ORIGINAL ARTICLE

# Altered CD8+ T-Cell Lymphocyte Function and TC1 Cell Stemness Contribute to Enhanced Malignant Tumor Properties in Murine Models of Sleep Apnea

Mahzad Akbarpour, PhD[1]; Abdelnaby Khalyfa, PhD[1]; Zhuanghong Qiao, PhD[1]; Alex Gileles-Hillel, MD[1]; Isaac Almendros, PhD[1,2]; Ramon Farré, PhD[2]; David Gozal, MD, MBA[1]

*[1]Department of Pediatrics, Pritzker School of Medicine, The University of Chicago, Chicago, IL; [2]Unitat Unitat de Biofísica i Bioenginyeria, Facultat de Medicina, Universitat de Barcelona-CIBERES-IDIBAPS, Barcelona, Spain*

**Study Objective:** The presence of obstructive sleep apnea (OSA) in patients with cancer appears to be accompanied by poorer outcomes. However, the mechanisms underlying such association are unknown. Tumor infiltrating lymphocytes (TILs), including CD8+ T cells, function as cytotoxic T lymphocytes (CTLs) and mount immune responses to cancer by the release of cytolytic enzymes, including granzyme B (GzmB), perforin (Prf), and cytokines such as interferon (IFN)-γ.
**Methods:** Using established in vivo mouse models, we investigated CD8+ T cells and cancer stem cells (CSCs) in intermittent hypoxia (IH) and sleep fragmentation (SF) in the context of tumor environment.
**Results:** Both IH and SF promoted increased tumor growth and invasion toward adjacent tissues compared to controls. The number and frequency of GzmB-producing CD8+ T cells per milligram of tumor tissue was significantly reduced in IH-exposed mice with impaired cytolytic function in both the groups and correlated with tumor weight. We also found that Oct4+ and CD44+CD133+ expressing CSCs were considerably increased in IH and SF tumors, respectively.
**Conclusions:** Reductions in GzmB in intratumoral CD8+ T cells in combination with the changes in tumor microenvironment that maintain the ability of CSCs to self-renew and even confer this capability to the nonstem population are compatible with reduced immunosurveillance and adverse tumor outcomes in animal models of OSA.
**Keywords:** cytotoxic T-lymphocytes, granzyme, cancer stem cell, obstractice sleep apnea.

**Statement of Significance**
Intermittent hypoxia and sleep fragmentation alter effector T cell lymphocyte function to reduce cytotoxic activity against malignant cells while concurrently increasing the stemness of the tumor and increasing its malignant properties.

## INTRODUCTION

Obstructive sleep apnea (OSA) is characterized by repetitive obstructions of the upper airway during sleep that results in intermittent hypoxia (IH), increased inspiratory efforts, and sleep fragmentation (SF). Recent epidemiological studies have linked OSA with enhanced cancer aggressiveness and mortality.[1,2] Using tumor xenografts in murine models exposed to either IH and SF exposures, evidence has emerged on the role played by these constitutive perturbations that characterize OSA in enhancement of tumor proliferation, invasion, and metastasis.[3–5]

Among the potential mechanisms linking OSA and cancer, the role of immune cells is a very likely player,[6] with contributions of macrophages to this process having already been uncovered.[7] Cytotoxic T lymphocytes (CTLs) are adaptive immune agents responsible for tissue surveillance and the elimination of tumor cells effecting a cellular response against transformed cells. Tumor infiltrating lymphocytes (TILs), including CD8+ T cells, function as CTLs and mount immune responses against cancer through the release of cytolytic enzymes, including granzyme B (GzmB), perforin (Prf), and cytokines such as interferon (IFN)-γ,[8] and their molecular signatures can predict tumor recurrence and have important implications for treatment strategies.[9] However, hypoxia dampens lymphocyte activation and costimulation,[10] diminishes lymphocyte proliferation, and reduces the ability of activated T cells to produce cytokines or lytic enzymes. Thus, hypoxia is immunosuppressive, and metabolic reprogramming due to increased activity of hypoxia inducible factor 1α (HIF-1α) may contribute to the attenuated immune antitumoral responses.[11] Hypoxia not only affects protective immune responses but also promotes tumorigenesis by enhancing proliferation of cancer cells and increasing their programmed cell death protein ligand 1 surface expression.[12] However, recent reports suggested that hypoxia and increases in HIF-1α activity may also boost effector T cell function, especially production of the lytic enzymes, GzmB and Prf, such that the role of IH in immune deregulation remains speculative at best.[13]

Changes in cancer cell phenotype could also operate as another potentially unexplored mechanism in the context of OSA effects on cancer. Indeed, low levels of $O_2$ can increase the cancer stem cell fraction in tumor stroma and promote acquisition of a stem-like state. Furthermore, hypoxia promotes maintenance of cancer stem cell populations and reprograms nonstem cells toward stem cell behavior.[7] In the context of SF, the mechanisms involved in oncogenesis are less well established thus far. Indeed, in addition to involvement of macrophages via a toll-like receptor 4 pathway[5] and NADPH oxidase,[14] we have recently uncovered that circulating exosomes in plasma in the context of experimental SF exposures contain a set of differentially expressed microRNAs that foster the malignant properties of TC-1 solid tumors in mice.[15] However, it is likely that multiple additional mechanisms are involved.

To assess the effect of IH and SF on tumor infiltrating CD8+ T cells, we used a lung epithelial tumor model (TC1 cells). We hypothesized that the adverse effects of IH and SF on tumor proliferation and invasion would be mediated not only by TAMs but also via alteration in CTLs function and direct effect on cancer stem cells (CSCs) fraction. To determine whether CSCs and CTLs exhibit altered phenotype and/or function under IH and SF conditions, CSCs and TILs from tumors were assessed

for surface markers with flow cytometry and in vitro cytolytic activity.

## MATERIALS AND METHODS

### Cells and Reagents

Epithelial lung tumor cells TC1 (ATCC, CRL-2785) were purchased from American Type Culture Collection (Manassas, VA). All media and supplements were acquired from Gibco (Grand Island, NY). Tumor cells were cultured in RPMI supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 0.1 mM nonessential aminoacids, 100 U/Penicillin / 100 µg/mL streptomycin, 10% fetal bovine serum, and geneticin 0.4 mg/mL. All antibodies were obtained from BioLegend (San Diego, CA): CD3-FITC (clone 145-2C11), CD45-APC (clone 30-F11), CD45-FITC (clone 30-F11), CD44-PE/Cy7 (clone IM7), CD62L-PB (clone MEL 14), CD25-PerCP (clone PC61), Foxp3-APC (clone MF-14), CD39-PE (clone Duha 59), KLRG1-FITC (clone 2F1), CD4-APC/Cy7 (clone RM4-5), CD8-PercP (clone 53–6.7), FoxP3-PE (clone MF-14), GzmB-APC (clone GB11; BD Bioscience), CD133-PE (clone 315-2C11), and Oct3/4-FITC (clone 40/oct3; BD Bioscience,).

### Animals, In Vivo IH, and Epithelial Lung Tumor Model

This study was carried out in C57BL/6J male mice obtained from Jackson Laboratories (Bar Harbor, ME). All experimental procedures were approved by the Institutional Animal Care & Use Committee of the University of Chicago (Animal Care and Use Procedure Certification number 72190). Mice (8 weeks old) were placed in commercially designed environmental chambers (Oxycycler C42; Biospheryx, Parish, NY) and were subjected to IH ($n$ = 15), as described previously.[4] Briefly, animals were exposed to alternating cycles of 90 s (6% $FiO_2$ followed by 21% $FiO_2$, 20 cycles/hr) for 12 hr/day. This pattern produces oxyhemoglobin saturations in the 65%–72% range, thereby mimicking the oxyhemoglobin desaturation events frequently encountered in moderate to severe OSA patients.[16–18] A custom-designed and validated SF paradigm was used to induce SF ($n$ = 16).[5] Briefly, the comercially available SF device relies on automated intermittent mechanically induced arousals of otherwise freely behaving mice in a standard laboratory mouse cage, using a near silent motorized mechanical sweeper (Model 80391; Lafayette Instruments, Lafayette, IN). This method does not require human contact and intervention or the introduction of foreign objects and touching of the animals during sleep. The selected SF paradigm consisted in inducing arousals at 2-min intervals between each sweep, implemented during the light period (7:00 a.m. to 7:00 p.m.). Four to five mice were housed in each cage to prevent isolation stress. A control group ($n$ = 14) was exposed to continuous circulating room air (RA) which also served as sleep control. Mice were preexposed during 2 weeks to RA, IH, or SF and were then injected subcutaneously with $10^5$ TC1 cells in the right flank. Seventeen days after injection, tumors became palpable in SF and IH groups, and they become visible in RA group around Day 20 to 21, at which time, tumor volume (V) was estimated by measuring its length (L) and width (W) with an electronic caliper ($V = W^2 \times L / 2$) every 3 days. After 28 days from tumor injection, mice were euthanized, and the disruption of capsule with presence of invasion toward the skeletal muscle was assessed visually and confirmed by histology by a blinded investigator as described previously.[4] Tumors were then excised, weighted, and samples were obtained for flow cytometry and CTL assay.

### Assessment of T Cells and Stem Cell Markers

Tumor samples were used for flow cytometry analyses, immunohistochemistry (CD8 or GzmB staining), and/or for isolation of CD8+ T cells (CTLs). Single-cell suspensions were prepared from the digested tumor tissues by cycles of washing and resuspension. Briefly, tumors were mechanically disrupted and incubated in collagenase IV solution, 1 mg/mL for 1 hr at 37°C. Cells were filtered through a 100-mm nylon mesh cell strainer, and CD8 T cells were isolated by magnetic labeling following the manufacturer's procedure (EasySep Mouse CD8 Positive Selection Kit; StemCell, 18770) and prepared for flow cytometry analyses, quantitative polymerase chain reaction (qPCR; IFNy_NM_008337.3_ Fw:ttcttcagcaacagcaaggc,rev:actccttttccgcttcctga; GzmB_NM_013542.2_ Fw: aca aag gca ggg gag atc at, Rev: cga ata agg aag ccc cca ca and Perforin_NM_011073.3_ Fw: tct tgg tgg gac ttc agc ttt, Rev: tgc ttg cat tct gac cga gt) or cytolytic T cell assays. In addition, we used stemness cell markers, namely, Oct4+ and CD44+CD133+, to assess changes in tumor cells.[19] Flow cytometry results were analyzed using FlowJo software. Data are expressed as mean ± standard error of the mean. RA, IH, and SF conditions were compared with Student's $t$ tests, analysis of variance followed by post hoc tests, or nonparametric testing as appropriate. Statistical significance was assumed at $p < .05$.

## RESULTS

### IH and SF Increase Tumor Size and Invasiveness and Alter CTLs Population

Both IH and SF promoted in vivo increased tumor growth and invasion toward adjacent tissues compared to RA (Fig 1A). Indeed, RA tumors weighed 851.3 ± 182.2 [SE] vs. 1509.8 ± 246.1 in IH ($p < .01$) and 1364.0 ± 211.9 mg in SF ($p < .04$). CTLs were defined as $CD3^+CD8^+$ $GzmB^+$ T cells (Figure 1B and C). Trends toward decreases in the absolute number of total $CD3^+CD8^+$ T cells as a function of tumor mass emerged in both IH and SF tumors (Figure 1B). In addition, the proportion of intratumoral regulatory T (Treg) lymphocytes (identified as $CD3^+$, $CD8^-$, $CD4^+$, $CD25^+$, Forkhead box $P3^+$ [FoxP3]) was increased in both IH (17.4% ± 3.9% of $CD3^+$, $CD4^+$, $CD8^-$ cells) and SF (16.4% ± 3.1%) when compared to RA (8.7% ± 1.8%; $p < .05$ vs. IH or SF).

Among CTLs, GzmB, a specific cytotoxic marker for CTLs, showed significant decreases in absolute numbers of GzmB-producing CTLs in IH-exposed mice but not among those exposed to SF (Figure 1C). However, further characterization of CTLs by qPCR showed considerable reductions in GzmB, Prf, and IFN-γ expression levels in both IH and SF tumors compared to RA tumors (Figure 2; $n$ = 11–12/experimental group). In addition, a significant inverse correlation emerged between

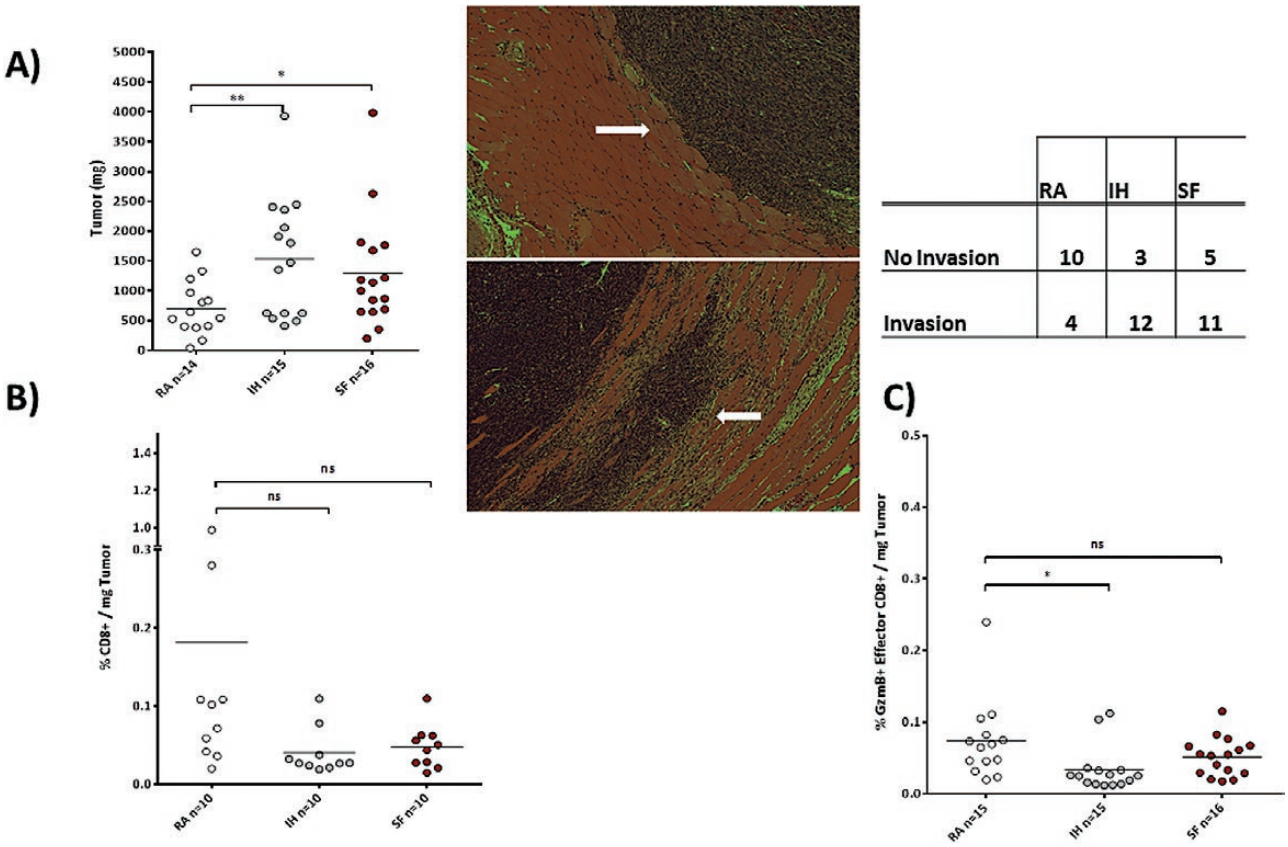


**Figure 1—**Mice were subjected to intermittent hypoxia (IH) consisting of alternating cycles of 90 s (6% $FiO_2$ followed by 21% $FiO_2$, 20 cycles/hr) for 12 h/day. This IH profile is associated with reproducible nadir of oxyhemoglobin saturations in the 68%–72% range. Another group of mice was exposed to sleep fragmentation (SF) via automated intermittent tactile stimulation of freely behaving mice in a standard laboratory mouse cage using a near-silent motorized mechanical sweeper.[5] Normoxic exposures in mice allowed to sleep normally served as controls (RA). (A) Individual TC1 tumor weights (IH vs. RA: $p < .01$, $n = 15$ and SF vs. RA: $p < 0.04$, $n = 16$) and the number of tumor-bearing mice with tumor invasion toward adjacent muscles (i.e., lateral external oblique and transversus abdominis) was more than triple in IH ($n = 12$) and SF ($n = 11$) than in RA ($n = 4$). (B) $CD8^+$ T cells showed no statistically significant differences in cell density in tumors from IH ($n = 10$) or SF ($n = 10$). (C) Flow cytometry analyses showed significant decreases in absolute numbers of granenzyme B (GzmB)-producing cytotoxic T lymphocytes (CTLs) infiltrating tumor in IH-exposed mice (IH vs. RA: $p < 0.02$, $n = 15$) but not among those exposed to SF.

GzmB expression in CTLs and tumor weight (Figure 2; $r = -0.48$; $p < .005$;).

### IH and SF Reduce CTLs Tumor Cytotoxicity

To further investigate the relevance of IH and SF effect on tumor cell escape from adaptive immunity, we used ex vivo-isolated CTLs from RA as effectors with tumor cells from RA, SF, or IH as targets in cytolytic assays (Figure 2E). These assays revealed that exposures of tumor-bearing mice to SF and IH resulted in significant decreases in CTL-mediated lysis and in increased escape of tumor cells from CTLs killing. This effect of SF and IH on resistance to CTLs-mediated lysis did not occur in RA tumor cells, since CTLs isolated from RA tumors effectively killed RA tumor cells but not IH- or SF-exposed tumor cells (Figure 2E).

### IH and SF Increase Stemness Markers in TC1 Tumor Cells

To determine the effect of IH and SF on CSCs, we performed FACS for known CSCs markers. We found that Oct4+ and CD44+CD133+ expressing CSCs were considerably increased in IH (Figure 3A) and SF tumors (Figure 3B), respectively.

## DISCUSSION

This study shows that IH and chronically fragmented sleep, highly prevalent conditions associated with a multiplicity of human disorders, lead to progressive tumor growth and invasiveness in mice models and that adverse effects are partly mediated by reduced intratumoral $CD8^+$ T cells function as effector CTLs and by maintaining the ability of CSCs to self-renew and even confer this capability to the nonstem cell population.

All immune cell types may be found in a tumor, including macrophages, dendritic cells, mast cells, natural killer cells, naive and memory lymphocytes, B cells, and effector T cells (including various subsets of T cell: T helper cells, T helper 1 (Th1) cells, Th2 cells, Th17 cells, Treg cells, T follicular helper cells, and cytotoxic T cells). Cytotoxic T cells are $CD3^+CD8^+$ effector T cells with cytotoxic granules that contain Prf and Gzms, which are released on interaction with target cells expressing cognate antigen. This process leads to the death of

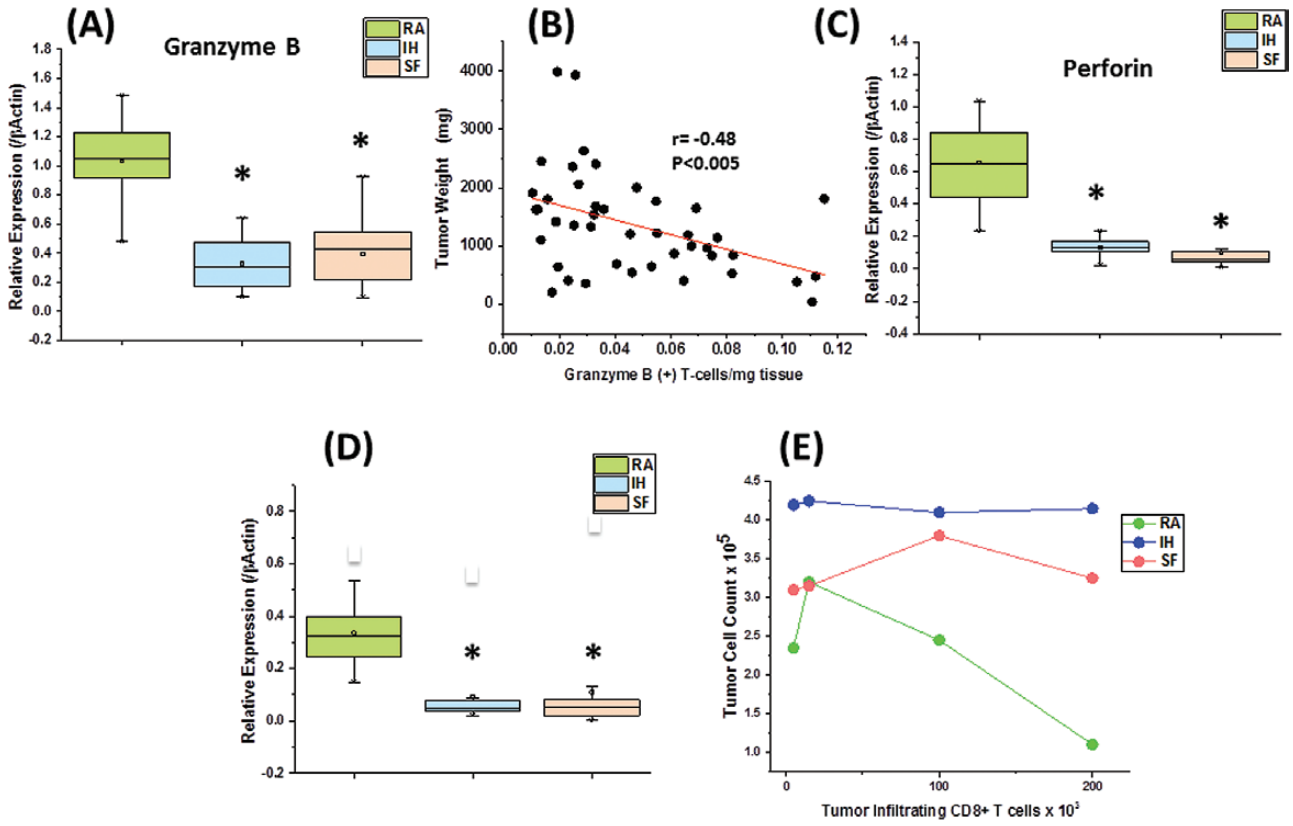


**Figure 2—**Characterization of CD8⁺ tumor infiltrating lymphocytes (TILs) by quantitative polymerase chain reaction (qPCR) showed reductions in granenzyme B (*GzmB*), *Perforin,* and interferon γ (*IFN*-γ) expression levels in both intermittent hypoxia (IH) and sleep fragmentation (SF) tumors (Panels A, C and D: $p < .001$ IH or SF vs. RA; $n = 11–12$/experimental group). A significant correlation emerged between tumor weight and the proportion of CD8⁺ TILs expressing *GzmB* (Panel B; $r = 0.48$; $p < .005$). Exposures of tumor-bearing mice to SF and IH resulted in significant decreases in cytotoxic T lymphocyte (CTL)-mediated lysis and in increased escape of tumor cells from CTLs killing. RA CTLs over RA tumor cells as target (green line), RA CTLs over SF tumor cells (red line), and RA CTLs over IH tumor cells (blue line; Panel E).

target cancer cells by apoptosis. As a corollary, the presence of a strong lymphocytic infiltration has been reported to be associated with good clinical outcomes in many different tumor types, including melanoma, head and neck, breast, bladder, urothelial, ovarian, colorectal, renal, prostatic, and lung cancer. Therefore, high densities of CD3⁺ T cells and CD8⁺ cytotoxic T cells were clearly associated with a longer disease-free survival (after surgical resection of the primary tumor) and/or overall survival.[20,21] CTLs recognize antigenic peptides presented by MHC class I molecules on the surface of target cells via the T cell receptor and CD8 and can mediate direct cytotoxic effects upon the release of Prf and GzmB.[22] CTLs are particularly efficient at controlling tumor progression when they produce high amounts of IFN-γ, a cytokine that has broad immunostimulatory effects and exert cytotoxic effects on endothelial cells from the tumor vasculature.[23]

The anticancer mediator GzmB controls the growth and spread of tumors, and reductions in GzmB in tumor-infiltrating CD8⁺ T cells are therefore compatible with reduced immunosurveillance and adverse tumor outcomes. We postulate that the presence of OSA in cancer patients may alter the tumor microenvironment and impair effector CTLs cell functionality, thereby enabling cancer cell escape.

Recent reports describing molecular connections between oxygen-regulated factors and pathways known to control stem cell function suggest a new mechanism, whereby hypoxia-induced factors may drive tumor growth through the generation or expansion of tumor-initiating cells, CSCs, and tumor invasion.[24,25] Oct4 is a member of a transcriptional network that regulates a large number of genes associated with cellular differentiation in embryonic stem cells.[26] The links between the HIFs, Notch, and Oct4 reveal specific molecular mechanisms, whereby oxygen responses can inhibit differentiation and, possibly, promote stem cell identity. They also raise the possibility of crosstalk between hypoxia and other stem cell signaling pathways. A number of experiments over the past decade support the idea that cancers can grow from a discrete subpopulation of malignant cells with stem cell properties (CSCs).[27,28] Oct4 and CD133 expression has also been detected in a variety of cancer cell lines and is induced by hypoxia in renal carcinoma cell line[29] and hepatocellular carcinoma.[30] Considering the association of hypoxia with CSCs[31] and the selective phenotypic shift of the tumor cell population toward increased cell growth and self-renewal, that is, stemness, following in vivo exposures to IH and SF reported herein, the changes in tumor microenvironment induced by IH and SF emerge as potential factors promoting

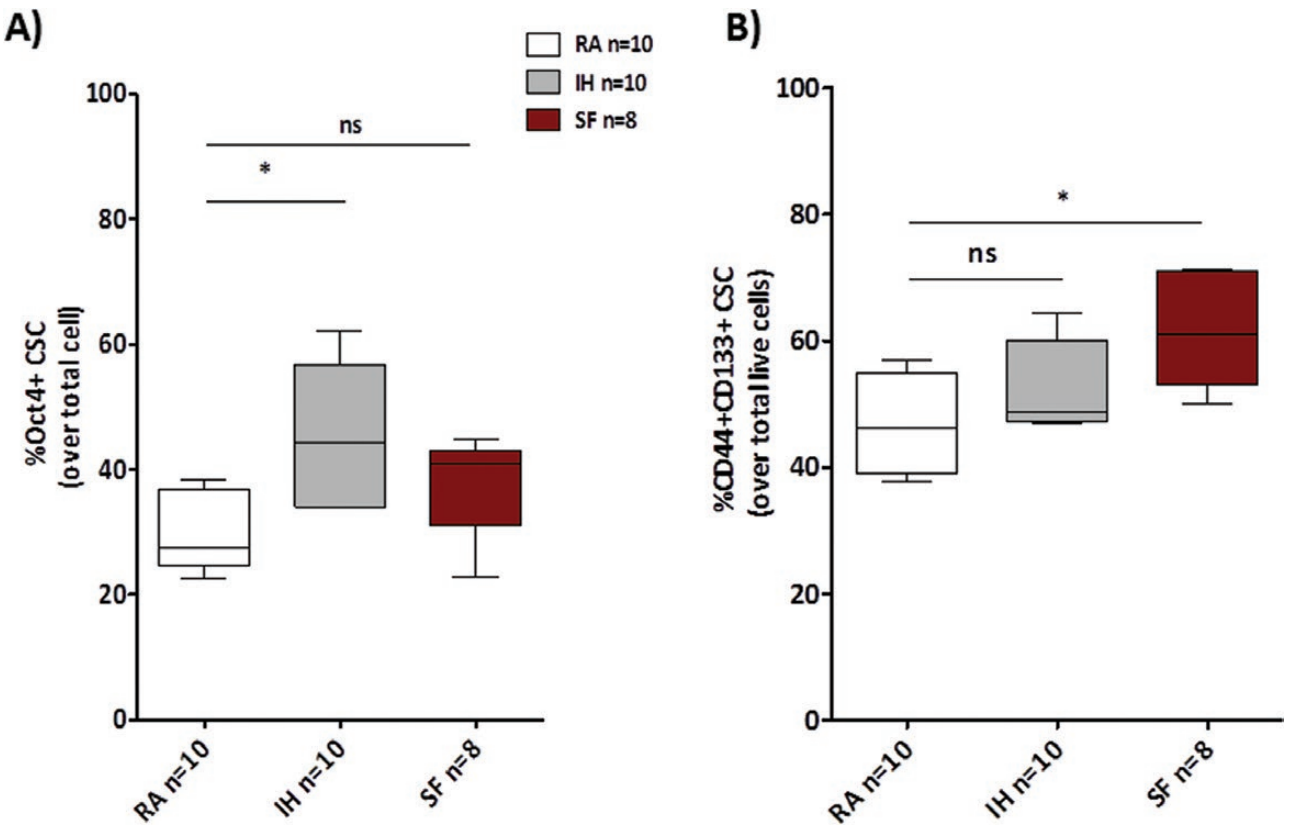


**Figure 3**—Stemness TC1 cancer cell markers in tumors following sleep fragmentation (SF) or intermittent hypoxia (IH) exposures. Significant increases in the frequency of $Oct4^+$ cells emerged in tumors harvested from mice exposed to IH (Panel A: $p < .04$ vs. RA; $n = 10$), while increased numbers of $CD44^+CD133^+$ cells emerged in SF-exposed mice (Panel B: $p < .03$ vs. RA; $n = 8$).

and maintaining the ability of CSCs to self-renew and even confer this capability to the nonstem population. We can propose that hypoxic tumor cells may promote the adoption of stem cell properties, including self-renewal and multipotency, by stimulating the expression or activity of Oct4, Notch, and other critical signaling pathways. However, there were limitations in the present study; the influence of IH and SF on self-renewal and differentiation was not investigated and expanded dissection of the mechanisms underlying the enhanced cellular proliferation and stemness, and altered CSCs phenotype within the tumor microenvironment in the context of IH and SF is needed.

In previous studies, we uncovered that both IH and SF, the two major constitutive elements of sleep apnea, independently promote several of the properties of tumors toward generation of adverse outcomes. In this setting, some initial contributors to such increased malignant properties in the context of sleep apnea were clearly related to altered immune function involving tumor-associated macrophages,[4,5,32] angiogenesis,[33] and surrounding adipose tissues.[34] However, the potential contributions of T cell lymphocytes in general, and more explicitly the potential reductions in the cytotoxic ability of CD8+ effector T-cell lymphocytes, was not explored and de facto constituted the major aim of the current study.

In summary, immune effectors and cancer cells are major components of the tumor microenvironment. In this environment, the immune system not only fails to defend the host against cancer but also often actively aids carcinogenesis by promoting CSC activity and facilitating malignant transformation and metastasis. The present results provide further biological plausibility to the putative deleterious effect of OSA in cancer patients.

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## SUBMISSION & CORRESPONDENCE INFORMATION

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Address correspondence to: David Gozal, MD, MBA, Section of Pediatric Sleep Medicine, Department of Pediatrics, Pritzker School of Medicine, Biological Sciences Division, The University of Chicago, KCBD, Room 4100, 900 E. 57th Street, Mailbox 4, Chicago, IL, 60637, USA. Telephone: (773) 702–3360; Fax: (773) 926-0756; Email: dgozal@uchicago.edu

## AUTHORS' CONTRIBUTION

MA participated in the conceptual framework of the project, performed experiments, analyzed data, and drafted components of the manuscript. AK, ZQ, AGH, and IA performed experiments and served as blinded observers. RF participated in data analysis and interpretation. DG conceptualized the project, provided critical input in all phases of the experiments, analyzed data, drafted the ulterior versions of the manuscript, and is responsible for the financial support of the project and the manuscript content. All authors have reviewed and approved the final version of the manuscript.

## DISCLOSURE STATEMENT

None declared.