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ORIGINAL ARTICLE

# Differential effect of intermittent hypoxia and sleep fragmentation on PD-1/PD-L1 upregulation

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## Abstract

Immunosurveillance is compromised in patients with obstructive sleep apnea (OSA) as reflected by overexpression of the programmed death cell receptor and its ligand (PD-1/PD-L1) coinhibitory axis. However, the contributions of intermittent hypoxia (IH) and sleep fragmentation (SF) are unclear. We therefore evaluated the expression of PD-1 and PD-L1 on immune cells from mice subjected to IH or SF, and in human cells exposed to IH, oxidative stress, or both conditions. Six-week-old male C57BL/6J mice were exposed to either IH or SF using previously established in vivo models. Moreover, human peripheral blood mononuclear cells (PBMC) were cultured overnight under normoxia, IH, hydrogen peroxide ($H_2O_2$), or both. Murine splenocytes and human PBMC were isolated, and labeled using surface-specific antibodies for flow cytometry analysis. Compared to control mice, IH induced higher expression of PD-L1 on F4/80 cells and of PD-1 on $CD4^+$ and $CD8^+$ T-cells, whereas no significant changes emerged after SF. In vitro models of IH and oxidative stress showed similar changes for expression of PD-L1 on human monocytes and PD-1 on $CD4^+$ T-cells. Furthermore, $H_2O_2$ increased PD-1 expression on $CD8^+$ T-cells, compromising their cytotoxic capacity assessed by perforin expression, similar to IH. No evidence of synergistic effects was apparent. Therefore, PD-1/PD-L1 upregulation reported in patients with OSA appears to be preferentially mediated by IH rather than SF.

### Statement of Significance

The increased risk of cancer associated to obstructive sleep apnea (OSA) has been attributed, among other potential mechanisms, to a deregulation of the immune surveillance system, which allows the release and progression of tumor cells. In fact, patients with severe OSA experience an up-regulation of the PD-1/PD-L1 coinhibitory axis, which suppresses T-cell cytotoxicity and has a major role in the cancer development. However, the contribution of major OSA components to PD-1/PD-L1 expression is not known. From in vivo and in vitro models, we show that while intermittent hypoxia exposures mimicking sleep apnea increase the expression of PD-1/PD-L1 in circulating immune cells, sleep fragmentation does not induce discernible alterations or show a synergistic effect on this pathway.

**Key words:** intermittent hypoxia; sleep fragmentation; oxidative stress; sleep apnea; immune system; cytotoxicity

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## Introduction

Obstructive sleep apnea (OSA) is characterized by repetitive obstructions of the upper airway during sleep that result in intermittent hypoxia (IH) and sleep fragmentation (SF). OSA has been associated with a higher risk of cancer, as well as poorer cancer outcomes [1–3]. In recent years, several experimental and clinical studies have largely demonstrated that OSA promotes cancer incidence, progression, aggressiveness, and mortality rates [4–11].

Among the potential mechanisms linking OSA and cancer, it has been suggested that IH-dependent immune deregulation may compromise the immune surveillance system and favor the release and progression of tumor cells [12]. Several reports have demonstrated that IH can induce altered polarization of tissue macrophages [13] as well as impaired maturation and activity of natural killer cells [14, 15]. Furthermore, IH also compromises the pathway of the programmed cell death receptor 1 (PD-1) and its ligand (PD-L1) [16], which plays a critical role in the development and progression of cancer and is currently one of the main targets for cancer immunotherapy [17, 18]. In patients with OSA, the PD-1/PD-L1 coinhibitory axis is up-regulated by the activation of hypoxia-inducible factor (HIF)-1, and such changes are associated with decreases in both the proliferative capacity of T-cells and their cytotoxicity [19, 20].

We should also point out that the other hallmark alteration of sleep in OSA, SF, can also accelerate tumor growth and invasiveness through the promotion of tumor-associated macrophage polarity changes and proinflammatory signaling [21]. In fact, it has been reported that SF is a powerful inducer of oxidative stress and inflammatory pathways [22, 23], with quick up-regulation of proinflammatory cytokine gene expression [24]. Moreover, in murine models, it has been described that both IH and SF can favor the escape of tumor cells by decreasing intratumoral $CD8^+$ T-cell cytotoxicity [25]. Although up-regulation of the PD-1/PD-L1 pathway can also be induced by an increased inflammatory response [17], no information is available about the effect of SF on its regulation.

Since detailed knowledge of the intermediate mechanisms and pathogenic pathways involved in the alteration of immune surveillance in sleep apnea might be important for both the identification of biomarkers and for the elaboration of future therapeutic strategies, we hypothesized that SF could also induce higher expression of the PD-1/PD-L1 axis or exert a synergistic effect with IH. Therefore, our objective was to determine the expression of PD-1 and PD-L1 using in vivo and in vitro models of IH and SF, either alone or in combination.

## Methods

### Mouse models of intermittent hypoxia and sleep fragmentation

The study was approved by the Ethics Committee for Animal Research of 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 14 mice were randomly assigned to IH exposure ($n$ = 7 mice) or normoxia ($n$ = 7 mice) for 6 weeks, as previously reported [26]. Briefly, IH was achieved by varying the nitrogen and oxygen concentrations in the mouse cages (26 cm long, 18 cm wide, and 6 cm high) via automated, computer-controlled gas exchange systems. Sixty hypoxic events/h (40 s of room air at 20% $O_2$ and 20 s of hypoxic air at 5% $O_2$) corresponding to criteria attributable to severe OSA were applied to the mice for 6 h/day during the light period (10 a.m.–4 p.m., corresponding to the usual sleep/rest period of mice). Using the very same setting, we independently verified that this model subjected the mice to intermittent hypoxemia: oxygen saturation levels ($SaO_2$) ranging from a maximum of 95.4 ± 0.1% to a minimum of 62.3 ± 3.5% [27]. Control mice breathing normoxic gas were placed in an identical system, but the hypoxic gas from the reservoir was replaced by room air.

### Study participants

Fifteen healthy volunteers aged 20–65 years were randomly selected from the reference population of our medical center. They were considered healthy if they were lifetime never-smokers, had no known respiratory or cardiovascular disease, and a diagnosis of OSA had been excluded by respiratory polygraphy or polysomnography. The exclusion criteria were: history of chest injuries; exposure to substances known to cause lung injury; respiratory symptoms during the previous 12 months (dyspnea, chronic cough, wheezing, or phlegm); occasional illness in the previous 3 months; morbid obesity (BMI > 40 kg/m$^2$); hypertension or hypotension; clinically relevant alterations during physical examination of the heart, lungs, and chest wall; abnormal chest radiographs; major ECG abnormalities; diabetes (self-reported or fasting glucose level >126 mg/dL); vaccination; and use of immune-modifying drugs, dietary supplements, diuretics, cardiac glycosides, or ß-adrenergic blocking agents. The study was approved by the local Ethics Committee (PI-1857), and informed consent was obtained from all subjects.

### Cell culture

Twenty milliliters of blood were obtained from the healthy volunteers between 8 a.m. and 9 a.m. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Plus gradient (GE Health Care Bio-Sciences, Buckinghamshire, UK) [30]. The monocytes were enriched by adherence for 1 h in media culture without fetal bovine serum, as we have previously described [30]. Roswell Park Memorial Institute (RPMI) medium (Invitrogen, Carlsbad, CA) was used for the cell cultures.

### In vitro models of intermittent hypoxia and oxidative stress

PBMC from each healthy volunteer were plated in 6-well plates and then randomly exposed to normoxia (21% $O_2$, 5% $CO_2$, 37°C), IH, oxidative stress, or the combination of these challenges. All protocols were maintained overnight. The IH exposure was performed in custom-designed incubation chambers attached to an external $O_2/N_2$ computer-driven controller using BioSpherix-OxyCycler-42 (Redfield, NY). This system is capable of generating periodic changes in $O_2$ concentrations that control air gas levels in each chamber, while individually maintaining $CO_2$ as previously described in detail [31]. Our IH model cycling $O_2$ saturation in the medium at 1% for 2 min, followed by 20% for 10 min with $CO_2$ maintained at 5%. The oxidative stress assay was conducted using $H_2O_2$ (Sigma–Aldrich, Darmstadt, Germany) at different concentrations, as previously described [32]. On the following day, we evaluated the reactive oxygen species (ROS) production using a CellROX Reagent (Life Technology, Pasley, UK) at a final concentration of 5 µM for 30 min at 37°C, following the manufacturer's instructions. Additionally, we evaluated cell

survival using 7AAD (BD-Pharmigen; BD Biosciences) by flow cytometry. After verifying both in normoxia and in IH that 30μM of $H_2O_2$ increased the expression of ROS on CD14⁺ without compromising cell viability assessed by 7-amino actinomycin D (7-AAD) fluorescence (Figure 1, Supplementary Material), this concentration was selected for all subsequent experiments.

### Flow cytometry analysis

PBMC were isolated and surface stained with the specific antibodies (CD14, PD-L1, PD-1, and perforin [PF]; BD Bioscience, Ghent, Belgium). The isolated splenocytes from the mice were labeled using surface-specific antibodies (F4/80, PD-L1, and PD-1, BD Bioscience). Moreover, the live/dead cell discrimination was performed using 7AAD (0.25μg/sample) 5–10 min prior to acquisition, which was conducted using FACSCalibur flow cytometer (BD Biosciences), and collected data were analyzed using FlowJo v10 (FlowJo, Ashland, OR).

### Statistical analysis

Data are presented as mean ± SEM. In the animal models, comparisons between the experimental and control situations were performed using the Mann–Whitney test. Comparisons among the four subgroups of the in vitro experiments were performed using analysis of variance with post hoc Dunnett tests. For all analyses, a $p$ value less than 0.05 was considered statistically significant. The analyses were conducted using Prism 5.0 (GraphPad) and SPSS 23.0 (Chicago, IL) software.

## Results

### PD-L1/PD-1 immune checkpoint expression in the in vivo mouse models

Animals under IH exhibited augmented PD-L1 expression on F4/80 cells compared to controls (26.58 ± 8.26 vs 34.17 ± 3.3%, $p = 0.0028$, Figure 1A). Moreover, we also observed a consistently higher PD-1 expression on T-cells in the IH model. Both the expression of PD-1 on CD4⁺ and on CD8⁺ was higher after IH than in normoxia (17.68 ± 3.72 vs 35.92 ± 5.31%, $p = 0.0159$ and 31.4 ± 1.92 vs 38.6 ± 4.32%, $p = 0.0286$, respectively) (Figure 1B and C).

In contrast, SF exposures were not associated with a detectable significant effect in PD-L1 expression on F4/80 cells compared to control animals (27.78 ± 5.78 vs 31.46 ± 3.88%, $p = 0.138$) (Figure 2A). Similarly, no significant changes in the PD-1 expression on any of the T-cell subsets were detected in the SF-exposed mice. PD-1 expression on CD4⁺ did not differ between animals under SF and control animals (16.88 ± 6.15 vs 17.07 ± 8.12%, $p = 0.847$) (Figure 2B), and PD-1 expression on CD8⁺ was similar for the two experimental groups as well (32.15 ± 11.11 in SF vs 34.65 ± 12.87% in controls, $p = 0.603$) (Figure 2C).

### PD-L1/PD-1 immune checkpoint expression in the in vitro models

As the lack of the suppressive effect of SF on PD-L1/PD-1 expression in the mouse model could reflect a species differentiation between mice and humans, we decided to perform an in vitro oxidative stress model using human monocytes from peripheral blood, mimicking the effects of OSA, since both IH and SF are associated with increased oxidative stress. The application of this model allows us to compare the in vivo effect of oxidative stress on PD-1/PD-L1 expression with that obtained in cells cultured under normoxia, IH, or the combination of IH and oxidative stress (Figure 3).

In agreement with the mouse model, human PBMC cultures under IH conditions increased PD-L1 expression on human monocytes compared to normoxia (26 ± 9.26 vs 4.75 ± 2.41%, $p = 0.0086$) as well as the PD-1 expression in both CD4⁺ (29.98 ± 6.99 vs 15.41 ± 3.45%, $p = 0.0035$) and CD8⁺ T-cells (34.82 ± 8.23 vs 20.74 ± 5.86%, $p = 0.0321$). However, $H_2O_2$ exposures elicited no detectable changes in PD-L1 expression in human monocytes (4.75 ± 2.4 vs 10.44 ± 5.85%) and no changes in PD-1 expression in CD4⁺ T-cells (16.79 ± 8.49 vs 15.41 ± 3.45%). However, PD-1 expression on CD8⁺ T-cells was significantly increased under oxidative stress conditions (27.48 ± 13.79 vs 20.74 ± 5.86%, $p = 0.015$) (Figure 3). Finally, these experiments did not reveal any evidence for a synergistic effect of oxidative stress on the IH-induced PD-1/PD-L1 overexpression in any of the three populations of immune cells (Figure 3).

### Effect of IH and oxidative stress on cytotoxic function of CD8⁺ T-cells

To study the relevance of the PD-1 overexpression on CD8⁺ T-cells induced by both IH and oxidative stress, we analyzed the expression of perforin, one of the primary surface markers of cytotoxic function. We found significantly decreased expression of perforin on human CD8⁺ T-cells cultured under IH or under $H_2O_2$ conditions, as well as following exposures to the combination of both IH and $H_2O_2$ (Figure 4). Collectively, these data show that both IH and oxidative stress can impair the in vitro cytotoxic activity of human CD8⁺ T-cells.

## Discussion

The results of the murine experiments show that while IH exposures mimicking sleep apnea increase the expression of PD-1/PD-L1 in circulating immune cells, SF does not induce discernible alterations in this pathway. These results confirm that IH strongly mediates up-regulation of PD-1/PD-L1, in agreement with previous evidence indicating that PD-L1 production is dependent on HIF-1 binding chromatin sites related to the PD-L1 promoter in human monocytes [33]. As a whole, our findings establish that the overexpression of the PD-1/PD-L1 axis identified in patients with severe OSA [19, 20] is dependent on episodic hypoxia, as previously corroborated by in vivo and in vitro models, as well as by HIF-1 transfection [19], while it seems to be less dependent on the alterations caused by SF. It should be mentioned that a possible difference between the IH and SF models is the potential level of stress induced in the animals. Indeed, it has been reported that IH increases corticosterone in plasma [34], while SF does not [28].

Furthermore, our in vitro model confirms that oxidative stress, a common consequence of both IH and SF, has no effect on the expression of PD-L1 in monocytes or PD-1 in CD4⁺ T-cells. However, oxidative stress induces increased expression of PD-1 in CD8⁺ T-cells, the latter translating into reduced cytotoxic capacity

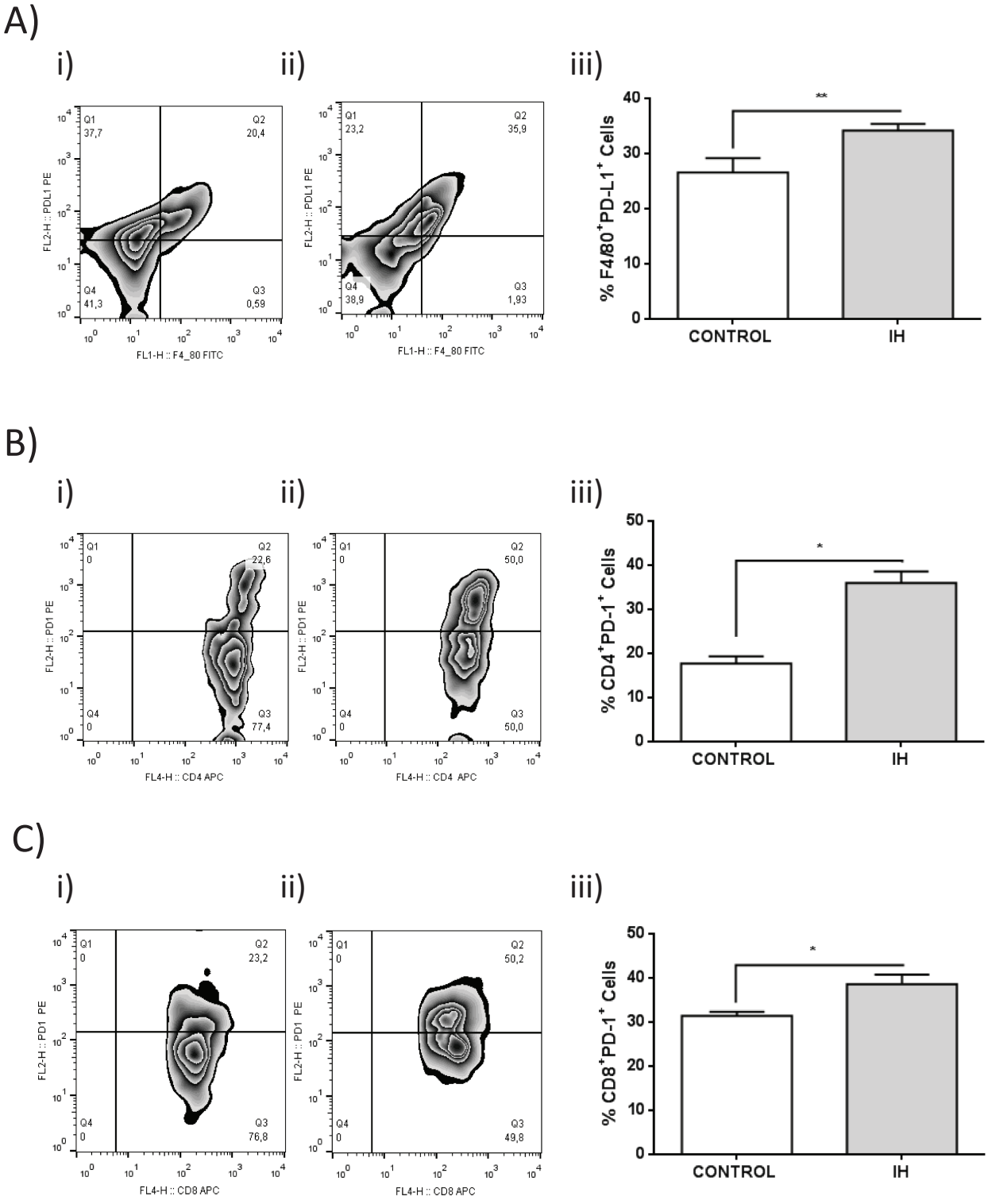


**Figure 1.** Expression of programmed cell death receptor (PD-1) and its ligand-1 (PD-L1) in the intermittent hypoxia murine model. Isolated spleen cells from mice under normoxia ($n = 6$) or under intermittent hypoxia ($n = 7$) were analyzed using flow cytometry. (A) Representative dot blots of PD-L1 expression on F4/80$^+$ cells for (i) normoxic and (ii) intermittent hypoxia conditions; (iii) percentage distribution of PD-L1$^+$ on F4/80$^+$ cell subset. (B) Representative dot blots of PD-1 expression on CD4$^+$ T-cells for (i) normoxic and (ii) intermittent hypoxia conditions; (iii) percentage distribution of PD-L1$^+$ on CD4$^+$ T-cells. (C) Representative dot blots of PD-1 expression on CD8$^+$ T-cells for (i) normoxic and (ii) intermittent hypoxia conditions; (iii) percentage distribution of PD-1$^+$ on CD8$^+$ T-cells. Error bars: SEM. $^*p < 0.05$ and $^{**}p < 0.01$ using the Mann–Whitney *U*-test.

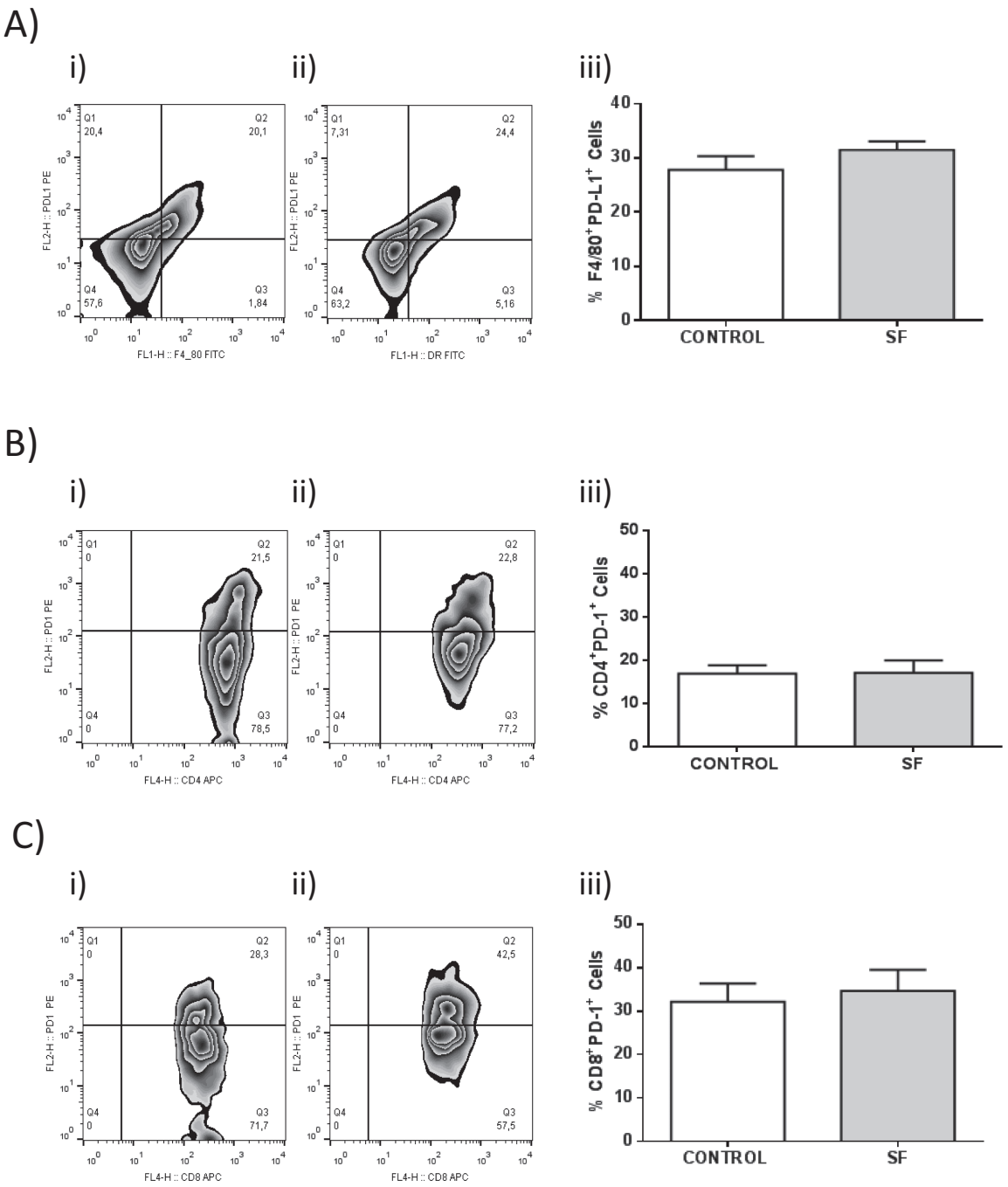


**Figure 2.** Expression of programmed cell death receptor (PD-1) and its ligand-1 (PD-L1) in the sleep fragmentation murine model. Isolated spleen cells from mice under control conditions ($n = 7$) and after sleep fragmentation ($n = 7$) were analyzed using flow cytometry. (A) Representative dot blots of PD-L1 expression on F4/80$^+$ cells for (i) control and (ii) sleep fragmentation; (iii) percentage distribution of PD-L1$^+$ on F4/80$^+$ cell subset. (B) Representative dot blots of PD-1 expression on CD4$^+$ T-cells for (i) control and (ii) sleep fragmentation; (iii) percentage distribution of PD-1$^+$ on CD4$^+$ T-cells. (C) Representative dot blots of PD-1 expression on CD8$^+$ T-cells for (i) control and (ii) sleep fragmentation; (iii) percentage distribution of PD-1$^+$ on CD8$^+$ T-cells. Error bars: SEM. Comparisons performed using the Mann–Whitney *U*-test.

of CD8$^+$ T-cells, the primary effectors of the adaptive immune response against cancer, as a consequence of the well-known effect of the PD-1/PD-L1 immune checkpoint on T-cell priming [35, 36]. This finding reinforces that CD8$^+$ T-cells are particularly susceptible to PD-1 overexpression, probably due to changes in cellular metabolism, and coincides with the previous description in patients with severe OSA [19] and in subjects with other hypoxic disorders [33], whereby increased PD-1 expression imposes an immunosuppressive effect on CD8$^+$ T-cells, decreasing both their proliferative rates and cytotoxic capacity. In turn, the difference in the response to $H_2O_2$ among the different immune cell subsets could be explained by a different sensitivity to oxidative stress. Classically, it is known that, in the presence of ferrous ion, $H_2O_2$ acts as a substrate for the generation of the highly reactive hydroxyl radical $OH^-$, whose effect on the immune cells is concentration-dependent. In this context, CD8$^+$ T-cells are highly susceptible, B-cells highly resistant and CD4$^+$ T-cells are moderately susceptible [37], probably due to differences in the cells' redox status [38].

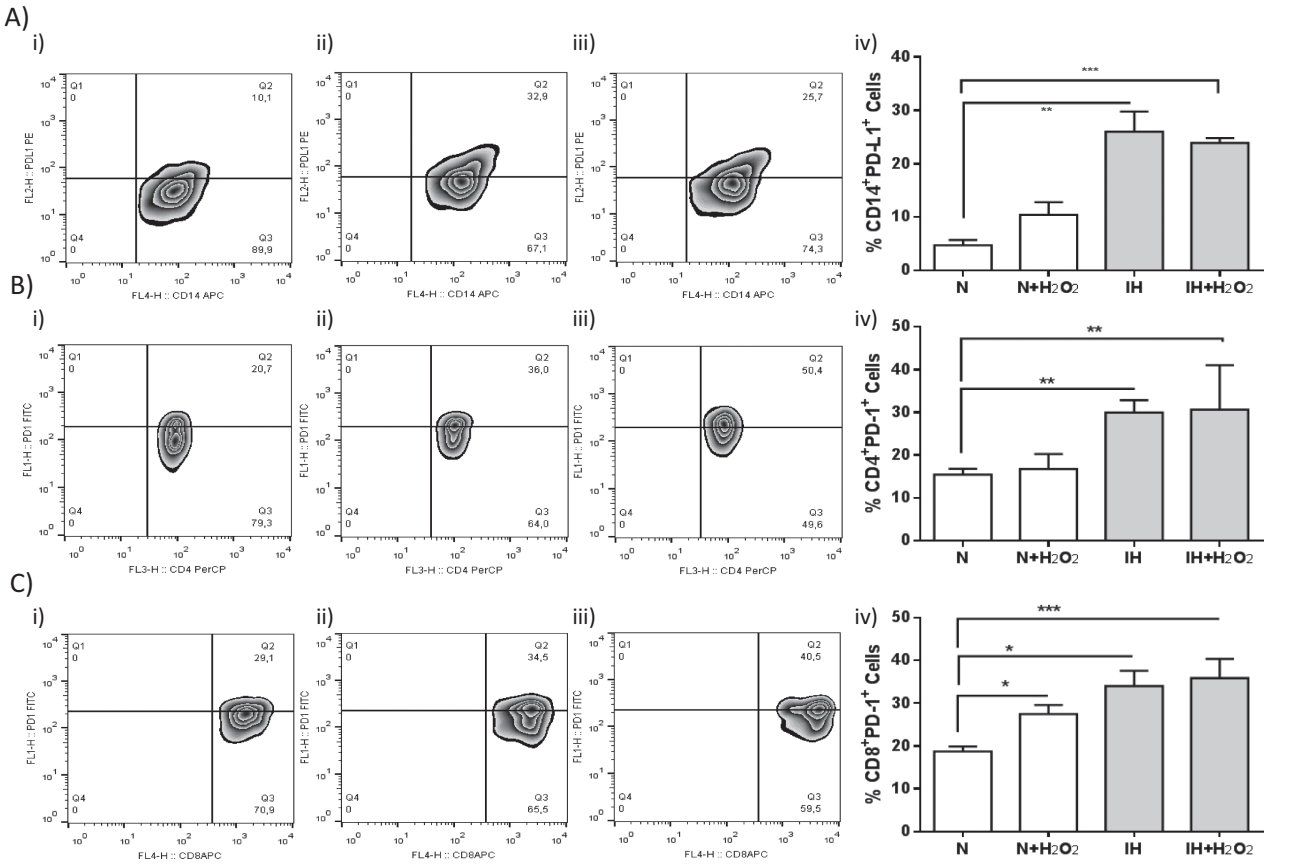


**Figure 3.** In vitro expression of programmed cell death receptor (PD-1) and its ligand-1 (PD-L1). Peripheral mononuclear blood cells were isolated from 15 healthy volunteers and randomly cultured under room air (white bars) with or without hydrogen peroxide ($H_2O_2$) treatment and intermittent hypoxia (gray bars) with or without $H_2O_2$ treatment. (A) Representative dot blots of PD-L1 expression on $CD14^+$ cells for (i) normoxia; (ii) normoxia with $H_2O_2$ treatment; (iii) intermittent hypoxia; (iv) intermittent hypoxia with $H_2O_2$ treatment; (v) percentage distribution of $PD\text{-}L1^+$ on $CD14^+$ cells in the four experimental conditions. (B) Representative dot blots of PD-1 expression on $CD4^+$ T-cells for (i) normoxia; (ii) normoxia with $H_2O_2$ treatment; (iii) intermittent hypoxia; (iv) intermittent hypoxia with $H_2O_2$ treatment; (v) percentage distribution of $PD\text{-}1^+$ on $CD4^+$ T-cells in the four experimental conditions. (C) Representative dot blots of PD-1 expression on $CD8^+$ T-cells for (i) normoxia; (ii) normoxia with $H_2O_2$ treatment; (iii) intermittent hypoxia; (iv) intermittent hypoxia with $H_2O_2$ treatment; (v) percentage distribution of $PD\text{-}1^+$ on $CD8^+$ T-cells in the four experimental conditions. Error bars: SEM. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ using ANOVA with post hoc multiple comparisons by the Dunnett test.

The discordance between the expression of PD-1 on $CD8^+$ T-cells induced by SF in mice and $H_2O_2$ in human cells could be interpreted as inherent aspects of the in vitro oxidative stress model used. Although the dose of $H_2O_2$ applied to cell cultures does not compromise the viability or proliferation of the immune cells evaluated, it may be markedly higher than that achieved in vivo as a result of SF episodes. In addition, treatment with $H_2O_2$ only reproduces one of the consequences of SF or IH, but does not allow us to evaluate other essential aspects, such as the increase in sympathetic tone or cellular metabolism. This is particularly relevant as it has been demonstrated that norepinephrine regulates T-cell function via alterations in mitochondrial metabolism and redox status [39], modifying their cytokine profiles [40], so that they could also modulate the response of these cells to $H_2O_2$.

Our experiments coincide in determining that oxidative stress does not exhibit synergism with IH in the induction of PD-1/PD-L1 overexpression. Notwithstanding, ROS can increase both the stability of HIF-1 and its transcriptional activity by inhibiting prolyl hydroxylases [41] and the phosphorylation of coactivator p300 by a $Ca^{2+}$/calmodulin-dependent protein kinase [42, 43], respectively. Moreover, HIF-1 translation is increased by ROS-dependent activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which affects the 5'-UTR (untranslated region) of HIF-1 mRNA [43, 44]. Under the influence of ROS, directly generated by exogenous $H_2O_2$, overexpression of NADPH oxidase subunit NOX4 induces an increase of HIF-1 protein following enhanced HIF-1 mRNA levels in pulmonary artery smooth muscle cells [45].

Therefore, high levels of ROS could promote HIF-1 activity and contribute to a greater expression of the PD-1/PD-L1 axis. However, the results from our in vitro model also do not confirm this assumption, since the expression of PD-1 or PD-L1 does not increase any further in the cells subjected to IH when $H_2O_2$ is then added. Thus, we should consider whether there is a ceiling effect in the modulation of PD-1/PD-L1 induced by HIF-1, or whether HIF-1 levels remain high throughout all the hypoxia–reoxygenation cycles. Likewise, it is interesting to consider that the IH-induced intracellular calcium accumulation activates the protein kinase C isoforms, which in turn activate mTOR, a kinase that promotes HIF-1 protein synthesis [41, 46], suggesting that mTOR activation maintains persistent elevation of HIF-1 during reoxygenation [41].

Our results disagree with previous studies conducted in mice injected with tumor cells which found that SF induces a suppressive effect on tumor-infiltrating lymphocytes [21]. In

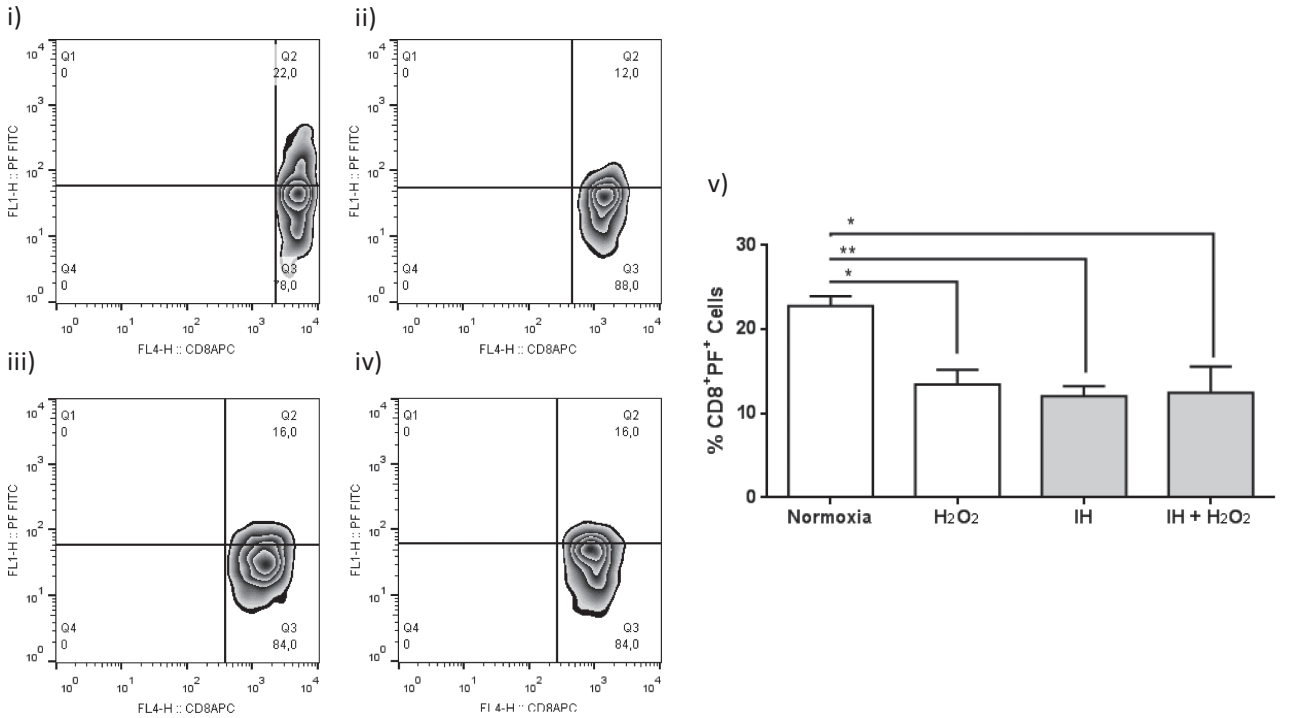


**Figure 4.** In vitro effect of intermittent hypoxia and oxidative stress on CD8⁺ T-cell activity. Peripheral mononuclear blood cells isolated from healthy volunteers ($n$ = 6, randomly selected) were cultured overnight under room air, hydrogen peroxide ($H_2O_2$) treatment, intermittent hypoxia, or intermittent hypoxia with $H_2O_2$ treatment. T-cells were analyzed by flow cytometry and gating strategy was used for the detection of CD8⁺ T-cells. The percentage of perforin (PF)⁺ CD8⁺ T-cells is shown. Representative dot blots of PF expression in CD8⁺ T-cells for (i) normoxia as control; (ii) intermittent hypoxia; (iii) normoxia with $H_2O_2$ treatment; (iv) intermittent hypoxia with $H_2O_2$ treatment; (v) percentage distribution of PF⁺ on CD8⁺ T-cells in the four experimental conditions. Error bars: SEM. $^*p < 0.05$ and $^{**}p < 0.01$ using ANOVA with post hoc multiple comparisons by the Dunnett test.

addition to being exogenous tumor cells and the possible concurrence of other local factors, such as necrosis and hypoxia of the tumor tissue, we should realize that these findings represent a different scenario. In these studies, the effect of SF on the local immune response was evaluated once the immunosurveillance system has been evaded, and an established tumor is already growing. Therefore, it is possible that the effect of SF is different systemically versus locally when a neoplastic process has already been established. Moreover, although SF is a potent inducer of oxidative stress and inflammatory pathways through the increase in NADPH oxidase 2 [22], there is much evidence to suggest that SF-induced increases in NADPH oxidase would be restricted to the central nervous system and other metabolically active tissues, such as visceral fat [47] or, hypothetically, peritumoral tissues [48]. Experimental SF models have demonstrated an upregulation of pro- and anti-inflammatory cytokine gene expression, but this response is not dependent on SF intensity and it is heterogeneous in various tissue locations [24]. Thus, while SF favors the polarization of macrophages toward an M2 phenotype in the periphery of the tumor tissue [22], in adipose tissue it promotes M1 polarization [24]. Moreover, it has been reported that serum corticosterone levels are similar in control and SF groups [49], reflecting insignificant systemic repercussions. On the other hand, it should also be considered that once a tumor process is established, PD-L1 expression in cancer cells is regulated by several signaling pathways, epigenetic factors, and other transcriptional factors, in addition to HIF-1 [18, 50]. However, even assuming the participation of other mechanisms, IH seems to maintain an important role in the regulation of the PD-1/PD-L1 axis in patients with already established cancer. In fact, we have recently described that the presence of OSA increases serum levels of soluble PD-L1 in patients with melanoma and that these levels are related to greater tumor aggressiveness and invasiveness [51].

We are aware that our study presents several limitations. First, in light of the impossibility to evaluate the individualized contribution of the main alterations triggered by sleep apnea on the over-regulation of the PD-1/PD-L1 axis in patients with OSA, we had to resort to both animal and in vitro models that tried to replicate the alterations presented by patients with severe OSA. Second, to simulate one of the main consequences of SF, we have used an in vitro model of oxidative stress induced by a previously validated $H_2O_2$ concentration [32], with the caveat that it is also induced by IH. However, it has already been mentioned that $H_2O_2$ does not reproduce all the mechanisms activated by either SF or IH, such as the increase in sympathetic tone. Third, our study focuses on the contribution of IH and SF to the overexpression of PD-1/PD-L1 in immune cells not yet exposed to tumor cells, since this immune checkpoint is the main mechanism for suppression of the immunosurveillance system in the event of exposure to neoplastic cells. Therefore, it may not represent the behavior in patients who already have an established tumor. And, fourth, the evaluation of the effect of IH and SF on this immune checkpoint is only intended to help identify the pathways by which immunosurveillance may be compromised in patients with severe OSA. Accordingly, based on the results of the present study, a recommendation cannot be made for the selection of patients at risk or for the establishment of an early therapeutic intervention.

In conclusion, our results confirm that IH compromises the activity of the circulating immune cells by increasing the

expression of the PD-1/PD-L1 axis, whereas SF is void of this effect. These findings may contribute toward better defining the pathogenic pathways of involvement of the immunosurveillance system in patients with OSA and confirm the predominant effect of IH on cancer risk in sleep apnea, as suggested by several clinical studies [4, 6, 7]. Therefore, adequate individualization of the risk of these patients may require the development and validation of strategies centered around OSA patients with more severe nocturnal hypoxemia.

## Supplementary Material

Supplementary material is available at *SLEEP* online.

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## Authors' contributions

CCZ, IA, RF, DG, and FGR conceived the study; CCZ, IA, EDG, VT, RC, RG, and ELC performed the experiments; CCZ, IA, ELC, RF, DG, and FGR contributed to data analysis and interpretation; CCZ and FGR contributed to drafting the manuscript; all authors reviewed and approved the final version of the manuscript.

*Conflict of interest statement*: The authors have no conflicts of interest to declare.

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