

ORIGINAL STUDY

Lung cancer aggressiveness in an intermittent hypoxia murine model of postmenopausal sleep apnea

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Abstract

Objective: Intermittent hypoxia (IH)—a hallmark of obstructive sleep apnea (OSA)—enhances lung cancer progression in mice via altered host immune responses that are also age and sex-dependent. However, the interactions of menopause with IH on tumor malignant properties remain unexplored. Here, we aimed to investigate lung cancer outcomes in the context of ovariectomy (OVX)-induced menopause in a murine model of OSA.

Methods: Thirty-four female mice (C57BL/6, 12-week-old) were subjected to bilateral OVX or to Sham intervention. Six months after surgery, mice were pre-exposed to either IH or room air (RA) for 2 weeks. Then, 10⁵ lung carcinoma (LLC1) cells were injected subcutaneously in the left flank, with IH or RA exposures continued for 4 weeks. Tumor weight, tumor invasion, and spontaneous lung metastases were assessed. Tumor-associated macrophages (TAMs) were isolated and subjected to flow cytometry polarity evaluation along with assessment of TAMs modulation of LLC1 proliferation in vitro. To determine the effect of IH and OVX on each experimental variable, a two-way analysis of variance was performed.

Results: IH and OVX promoted a similar increase in tumor growth (~2-fold; $P = 0.05$ and ~1.74-fold; $P < 0.05$, respectively), and OVX-IH further increased it. Regarding lung metastasis, the concurrence of OVX in mice exposed to IH enhanced the number of metastases (23.7 ± 8.0) in comparison to those without OVX (7.9 ± 2.8 ; $P < 0.05$). The pro-tumoral phenotype of TAMs, assessed as M2/M1 ratio, was increased in OVX (0.06 ± 0.01 ; $P < 0.01$) and IH (0.06 ± 0.01 ; $P < 0.01$) compared with sham/RA conditions (0.14 ± 0.03). The co-culture of TAMs with naive LLC1 cells enhanced their proliferation only under IH.

Conclusion: In female mice, both the IH that is characteristically present in OSA and OVX as a menopause model emerge as independent contributors that promote lung cancer aggressiveness and seemingly operate through alterations in the host immune response.

Key Words: Animal models – Cancer progression – Intermittent hypoxia – Menopause – Obstructive sleep apnea – Ovariectomy.

Obstructive sleep apnea (OSA), the most common sleep-related breathing disorder across the lifespan, is characterized by increased collapsibility of the upper airway resulting in intermittent hypoxemia (IH) and sleep fragmentation (SF). The prevalence of OSA in adults increases with age with a clear male predominance that is attenuated after menopause.¹⁻⁴ Untreated OSA has been associated with a wide array of morbid end-organ

consequences including cardiovascular, cognitive, and metabolic disorders, and more recently with the possibility of increased cancer incidence and mortality.⁵ In animal models mimicking the chronic exposures to IH that are the hallmark of OSA, enhanced tumor progression and invasiveness have been reported in melanoma,⁶⁻⁹ lung,¹⁰⁻¹² and kidney cancer.¹³ Moreover, the cumulative findings emanating from epidemiological, clinical, and basic studies on the relationship

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between cancer and OSA suggest that this interplay can be strongly modulated by age, sex, and cancer type.¹⁴⁻¹⁹ Thus, it is important to understand how these factors modulate cancer aggressiveness to better interpret the epidemiological and clinical findings to date, and to better identify underlying mechanisms.

In terms of chronological age, Torres et al²⁰ have recently shown that aging appears to be protective in the context of IH-driven cancer aggressiveness in a mouse model of lung cancer. These findings may reside in declines affecting the immune system function that characteristically occur during aging.²¹ Indeed, such changes have been associated not only with higher cancer incidence²² but also with slower cancer progression in advanced age.²³ When considering the potential role of sex, particularly among women, OSA-cancer relationships are of particular interest in light of the marked increases in OSA prevalence that emerges after the onset of menopause.^{2-4,24} However, notwithstanding the strong possibility that sex is a major determinant of OSA-associated morbidities,^{18,25} the number of studies exploring sex differences is still very scarce. Furthermore, the vast majority of mechanistic studies on IH and cancer have included only male animals. Torres et al²⁰ showed that IH increased lung cancer aggressiveness through changes in the immune system and that such changes were similar in male and female mice.¹⁰⁻¹² However, this study did not incorporate the changes that occur in female menopause in the experimental design, such that the potential effects of menopause-derived changes on IH-induced cancer aggressiveness remain unexplored. In the present work, we overcome this limitation and explore both the isolated and combined effects of IH (as a surrogate model of OSA) and of long-term gonadectomy (a well established menopause model) on lung cancer aggressiveness characteristics.

METHODS

Animals

A total of 34 C57Bl/6j female, 12-week-old mice (Charles River, Écully, France) were included. Animals were kept under controlled light, temperature, and humidity conditions in the vivarial facilities of the University of Barcelona and received standard diet and water ad libitum. All procedures were approved by the Ethical Committee of the University of Barcelona (DAAM 8168).

Ovariectomy

Mice were randomly allocated to bilateral ovariectomy (OVX) (n = 18) or sham surgery (n = 16) intervention under anesthesia, as previously described.²⁶ Briefly, under aseptic conditions, a short incision was made in the skin of the lumbar region, which was previously cleaned and shaved. A muscle wall incision was made on both sides to exteriorize each ovary and oviduct. The oviduct was ligated near the ovary, which was then removed. The ligated oviduct was placed again into the peritoneal cavity and the skin was stitched with sterile silk suture. Sham intervention consisted of performing the same

procedure but without ligating the oviduct and proceeding with ovary excision. All animals received analgesic treatment for 3 days after the intervention. Exposures to IH and tumor induction were started 6 months after gonadectomy. Effectiveness of ovariectomy was tested by excising and weighting the uteri of all mice after their sacrifice at the end of the experimental procedures.

Intermittent hypoxia exposures

Mice in each group (OVX and sham) were randomized into equal subgroups to receive either IH or room air (RA) using an experimental setting previously described.⁶ Briefly, animals were kept within chamber (26 cm long, 18 cm wide, 6 cm high) in which gas flow was circulated continuously. IH consisted of cycles of 20 seconds at 5% O₂ followed by 40 seconds at 21% O₂ achieved by means of an electrically controlled valve that switched from a reservoir of hypoxic gas mixture or a reservoir containing RA. IH was applied for 6 hours/day 1 during the light period (10:00-16:00). As control group, RA animals breathed air at 21% O₂ throughout. All animals received 14 days exposures to IH or RA before lung tumor induction.

Lung adenocarcinoma induction

After IH or RA pre-exposures, all mice were implanted with 10⁵ mouse Lewis lung carcinoma cells (LLC1) (American Type Culture Collection, Manassas, VA) by a subcutaneous injection in the right flank. Before injection, LLC1 were grown with high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), and antibiotic/antimycotic solution at final concentrations of 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.250 µg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO). Cells were cultured in 75 cm² tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland) and were maintained in a standard humidified incubator at 20% O₂, 5% CO₂, and 37°C. After tumor injection, IH or RA exposures were continued for 4 weeks.

Assessment of tumor growth, invasiveness, and metastatic potential

At the end of the experiment, animals were anesthetized and euthanized. Tumor invasiveness to the surrounding tissues, namely muscle or bone, was assessed visually as previously reported.¹¹ Then, tumors were carefully excised and weighed.

Metastatic potential was assessed by counting the number and extension of lung metastases by histology. To this end, all lung lobes were excised, conserved in formalin, and then paraffin-embedded. Histological mid-sections of each pulmonary lobe were prepared and stained with hematoxylin and eosin. The number of total metastases was counted, and the proportion of metastatic area per healthy lung area was quantified under double-blinded conditions by light microscopy.

Quantification of tumor-associated macrophages and assessment of their phenotype

A piece of the flank-located primary tumor of ≈ 0.3 mg was minced and digested with collagenase type IA (Gibco, Gaithersburg, MD) for 1 hour at 37°C to assess the total number of tumor-associated macrophages (TAMs) and their density (cells/g tumor) from several segments of each tumor. TAMs were identified by flow cytometry as CD45+, CD11b+, F4/80+ cells.¹⁰

Isolation of tumor-associated macrophages and proliferation in vitro assay

Another piece of tumor of ≈ 0.3 mg was minced and digested with collagenase type IA (Gibco, Gaithersburg, MD) for 1 hour at 37°C to purify and count TAMs using magnetic beads coupled to anti-CD11b antibody (EasySep TM Mouse CD11b Positive Selection Kit, StemCell Technologies, Vancouver, Canada). A tumor proliferation assay was performed with the isolated TAMs and consisted in coculturing in standard medium 50,000 naïve LLC1 cells with 200,000 TAMs isolated from each individual mouse. After 48 hours, cells were labeled with CD45 antibody (Biolegend, San Diego, CA), and both TAMs and LLC1 populations were counted by flow cytometry (FACS Canto II cytometer) using FACS Diva 5.5 software (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, San Carlos, CA). A control single culture group, without TAMs, was also performed.

Circulating follicle-stimulating hormone measurements

Circulating follicle-stimulating hormone (FSH) concentrations were assayed in plasma samples using a competitive inhibition enzyme immunoassay technique (Mouse FSH ELISA Kit, CSB-E06871m; Cusabio, Hubei Province, China).

Statistical analysis

All results are shown as mean \pm standard error. To compare the different variables between groups, two-way analysis of variance (variables: OVX vs sham; and RA vs IH) was performed, using statistical software (SigmaPlot 11.0; Systat Software, Inc, San Jose, CA). Student-Newman-Keuls post hoc tests were employed for multiple comparisons. A P value ≤ 0.05 was considered for statistical significance.

RESULTS

As depicted in Fig. 1A, OVX and IH exerted opposite effects on animal body weight. While animals undergoing OVX experienced weight gain 6 months after surgery (26.30 ± 0.78 vs 24.09 ± 0.43 g; $P = 0.01$), OVX mice subjected to IH resulted in weight loss (23.92 ± 0.55 g; $P < 0.01$). As expected, OVX resulted in a drastic reduction of uterine weight in both RA (15.5 ± 0.8 mg; $P < 0.001$) and IH (19.3 ± 2.5 mg; $P < 0.001$) groups in comparison with sham groups (109.6 ± 8.8 and 84.1 ± 7.2 mg, respectively) (Fig. 1B). Circulating FSH in sham (1.11 ± 0.09 ng/mL)

and OVX (1.24 ± 0.05 ng/mL) mice was significantly increased by IH (1.39 ± 0.07 , $P < 0.05$; and 1.62 ± 0.08 , $P < 0.001$, respectively) (Fig. 1C).

Exposures to IH enhanced lung cancer progression in OVX and sham mice (Fig. 1D). Specifically, the growth ratio induced by IH in sham (~ 2 -fold; $P = 0.05$) and OVX (~ 1.74 -fold; $P < 0.05$) mice was similar. Moreover, IH promoted tumor invasion to surrounding tissues in OVX ($P < 0.001$) and sham ($P < 0.01$) mice, whereas no effects of OVX emerged as far as tumor invasion (Fig. 1E). Furthermore, most mice presented lung metastases from primary tumors regardless of the experimental group (Fig. 1F). Interestingly, among mice presenting lung metastasis, those subjected to IH in concurrence with OVX exhibited a more aggressive phenotype with increased number of metastases compared with those without OVX (23.7 ± 8.0 vs 7.9 ± 2.8 ; $P < 0.05$, respectively) (Fig. 1G).

Application of IH or OVX induced increased recruitment of TAMs to the tumor as previously reported (Fig. 2A). However, no significant changes were observed when the TAMs population was adjusted for tumor mass (data not shown). To assess whether IH and OVX induce a different TAM phenotype, we isolated TAMs from the tumors, and assessed cell surface expression of CD86 (M1 antitumoral marker) and CD206 (M2 pro-tumoral marker)¹⁰ (Fig. 2B). Exposures to IH and OVX induced a similar down-regulation of CD86 ($\sim 33\%$ and 40% , respectively) (Fig. 2C), whereas no changes on CD206 expression were detected for both exposure paradigms (Fig. 2D). Taken together, changes in M1 and M2 TAM polarity indicate that IH and OVX independently induced a shift towards a pro-tumoral phenotype (Fig. 2E). Specifically, the M1/M2 ratio in sham RA mice (0.14 ± 0.03) was reduced in response to IH (0.06 ± 0.01 ; $P < 0.01$) and OVX (0.06 ± 0.01 ; $P < 0.01$) individually. However, the concurrence of IH and OVX did not result in a synergistic effect on TAMs polarity phenotype, resulting in similar changes to those induced by isolated IH or OVX. TAMs isolated from tumors of mice exposed to IH increased tumor proliferation when co-cultured with naïve lung cancer cells, as previously reported¹⁰ (Fig. 2F). In contrast, TAMs isolated from OVX mice did not accelerate tumor growth in vitro, suggesting that OVX-induced changes in lung cancer aggressiveness could be mediated via different pathways from those mediated by IH.

DISCUSSION

This study shows that IH and OVX constitute two independent factors that promote lung cancer aggressiveness in female mice. Specifically, both experimental paradigms promoted accelerated tumor growth and induced macrophage polarization toward a pro-tumoral phenotype. Furthermore, although no synergistic effects on tumor growth emerged between IH and OVX, both IH and OVX differentially contributed to lung cancer invasiveness and metastasis. Indeed, whereas cell invasion was exclusively enhanced by IH, the progression of lung metastases was affected only by OVX.

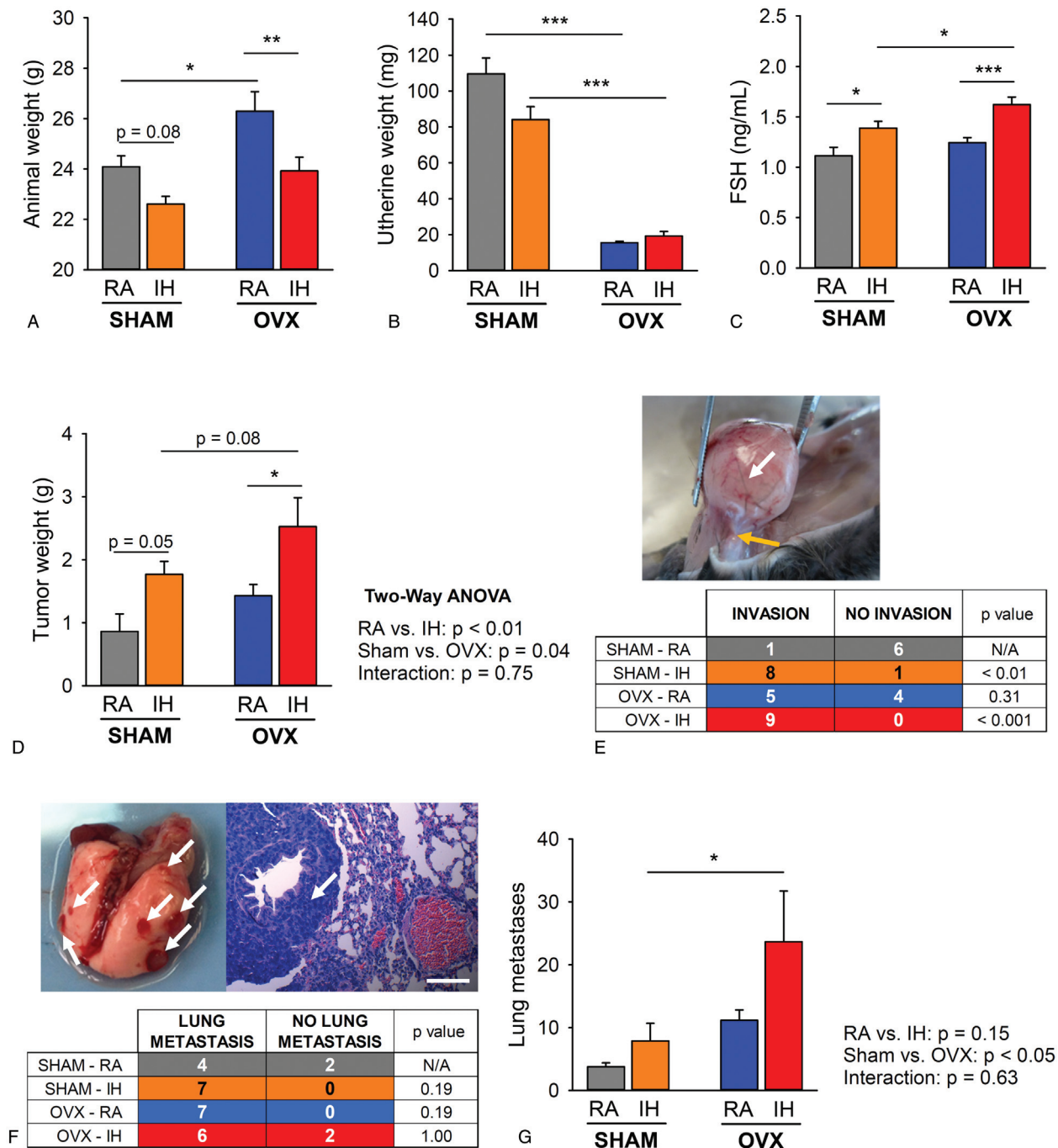


FIG. 1. Effects of intermittent hypoxia (IH) and ovariectomy (OVX) in LLC1 murine cancer outcomes. Body (A) and uterine (B) weights at the end of the study. (C) Plasma levels of follicle-stimulating hormone (FSH). (D) IH and OVX independently induced tumor growth but no synergistic effects are apparent. (E) Disruption of the tumor capsule with the presence of invasion toward the skeletal muscle (yellow arrow) of the primary tumor (white arrow) and indicative of lung cancer invasiveness toward surrounding tissues was present in IH, but not in OVX. (F) Although no differences on number of animals presenting lung metastasis were found between experimental groups, the number of metastatic lung nodules (white arrows) (G) was increased only in OVX mice among mice who exhibited metastatic nodules. Scale bar 100 μm . * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Obstructive sleep apnea is a sleep disorder commonly found among middle-aged obese men, but its occurrence in women is increasingly recognized. Furthermore, the prevalence of OSA in women rises markedly after onset of menopause, even after adjusting for body mass index (BMI) and neck circumference.²⁴ Despite such epidemiological evidence, preclinical

studies in different animal models of OSA have been virtually and exclusively carried out in males, with most experimental findings being then extrapolated to both sexes. However, data from various clinical studies show that some types of cancer seem to respond differentially to OSA depending on chronological age and sex.¹⁸ In addition to the effects of aging, women

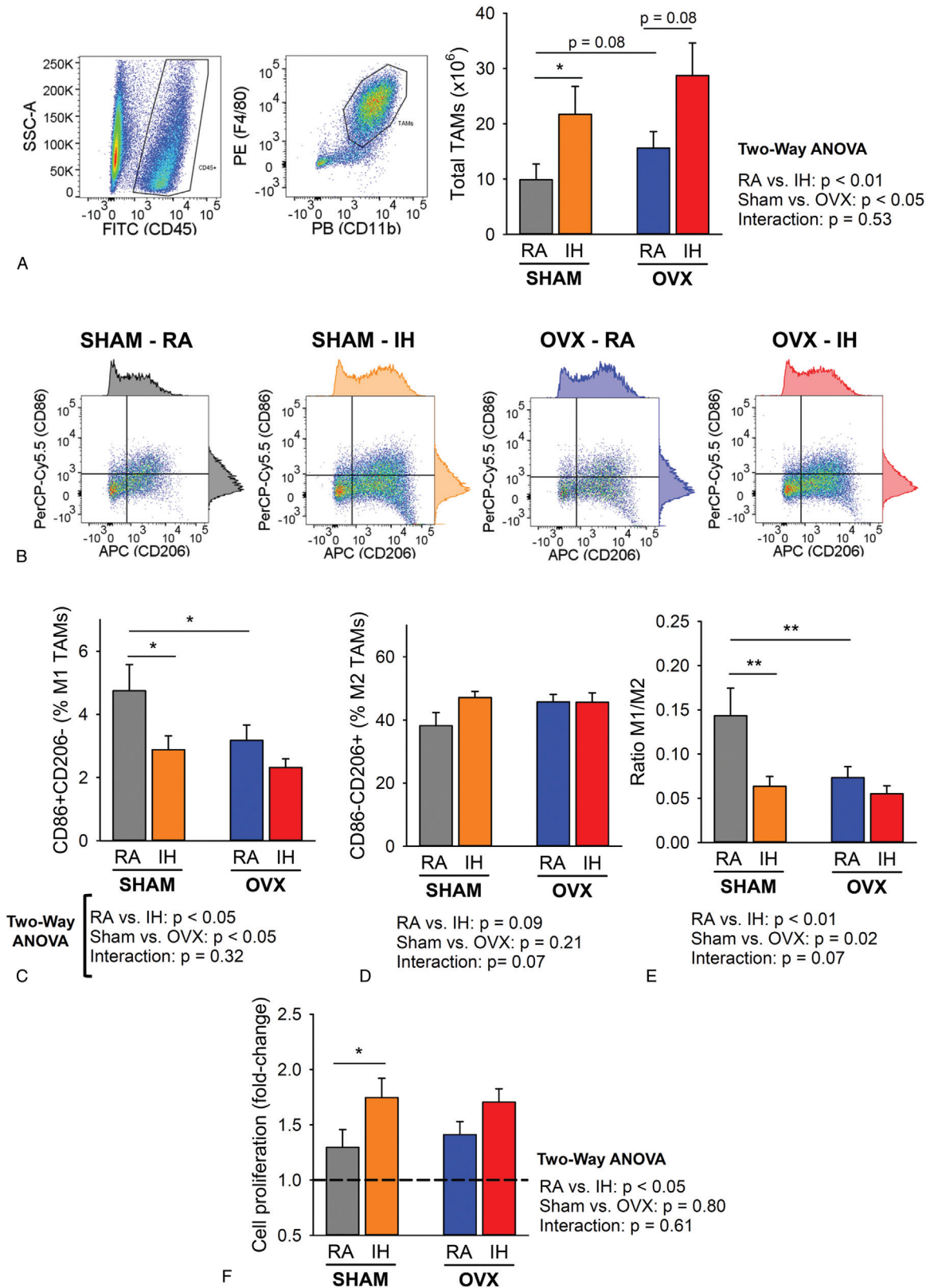


FIG. 2. Macrophage polarization and function under intermittent hypoxia (IH) challenge and ovariectomy (OVX). (A) Identification and selection of tumor-associated macrophages (TAMs) by flow cytometry (right). IH and OVX enhanced macrophage recruitment toward the tumor (left). (B) Representative surface expression of CD86 (M1) and CD206 (M2) in TAMs and histograms by flow cytometry. (C) IH and OVX induced a significant reduction of CD86 (M1 marker). (D) No changes were observed in CD 206 (M2), and (E) a significant reduction was observed in both conditions in the ratio M1/M2. (F) Isolated macrophages increased lung cancer cell proliferation in vitro only under IH conditions. * $P < 0.05$; ** $P < 0.01$.

experience important physiological and hormonal changes after menopause. However, only humans, short-finned pilot whales, and killer whales develop menopause. For this reason, OVX has been adopted as the gold standard approach for evaluating gonadal hormone effects and as a valid menopause model resulting in the cessation of gonadal function in female mice.²⁷ Under both conditions, that is, gradually decreased gonadal function (menopause) or acute depletion (gonadectomy), estrogen production is reduced leading to an increase in gonadotropin-releasing hormone (GnRH), FSH, and luteinizing hormone (LH).²⁸ It has been shown that there is a fast increase in FSH levels after OVX, and that after 12 weeks from the surgical procedure, FSH levels gradually decrease.^{29,30} Also, the cognitive alterations generated by OVX are strongly age-dependent and are reversed by estradiol therapy,³¹⁻³³ suggesting that aging is an important factor to consider during menopause/OVX hormonal changes. Thus, the experimental model presented here was carefully designed, and incorporated the aforementioned considerations to better elucidate the role of menopause in OSA and lung cancer. Indeed, OVX surgery was conducted 6 months before IH exposures to avoid any potential acute hormonal and physiological responses to gonadectomy including the FSH peak previously described after 12 weeks from surgery,^{29,30} which could explain the different biological effects of short versus long-term OVX.³⁴ Moreover, IH exposures and tumor inoculation were performed at the chronological age of ~10 months, which is equivalent to 38 to 47 years old in humans,³⁵ when the hormonal response to OVX³¹⁻³³ and IH-induced cancer progression could be different from earlier ages.²⁰ Interestingly, these two experimental conditions could explain the relatively minor increase in FSH levels detected in OVX mice when compared with controls.

Exposure to IH promoted an accelerated tumor growth in both sham and OVX mice. In sham mice, the magnitude of IH-induced tumor progression observed in middle-aged female mice is similar to the magnitude previously reported in young adult male mice¹⁰⁻¹² and young adult female mice.²⁰ In fact, the effects of IH on lung cancer progression found in this study, including tumor growth and invasion, were strikingly similar to those observed in 2-month-old mice, and markedly contrasted with the absence of IH effects on tumor growth reported in 20-month-old male mice.²⁰ Thus, this work supports the notion that IH-induced tumor growth is not affected by sex in mice with preserved gonadal function.

After menopause, hormonal changes could also participate in cancer outcomes in addition to aging. Traditionally, high serum values of FSH and LH have been associated with the poorest cancer outcomes by increasing cancer cell proliferation, migration, inhibiting tumor cell apoptosis, and enhancing tumor invasion and neovascularization.³⁶⁻³⁸ Application of IH in sham and OVX mice resulted in an unexpected increase in plasma FSH levels (Fig. 1B). This is an intriguing finding, because it suggests a novel potential mechanism linking OSA with cancer progression. In fact, it could account

for the increased levels of FSH reported in chronic obstructive pulmonary disease (COPD)—a respiratory disease characterized by episodic hypoxia³⁹—and the reductions in FSH levels observed after 4 months of long-term oxygen therapy. Taken together, the hormonal changes induced by OVX and by IH could explain per se the increased lung cancer tumor progression and invasiveness reported in this work.

In contrast to tumor growth and invasion, both IH and OVX did not increase the metastatic potential in lung cancer. At the end of the experiment, almost all the mice presented lung metastases regardless of the experimental group to which they were assigned. These results reproduce previous findings reported in male mice subjected to the same IH paradigm and lung cancer model,¹² but are at variance with the findings observed in melanoma.⁸ However, analysis of those mice presenting lung metastasis revealed that OVX developed a significantly higher number of metastatic nodules, suggesting that OVX enhances the metastatic potential of lung carcinoma.

Macrophages are a highly diverse population of white blood immune cells that regulate innate and adaptive immunity in cancer. The recruitment of these immune cells has been observed in a wide range of cancer types, and correlates with poor prognosis.⁴⁰ Here, we show that animals exposed to IH presented a higher total number of macrophages recruited into the primary lung tumor, but no differences between groups were found when TAMs were normalized for the changes in tumor mass. These findings are in agreement with previous studies carried out in young male¹⁰ and female²⁰ mice bearing lung tumors exposed to IH. In addition, macrophages are typically classified into two distinctive polarity-related phenotypes. While M1 macrophages exhibit antitumor immune activity, M2 macrophages promote tumor progression, and have been associated with adverse overall survival rates among non-small cell lung cancer patients.⁴¹ The results obtained here in response to IH in middle-aged female mice are virtually identical to these previous findings.¹⁰ Furthermore, the reduction in expression of the CD86 surface marker in OVX and IH conditions resulted in a pro-tumoral phenotype of TAMs, while the concurrence of both conditions, IH and OVX, did not yield any additive effects on CD86 expression. When TAMs isolated from all four experimental groups were co-cultured with naïve lung cancer cells to assess their capacity to modulate their proliferative rates,¹⁰ only TAMs isolated from mice exposed to IH promoted increases in tumor proliferation LLC1 cells in vitro as previously reported for young male mice,^{10,12} but no effects were detectable in response to OVX.

Clinical epidemiological studies on the effects of OSA on lung cancer are limited to only a few recent papers showing that OSA is very prevalent in patients presenting with lung cancer. Both studies showed that nocturnal hypoxemia is an important risk factor which increases almost three-fold the risk of positive screening findings.⁴² Furthermore, about 50% of lung cancer patients had moderate to severe OSA (AHI >15 events per hour of sleep).⁴³ However, these studies have

important limitations, such as the co-existence of chronic pulmonary disease and the smoking history of these patients. The available evidence on sex differences in the association between cancer and OSA is also very scarce. In a retrospective, multicenter, longitudinal cohort study that included 4,910 patients, Campos-Rodríguez et al¹⁹ showed that the association between OSA and cancer incidence was stronger in patients younger than 65 years, and was only significant in men. Gozal et al¹⁸ assessed the incidence of different types of cancer based on a cohort of ~5.6 million individuals included in an employee-sponsored health insurance database, and showed that OSA appears to increase specific cancer types, whereas aging and sex can modulate these relationships. However, in contrast to the study by Campos-Rodríguez et al, this study did not find increased lung cancer risk in OSA patients. A more recent study from the European Sleep Apnoea Database (ESADA) showed that OSA is associated with increased cancer prevalence, and that such association is particularly enhanced in women.⁴⁴ This study revealed that the most prevalent types of cancer in females were breast cancer, gynecological, thyroid, lymphoma, lung, and colon cancer, and melanoma. However, as in previous studies, the authors did not analyze the role of some determinant factors in cancer such as hormonal changes and cigarette smoking status. As such, many questions remain unanswered in relation to these confounders and preclude immediate extrapolation from the murine experiments presented herein and human epidemiological cohorts.

Potential clinical value

This study provides novel insights on the effects of hormonal changes caused by surgical ovariectomy as a model of menopause on the relationship between lung cancer and OSA. The experimental setup consisting of surgical OVX and IH avoided most of the major confounding factors in patients with lung cancer (smoking status, COPD, emphysema) and OSA (hypertension, diabetes, obesity). Most importantly, the effects of OVX on cancer outcomes observed in this work opens intriguing leads worthy of exploration in future studies. For instance, the use of different histological subtypes of human lung cancer cells could be of great interest because proliferative responses of lung cancer cells to different hypoxic patterns is cell type-dependent.⁴⁵ Moreover, exploring how IH can modulate the increased cancer risk associated with hormone therapy may also have important translational implications.⁴⁶

CONCLUSIONS

Intermittent hypoxia and bilateral gonadectomy, as a menopause model, emerge as independent contributors promoting lung cancer aggressiveness through alterations in the host immune response. These data provide biological plausibility to the age and sex differences observed in previous clinical and epidemiological studies. Thus, these novel insights could serve as proof-of-concept for the design of future clinical and

translational studies investigating the role of menopause on cancer and OSA.

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