant upregulation of PD-1 expression on CD8^{+} T-cells under intermittent hypoxia conditions compared with normoxic CD8^{+} T-cells (figure 4b).

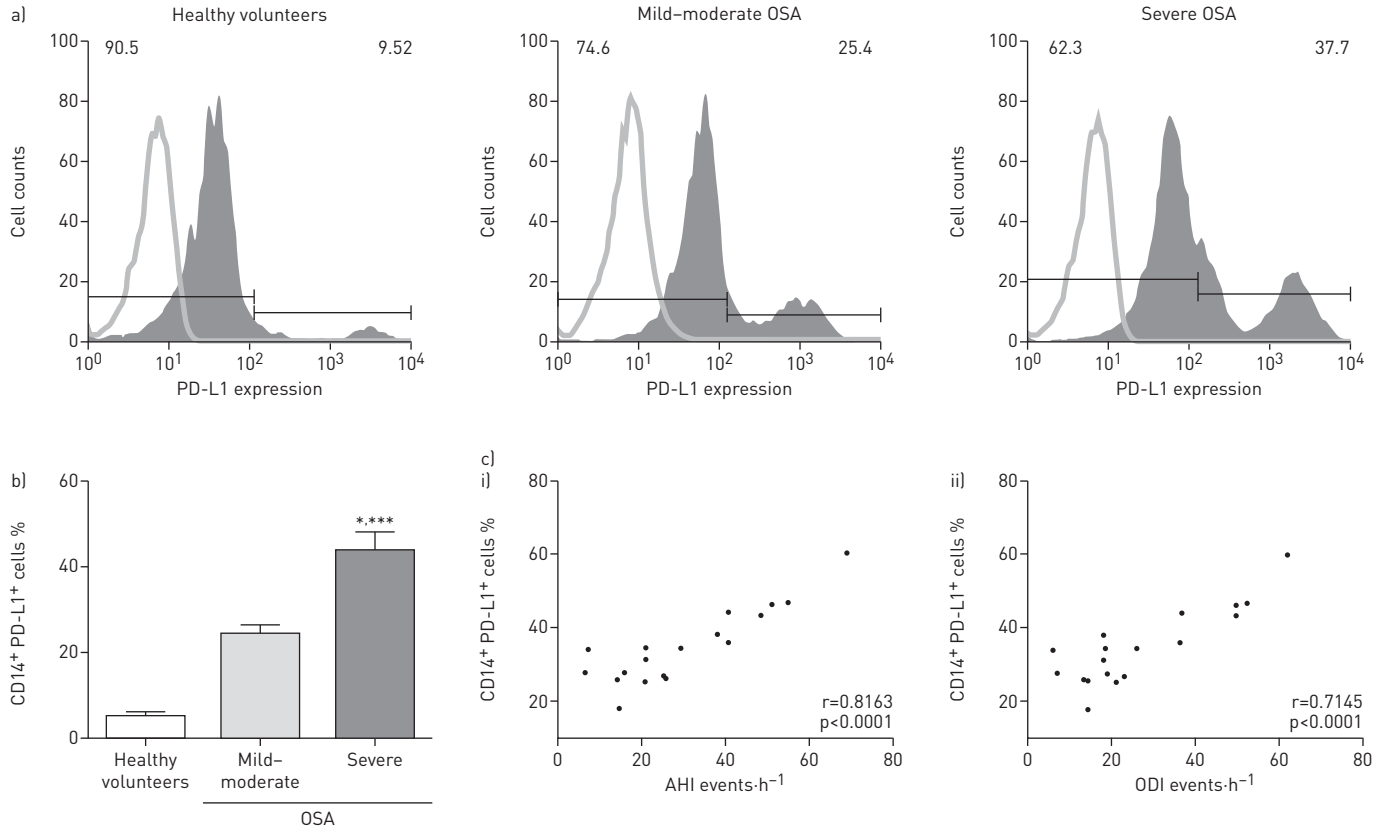

FIGURE 1 Programmed cell death ligand-1 (PD-L1) is overexpressed on monocytes from patients with obstructive sleep apnoea (OSA). $CD14^+$ monocytes isolated from randomly selected healthy volunteers (n=20) and patients with mild–moderate OSA (n=18) and severe OSA (n=12) were analysed using flow cytometry. a) Representative histograms of PD-L1 expression on $CD14^+$ gated cells. The shaded histograms represent the isotype control and percentage of expression is shown within the chart area of each histogram. b) Comparisons between groups were performed by ANOVA using the Kruskal–Wallis test with Dunn's multiple comparison. Error bars: SEM. *: p<0.05 *versus* mild–moderate OSA; ***: p<0.001 *versus* healthy volunteers. c) Relationship between the PD-L1 expression levels on $CD14^+$ cells from patients with OSA and i) apnoea–hypopnea index (AHI) and ii) oxygen desaturation index (ODI). Spearman's correlation coefficients (r) and p-values are shown.

Moreover, we analysed PD-1 expression on T-cells from mice exposed to intermittent hypoxia conditions. The PD-1 expression on $CD4^+$ and $CD8^+$ T-cells of animals under intermittent hypoxia were higher than on cells from animals maintained in normoxia (figure 4c and d). Although the behaviour of $CD8^+$ T-cells under intermittent hypoxia is similar to human T-cells, discrepancies in $CD4^+$ might be attributed to the fact that $CD4^+$ T-cells from mice are more susceptible to intermittent hypoxia, or different pathways between humans and mice govern PD-1 expression on $CD4^+$ T-cells.

Blocking PD-L1/PD-1 decreases T-cell proliferation in patients with OSA

To assess the consequences of activating the PD-L1/PD-1 checkpoint, we evaluated T-cell proliferation in the three study groups using isolated PBMCs stimulated or not with PWD for 4 days. Although $CD4^+$ T-cell proliferation significantly decreased in the patients with severe OSA compared with the healthy volunteers (figure 5a), $CD8^+$ T-cell proliferation was significantly lower in the patients with mild–moderate or severe OSA than in the healthy volunteers (figure 5b). We performed an *ex vivo* PD-L1/PD-1 blocking assay using anti-PD-1 monoclonal antibody to evaluate the T-cell response. We separated and co-cultured PBMCs with autologous isolated lymphocytes from the healthy volunteers and the patients with OSA with or without α-PD-1 antibody, and we assessed the T-cell suppressive function using an *ex vivo* assay. Interestingly, the blockade of PD-L1/PD-1 in the patients with severe OSA significantly abrogated the suppressive activity of monocytes in response to $CD4^+$ and $CD8^+$ T-cells (figure 5). However, T-cell proliferation in the patients with mild–moderate OSA showed a prominent difference after PD-L1/PD-1 blocking only on autologous $CD8^+$ T-cells. Moreover, we did not find a significant difference on $CD4^+$ and $CD8^+$ T-cells from healthy volunteers after the PD-L1/PD-1 blocking assay. Collectively, these data suggest a T-cell suppressive effect through PD-L1 expression on OSA monocytes. Moreover, this PD-L1 immunosuppressive effect appears to be related to OSA severity.

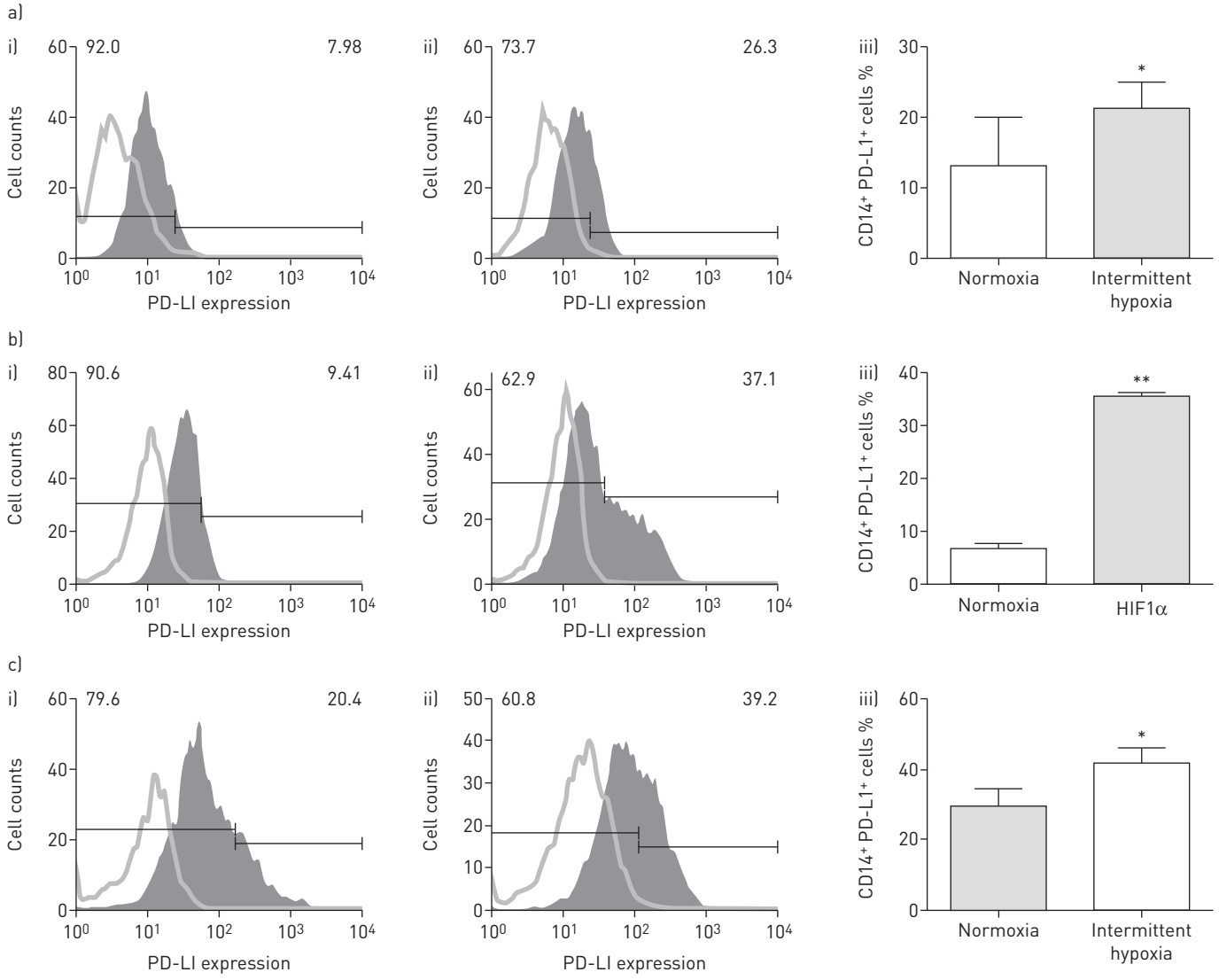

FIGURE 2 Programmed cell death ligand-1 (PD-L1) is overexpressed on monocytes under intermittent hypoxia. a) $CD14^+$ monocytes were isolated from randomly selected healthy volunteers and cultured under normoxia or intermittent hypoxia. $CD14^+$ monocytes were analysed using flow cytometry. Representative histograms of PD-L1 expression on $CD14^+$ cells gated under i) normoxia and ii) intermittent hypoxia; iii) statistical analysis. b) $CD14^+$ monocytes isolated from healthy volunteers were nucleofected with control plasmid (0.5 µg; n=7) or HIF1α plasmid (0.5 µg; n=7) for 16 h. $CD14^+$ monocytes were analysed using flow cytometry. Representative histograms of PD-L1 expression on $CD14^+$ cells from i) control and ii) HIF1α groups; iii) statistical analysis. c) Isolated spleen cells from mice under normoxic conditions (n=6) and the intermittent hypoxia model (n=7) were analysed using flow cytometry. Representative histograms of PD-L1 expression on $F4/80^+$ cells for i) normoxic and ii) hypoxic groups; iii) statistical analysis. The shaded histograms represent the isotype control and percentage of expression is shown within the chart area of each histogram. Error bars: SEM. *: $p<0.05$ and **: $p<0.01$, using the Mann–Whitney test.

T-cell activity is suppressed in patients with OSA

In order to study the relevance of the PD-L1/PD-1 immune checkpoint on T-cell activity, we analysed the T-cell activator marker (CD69) on both $CD4^+$ and $CD8^+$ T-cell subsets from patients with OSA. We found no differences in CD69 expression on either $CD4^+$ or $CD8^+$ T-cells (figure 6a and b).

To complete the T-cell activation study, we analysed the expression of perforin, one of the primary surface markers of cytotoxic function. We found significantly decreased expression of perforin on $CD8^+$ T-cells from the patients with severe OSA compared with the healthy volunteers (figure 6c). Collectively, these data show that the cytotoxic activity of $CD8^+$ T-cells is impaired in patients with severe OSA.

Myeloid-derived suppressor cells are increased in patients with OSA

Figure 7 shows the distribution of myeloid-derived suppressor cells (MDSCs) from healthy volunteers and patients with mild–moderate and severe OSA. The MDSC population increased in the patients with severe OSA compared with the healthy volunteers and the patients with mild–moderate OSA (figure 7a). To

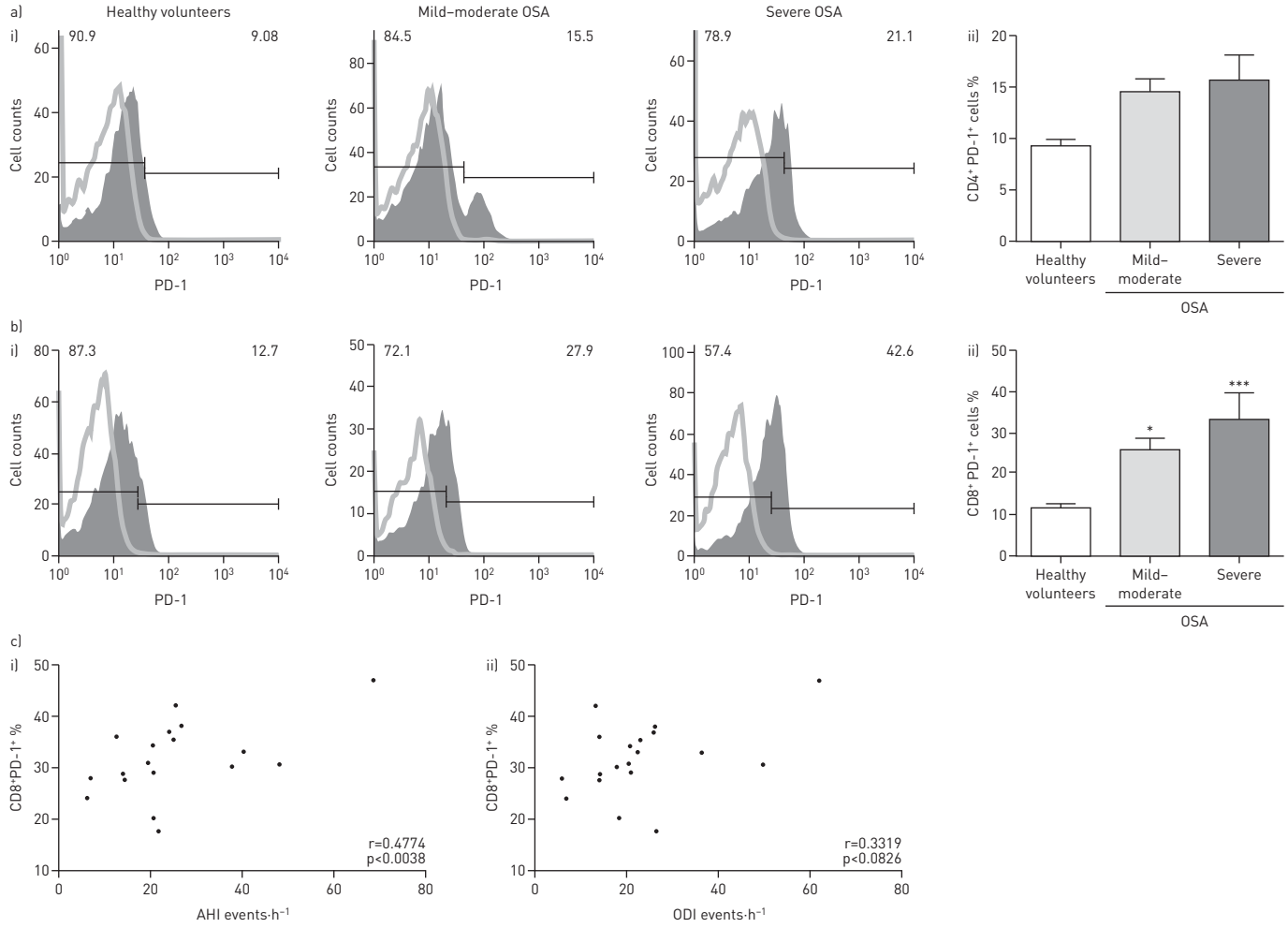

FIGURE 3 Programmed cell death receptor (PD-1) is overexpressed on T-cells from patients with obstructive sleep apnoea (OSA). Peripheral blood mononuclear cells (PBMCs) from healthy volunteers and randomly selected patients with mild–moderate OSA and severe OSA were analysed using flow cytometry. a) i) Representative histograms of PD-1$^+$ expression on CD4$^+$ T-cell subsets; ii) percentage distribution of PD-1$^+$ on CD4$^+$ T-cell subset for healthy volunteers (n=10), patients with mild–moderate OSA (n=14) and severe OSA (n=7); error bars: SEM. b) i) Representative histograms of PD-1 expression on CD8$^+$ T-cells; ii) percentage distribution of PD-1$^+$ on CD8$^+$ T-cell subset. The shaded histograms represent the isotype control and percentage of expression is shown within the chart area of each histogram. *: p<0.05 *versus* healthy volunteers; ***: p<0.001 *versus* healthy volunteers by ANOVA using the Kruskal–Wallis test with Dunn's multiple comparison; error bars: SEM. c) Correlation between the percentage of CD8$^+$ T-cells from patients with OSA that expressed PD-1 and i) the apnoea/hypopnoea index (AHI) or ii) oxygen desaturation index (ODI). Spearman correlation coefficients (r) and p-values are shown.

assess the generation of MDSCs in the patients with OSA, we studied RORC expression. We found increased RORC expression on monocytes isolated from the patients with mild–moderate OSA compared with the healthy volunteers, and an even greater overexpression on the monocytes from patients with severe OSA, which was superior to that of both the healthy volunteers and the patients with mild–moderate OSA (figure 7a).

Moreover, monocytes from healthy volunteers under intermittent hypoxia showed increased MDSC distribution compared with the monocytes under normoxic conditions. In addition, RORC expression was upregulated on monocytes from healthy volunteers subjected to the intermittent hypoxia model (figure 7b). In agreement with these data, we corroborated the increased MDSC and RORC expression on HIF1α overexpressed cells compared with siR-control cells (figure 7c). Additionally, in the murine intermittent hypoxia model, we found an MDSC pattern similar to that of humans (figure 7d).

Discussion

The primary finding of our study is the severity-dependent upregulation of PD-L1/PD-1 crosstalk in patients with OSA as a consequence of intermittent hypoxia. This upregulation induces suppressed autologous T-cell proliferation, decreases CD8$^+$ T-cell cytotoxic activity and increases MDSCs.

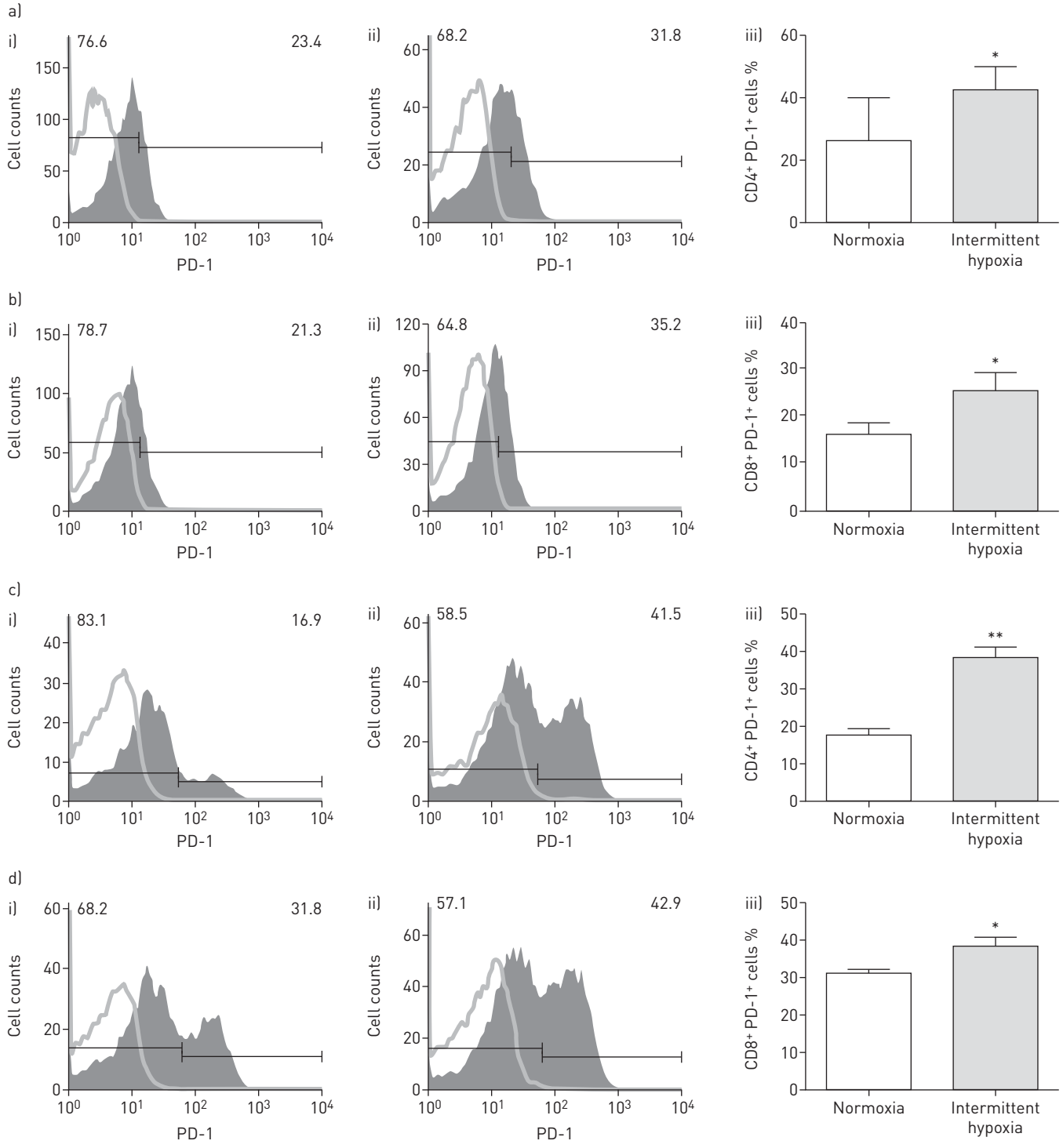

FIGURE 4 Programmed cell death receptor (PD-1) is overexpressed on T-cells under intermittent hypoxic conditions. a) Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers and cultured under normoxia or intermittent hypoxia. $CD4^+$ T-cells were analysed using flow cytometry. Representative histograms of $PD\text{-}1^+$ expression on $CD4^+$ T-cell subsets under i) normoxia and ii) intermittent hypoxia; iii) statistical analysis (n=7, randomly selected); error bars are SEM. b) $CD8^+$ T-cells were analysed using flow cytometry. Representative histograms of $PD\text{-}1^+$ expression on $CD8^+$ T-cell subsets under i) normoxia and ii) intermittent hypoxia; iii) statistical analysis (n=7, randomly selected). c) Isolated spleen cells from the normoxic mouse (n=6) group and the IH mouse model (n=7) were analysed using flow cytometry. i, ii) representative histograms of $PD\text{-}1^+$ expression on $CD4^+$ T-cells; iii) statistical analysis. d) i, ii) Representative histograms of PD-1 expression on $CD8^+$ T-cells. The shaded histograms represent the isotype ccontrol and percentage of expression is shown within the chart area of each histogram. iii) Statistical analysis. *: $p<0.05$ and **: $p<0.01$, by the Mann–Whitney test; error bars: SEM.

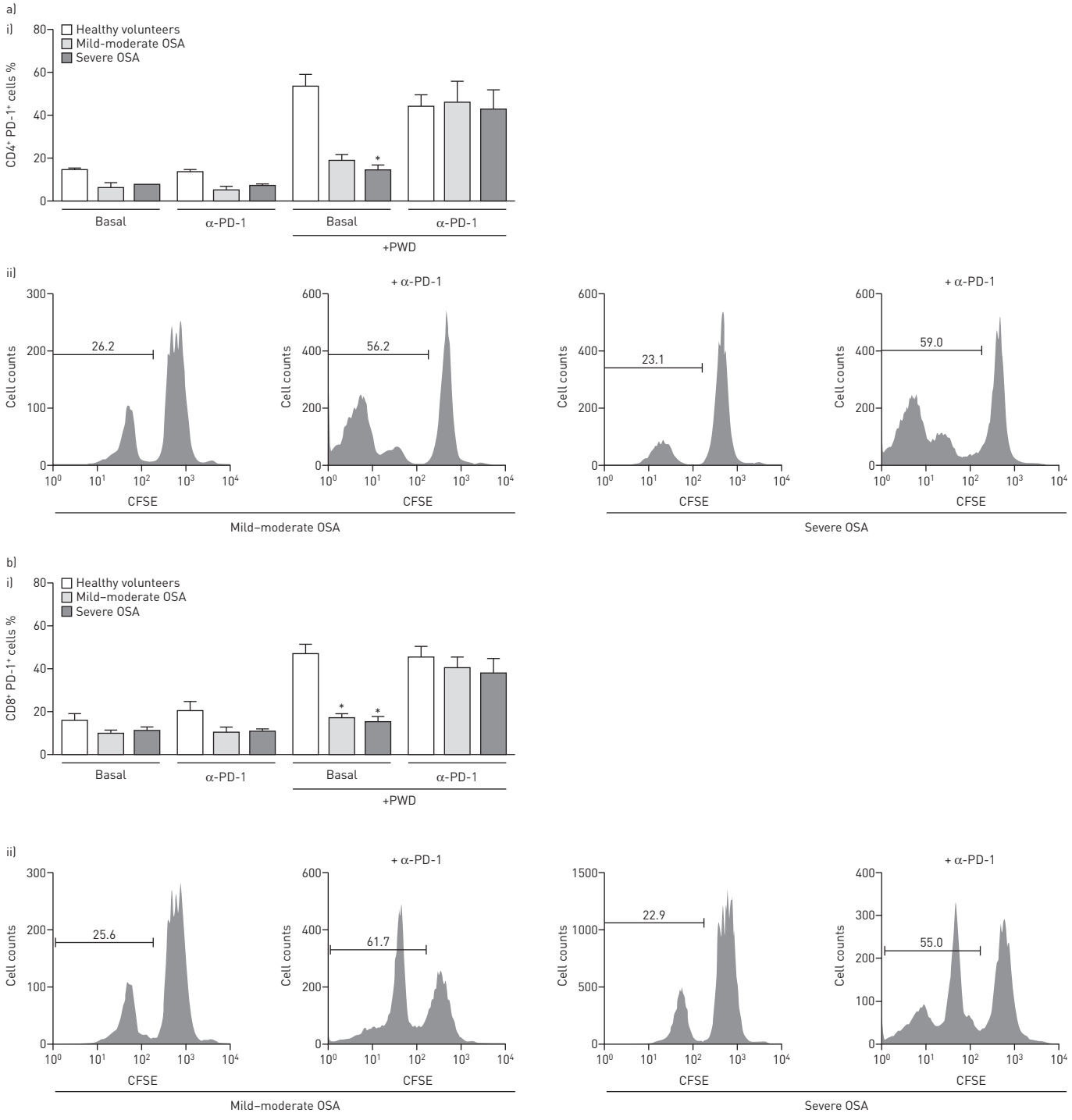

FIGURE 5 T-cell proliferation and programmed cell death receptor-1 (PD-1)/programmed cell death ligand (PD-L1) blocking assay from patients with obstructive sleep apnoea (OSA). Isolated monocytes from healthy volunteers (n=7 randomly selected) and patients with mild–moderate OSA (n=7, randomly selected) or severe OSA (n=7) were used to evaluate the T-cell proliferation after PD-1/PD-L1 blocking assay. The isolated monocytes were cultured with α-PD-1 or α-IgG antibodies. Afterwards, the cells were washed and the autologous carboxyfluorescein succinimidyl ester (CFSE)-labelled T-cells were added at a ratio 1:10, and cultured with pokeweed (PWD) (2.5 $\mu g \cdot mL^{-1}$) or without stimulus for 1 h. Next, cells were washed and cultured for 4 days. The T-cells were analysed using flow cytometry. Gating strategy for the detection of $CD4^+$ T-cells. a) i) Percentage of $CD4^+$ T-cell proliferation; error bars are SEM; ii) representative CFSE histogram of $CD4^+$ gated T-cells from patients with mild–moderate and severe OSA. b) i) Percentage of $CD8^+$ T-cell proliferation; error bars are SEM, ii) representative CFSE histogram of $CD8^+$ gated T-cells from patients with mild–moderate and severe OSA. *: $p<0.05$ *versus* healthy volunteers, by ANOVA using the Kruskal–Wallis test with Dunn's multiple comparison.

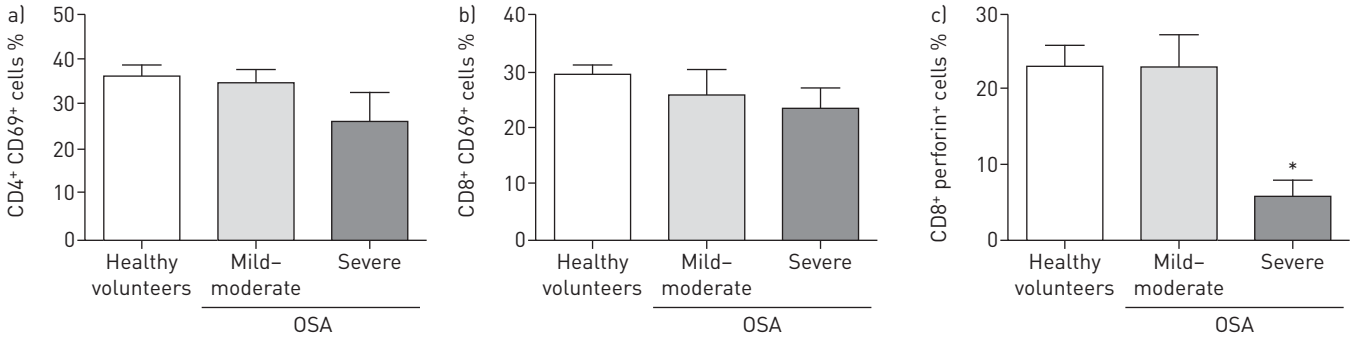

FIGURE 6 T-cell immunosuppressant activity in patients with obstructive sleep apnoea (OSA). Peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers (n=10, randomly selected) and patients with mild–moderate (n=10, randomly selected) and severe OSA (n=10, randomly selected) were analysed by flow cytometry. a) T-cells were stimulated with PMA (10 ng·mL^{-1}) for 16 h. The percentage of CD69$^+$ cells is shown. Gating strategy for the detection of CD4$^+$ T-cells and b) CD8$^+$ T-cells; c) T-cells were analysed by flow cytometry. Gating strategy for the detection of CD8$^+$ T-cells. The percentage of perforin$^+$ cells is shown. *: p<0.05, *versus* healthy volunteers, by ANOVA using the Kruskal–Wallis test with Dunn's multiple comparison; error bars are SEM.

PD-L1 overexpression on monocytes from patients with severe OSA, verified at both the transcriptional and translational levels, is closely related to the AHI and the ODI. This alteration was reproduced in the human *in vitro* and animal models of intermittent hypoxia, as well as by the transfection of HIF1α, suggesting that intermittent hypoxia enhances PD-L1 expression on these cells from patients with OSA. Accordingly, other authors have reported that hypoxic stress leads to the upregulation of PD-L1 [15, 19]. At least two hypoxia response element-binding sites have been identified in the PD-L1 promoter, and HIF activates PD-L1 transcription through binding to them [19].

However, our data do not allow us to conclude that intermittent hypoxia is the only trigger of PD-L1/PD-1 upregulation in patients with OSA. It is known that PD-L1 expression is also induced by pro-inflammatory cytokines, including interferon and vascular endothelial growth factor [7]. It has been also proposed that NF-κB plays a role in regulating PD-L1 expression on human monocytes [20]. Because OSA has been largely demonstrated to be associated with systemic inflammation and NF-κB upregulation [17, 21, 22], inflammation might play a potentially synergistic role with intermittent hypoxia in the induction of PD-L1 expression. We have confirmed that the intermittent hypoxia model induces inflammation and that levels of pro-inflammatory cytokines, such as tumour necrosis factor-α, interleukin-6 and -1β, are increased after intermittent hypoxia. However, when isolated monocytes from healthy donors were pretreated with an NF-κB inhibitor (PCTA1) before the intermittent hypoxia model, PD-L1 expression increased as a result of intermittent hypoxia conditions (online supplementary figure S3). In this study, we demonstrated that patients with OSA show enhanced expression of PD-1 on T-cells, associated with a lower T-cell activity than in healthy volunteers. Interestingly, the mouse model confirmed the suppression of effector CD8$^+$ T-cells [23] and engagement of the inhibitory receptor PD-1 by PD-L1 [24–26]. In addition, we have demonstrated that T-cell proliferation increases after blocking PD-1 on lymphocytes, allowing the cells to efficiently deliver the antigens to the T-cells. Moreover, our PD-1 blockade on patients with OSA under normoxic conditions might suggest the restored plasticity of the immune system. These results are concordant with previous reports showing that, when PD-1 expression and their ligands are persistently elevated, this elevation leads to a profound inability of T-cells to respond to activation signals [27].

Interestingly, we have found that patients with severe OSA experienced an increase in the number of MDSCs and greater RORC expression on their monocytes. MDSCs are a large group of myeloid cells at earlier stages of differentiation [12]. They play a major role in the orchestration of an immunosuppressive network in many pathological conditions, *via* suppression of CD8$^+$ T-cell cytotoxicity [15, 28]. Our *in vitro* and murine models demonstrated that intermittent hypoxia increased PD-L1 expression and enhanced the number of MDSCs and RORCs in monocytes, which is in line with previous studies reporting that hypoxia regulates function and differentiation of MDSCs, increasing PD-L1 expression [29]. In consequence, our data might indicate that intermittent hypoxia induces immunosuppressive factors, paralysing cytotoxic CD8$^+$ T-cells. In fact, an antigen-unspecific defect was observed in our patients with severe OSA, as well as the downregulation of perforin on their CD8$^+$ T-cells.

Although it was not a goal of our study, the upregulation of the PD-L1/PD-1 inhibitory pathway found in the patients with OSA might have important clinical consequences. PD-L1 is a critical mediator of regulatory T-cells, a key mediator in controlling antitumour immune suppression, tumour immune escape, metastasis and relapse [30]. Therefore, the overexpression of this immune checkpoint in patients with

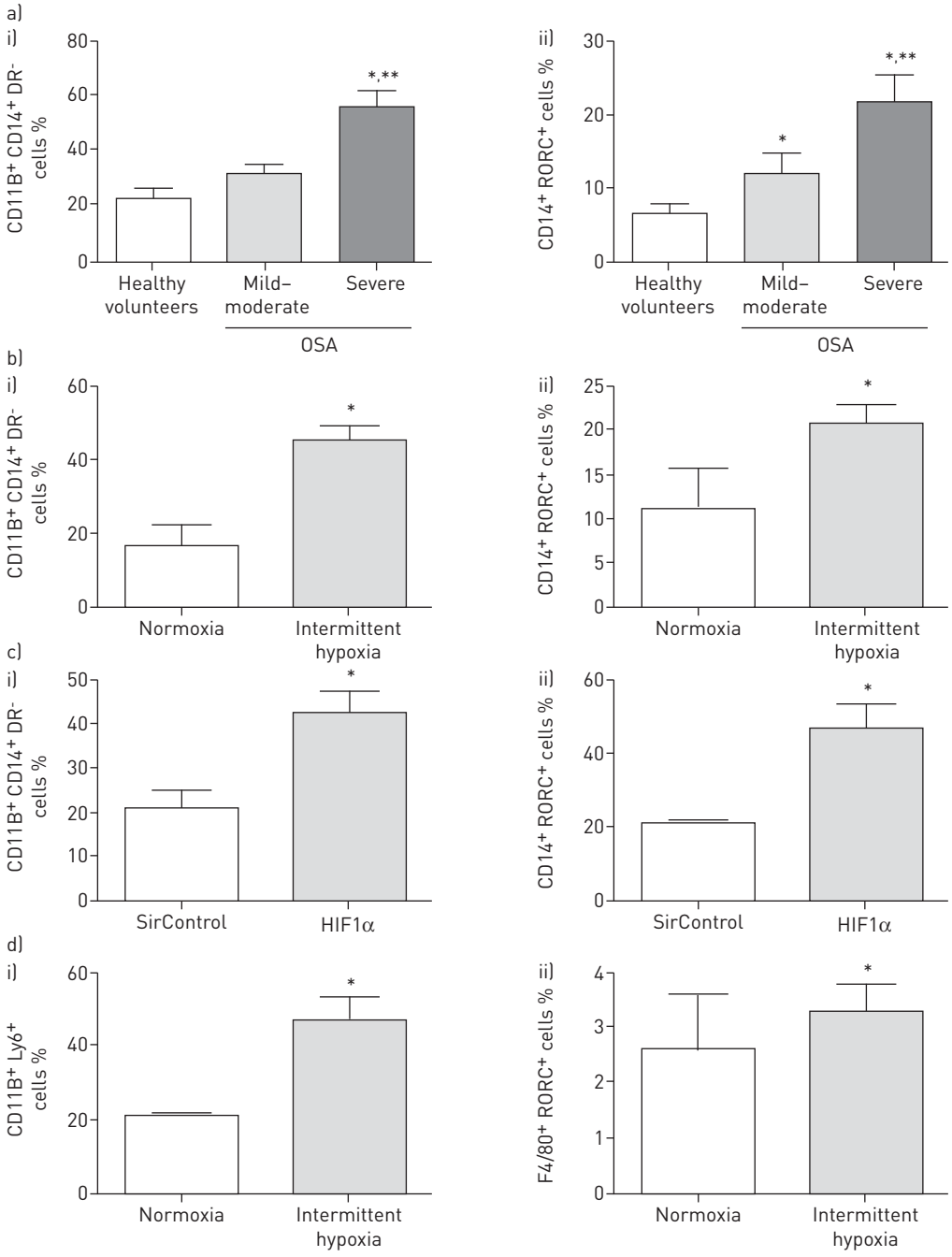

FIGURE 7 Myeloid-derived suppressor cell and retinoic acid related orphan receptor (RORC) expression analysis. a) Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers (n=20, randomly selected) and patients with mild–moderate OSA (n=18, randomly selected) and severe OSA (n=12, randomly selected); the cells were analysed by flow cytometry. i) percentage of $CD11B^+CD14^+DR^-$ cells; ii) gating strategy for the detection of $CD14^+$ cells; percentage of $RORC^+$ cells. *: p<0.05 *versus* mild-moderate OSA; **: p<0.01 *versus* healthy volunteers by ANOVA using the Kruskal–Wallis test with Dunn's multiple comparison; error bars are SEM. b) PBMCs isolated from healthy volunteers were cultured under normoxia (n=7) or intermittent hypoxia (n=7). i) Percentage of $CD11B^+CD14^+DR^-$ cells; ii) gating strategy for the detection of $CD14^+$ cells; percentage of $RORC^+$ cells. *: p<0.05, using the Mann–Whitney test; error bars are SEM. c) $CD14^+$ monocytes isolated from healthy volunteers were nucleofected with control plasmid (SirControl) (0.5 μg, n=7) or HIF1α plasmid (0.5 μg, n=7) for 16 h. i) percentage of $CD11B^+CD14^+DR^-$ cells; ii) gating strategy for the detection of $CD14^+$ cells; percentage of $RORC^+$ cells. *: p<0.05 using the Mann–Whitney test to compare normoxia; error bars are SEM. d) Isolated spleen cells from the normoxic mouse (n=6) group and the intermittent hypoxia mouse model (n=7) were analysed. i) percentage of $CD11b^+Ly6C^+$ cells. ii) gating strategy for the detection of $F4/80^+$ cells; percentage of $RORC^+$ cells. *: p<0.05 using the Mann–Whitney test to compare normoxia; error bars are SEM.

severe OSA might constitute a justification for the previously described higher incidence and aggressiveness of cancer. Increased PD-L1 has been associated with a higher incidence of some tumours [31] and with a poor prognosis in several cancers [7, 32], whereas the inhibition of PD-L1 with antibodies improves overall survival rates in patients with these cancers [7]. In turn, overexpression of PD-L1/PD-1 has also been linked to a poorer response to chemotherapy [33].

In addition to the effect of upregulation of the PD-L1/PD-1 pathway on T-cell activity, it is necessary to consider its role as an MDSC inducer, one of the major components of the immunosuppressive network responsible for immune cell tolerance in cancer [3]. At the tumour site, hypoxia modulates the MDSCs to differentiate into tumour-associated macrophages (TAMs) [34]. Moreover, we have reported that OSA monocytes exhibited a TAM-like phenotype [18]. In contrast, the MDSCs lacking HIF1α did not differentiate into TAMs within the tumour microenvironment [15]. In this regard, it has been demonstrated in a murine model that intermittent hypoxia increases the TAM population [35]. The increase of MDSCs detected in patients with severe OSA support the low proliferation of $CD8^+$ in response to a stimulus, as well as their low cytotoxicity capacity [15, 28].

Finally, the cytotoxic immune dysfunction induced by PD-L1/PD-1 upregulation in patients with OSA might also increase susceptibility to some chronic infections. Indeed, some virus or bacterial infections have been associated with augmented expression of the PD-L1/PD-1 pathway [36, 37].

In conclusion, the present study shows that intermittent hypoxia induces overexpression of the PD-L1/PD-1 immune checkpoint in patients with severe OSA, which reduces autologous T-cell proliferation and the cytotoxic activity of $CD8^+$ T-cells and increases the recruitment of MDSCs. Overall, these findings provide biological plausibility to recent clinical and epidemiological data suggesting that OSA is associated with an increased incidence and aggressiveness of cancer, as well as a higher risk of infections.

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