Mechanism of isoflurane-mediated breast cancer growth *in vivo*

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Abstract. Use of volatile anesthetics is associated with worse outcome following tumor resection surgery compared with the use of intravenous anesthetics. However, the underlying mechanism has not been clearly delineated yet *in vivo*. The EO771 cell-based congenic breast cancer model was used in the present study. Isoflurane directly binds to and inhibits two adhesion molecules, leukocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1). Similarly, exposure to sevoflurane, another volatile anesthetic and LFA-1 inhibitor, is associated with an increase in breast cancer size compared with non-exposure. Thus, the present study first examined the role of LFA-1 and Mac-1 in the EO771 breast cancer model. Both LFA-1 deficiency and inhibition enhanced tumor growth, which was supported by cytokine and eicosanoid data profiles. By contrast, Mac-1 deficiency did not affect tumor growth. The exposure to isoflurane and sevoflurane was associated with an increase in breast cancer size compared with non-exposure. These data suggested that isoflurane enhanced tumor growth by interacting with LFA-1. Isoflurane exposure did not affect tumor growth in LFA-1-deficient mice. In summary, the present data showed that LFA-1 deficiency facilitated breast cancer growth, and isoflurane, an LFA-1 inhibitor, also increased breast cancer growth.

Introduction

The effect of anesthetics on cancer has been a topic of clinical interest. A number of retrospective studies reported that the use of volatile anesthetic-based general anesthesia was associated with higher incidence of cancer recurrence and worse survival compared to the use of intravenous anesthetic-based anesthesia (1-3). These observations triggered researchers to investigate the underlying mechanism of how anesthetics affect cancer. So far clear volatile anesthetic targets have not been shown *in vivo*.

Breast cancer is the most frequently diagnosed cancer and the cause of mortality among all cancers in females (4). Although spontaneous tumor growth models or xenogeneic tumor implantation models have been used to study breast cancer in mice (5), they already have immunologically altered background. Instead, EO771 tumor cell implantation congenic model allows us to study tumor growth in fully immunocompetent mice. EO771 cells are breast adenocarcinoma cells derived from a spontaneous mammary tumor from a female C57BL/6 mouse (6) and have been used for congenic breast cancer model (7). Now it is well recognized that anesthetics can affect leukocyte functions (8,9). Thus, using congenic model to study the effect of anesthetics on tumor growth would be logical.

In this study, we examined the impact of commonly used volatile anesthetic isoflurane on tumor growth and its underlying mechanism *in vivo*. We previously reported that volatile anesthetic isoflurane directly binds to and inhibits critical adhesion molecules leukocyte function-associated antigen-1 (LFA-1) (10-12) and macrophage-1 antigen (Mac-1) (13). LFA-1 and Mac-1 are members of β2 integrins, which belong to a heterodimeric adhesion molecule family consisting of α- and β2-subunits. LFA-1 is also called αLβ2 or CD11a/CD18 and ubiquitously expressed on leukocytes (14). Mac-1 is also called αMβ2, CD11b/CD18 or complement receptor 3 (CR3) and expressed primarily on myeloid cells (15). Thus, we also examined the role of LFA-1 and Mac-1 in tumor growth.

Materials and methods

Mice. Wild type, CD11a (αL) knockout (KO) mice (16) and CD11b (αM) KO mice (17) on the C57BL6 background were obtained from Jackson Laboratory (Bar Harbor, Maine, USA). They were housed under specific pathogen-free conditions, with 12-h light and dark cycles. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital (Protocols 16-03-3120 and 00001574 'Anesthetics and tumor recurrence or metastasis').

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EO771 tumor implantation model. The experiments were performed between August 2016 and July 2018. EO771 cells were cultured in RPMI1640/10% FBS. On the day of tumor implantation, mCherry-EO771 cells (7) (mCherry-EO771 cells were kindly given by Dr. Johnstone at University of Melbourne) were collected and suspended in Matrigel matrix (Corning, Inc., Corning, NY). $1x10^5$ of EO771 cells per mouse suspended in 50 ul of Matrigel matrix were implanted at the 4th nipple fat pad in the morning of the experimental days (7). Given subcutaneous tumor injection is minimally invasive (18), for the injection, mice were placed in a quiet room and held in researcher's hand for injection with a 30G needle. No anesthetics were used as approved by the IACUC. Then, tumor size was monitored every other day. Mice behaved actively during our observation. Tumor volume was calculated ½(length x width^2), as previously described (19). For IVIS (*in vivo* imaging system) based tumor imaging, mice were implanted with cells labeled with firefly luciferase and subjected to intraperitoneal injection of Luciferin (15-150 mg/kg) 10 min before the measurement. During the imaging, mice were anesthetized with isoflurane (4% induction, 2-3% maintenance).

In some experiment, either 100 μg of isotype control or CD11a monoclonal antibody (mAb) (clone M17/4) was given on day 7 and day 10 after tumor implantation. Some mice were also exposed to 1% isoflurane (induction and maintenance) or 2.1% sevoflurane (induction and maintenance) for 4 h on day 7 after tumor implantation. Because the minimum alveolar concentration (MAC; the concentration at which 50% of mice do not respond to tail clamping) is 1.3% for isoflurane (20) and 1 MAC is 2.8% for sevoflurane (21), 2.1% sevoflurane matches the potency of 1% isoflurane. We intended to provide them to mice at clinically relevant doses, not for full general anesthesia. The total number of mice used was described in each Figure legend. We observed tumor growth for 2 weeks expect the experiment using CD11b KO mice. For CD11b KO mice experiment, we observed up to 3 weeks due to slower tumor growth. If tumors exhibited abrasion and fluid leakage, we euthanized and excluded mice from the study. In this study, we euthanized one mouse due to the leakage from the tumor bed. We observed redness (abrasion) at the leakage site but did not measure the size of the abrasion. At the end of observation, all mice were euthanized with CO_2 (30-70% of the chamber volume per minute, approximately 4-5 min). Euthanasia was confirmed by the lack of movement including respiration and heartbeat.

Tumor bed histology analysis. Tumor tissue beds were fixed using 4% paraformaldehyde. Hematoxylin & Eosin (H&E) staining was done using the Leica ST5020 Multi-staining machine in Boston Children's Hospital pathology core.

Eicosanoid measurements of mass at tumor bed. Tumor beds were removed and kept in -80°C freezer until use. Then, tumor mass was subjected to mechanical disruption for lipid extraction. The lipids were extracted with methanol and diluted with water containing 0.1% formic acid to yield a final methanol concentration of 20%. Reverse-phase mass spectrometry (MS)-based quantitation technique for eicosanoids was previously described (22). After addition of deuterium-labeled internal standards, the samples were loaded on Oasis HLB cartridge (Waters, Milford, MA). The column was washed with 1 ml of water, 1 ml of 15% methanol, and 1 ml of petroleum ether and then eluted with 0.2 ml of methanol containing 0.1% formic acid. Eicosanoids were quantified by reverse-phase HPLC-electrospray ionization-tandem MS method.

Reverse transcription-quantitative PCR (RT-qPCR). Tumor bed tissues were collected and kept in -80°C until use. Tissues were suspended in Trizol (Thermofischer, Waltham, MA) and homogenized. Then, samples were subjected to RNA purification per the company's protocol. A total of 1 μg RNA was then converted to first-strand cDNA. RT-qPCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific) on StepOnePlus System (Applied Biosystems, Waltham, MA). For data normalization, GAPDH was used as an internal reference, and the fold change in gene expression was calculated using the comparative Ct method (2-ddCt) (23). Primers used for RT-qPCR were TNF-α Forward CCCTCACACTCAGAT CATCTTCT, Reverse GCTACGACGTGGGCTACAG; IL-1β forward GCAACTGTTCCTGAACTCAACT, reverse ATCTTTTGGGGTCCGTCAACT; IL-6 forward GCTACC AAACTGGATATAATCAGGA reverse CCAGGTAGCTAT GGTACTCCAGAA; CXCR1 forward TCTGGACTAATC CTGAGGGTG, reverse GCCTGTTGGTTATTGGAACTC TC; G-CSF forward ATGGCTCAACTTTCTGCCCAG, reverse CTGACAGTGACCAGGGGAAC; GAPDH forward GCACAGTCAAGGCCGAGAAT, GAPDH reverse GCC TTCTCCATGGTGGTGAA.

In vitro EO771 cell growth assessment. We examined the growth of EO771 cells with or without isoflurane exposure. Isoflurane exposure was done in an airtight chamber as we previously performed (24,25). Isoflurane concentration was measured by infrared spectroscopy (Ultima, Datex Instrument Corp., Helsinki, Finland). Cells were detached by trypsin and the number of live cells was counted following trypan blue staining using a hemocytometer.

Statistical analysis. Data are presented as the mean ± SD. Unpaired Student's t-test and two-way mixed ANOVA with Bonferroni post hoc analysis were used. Statistical significance was defined as $P< 0.05$. All the statistical calculations were performed using PRISM5 software (GraphPad Software, La Jolla, CA).

Results

Isoflurane exposure facilitated breast cancer growth. We examined the effect of commonly used volatile anesthetic isoflurane on breast cancer growth (Fig. 1A). We administered 1% isoflurane for 4 h to mice at 7 days after EO771 implantation, mimicking the duration for patients receiving breast cancer resection. As expected, isoflurane significantly facilitated breast cancer growth (tumor size at day 13, 343.3 +/- 132.9 mm^3, maximum 683 mm^3 for no exposure and 686.7 +/- 265.8 mm^3, maximum 1,366 mm^3

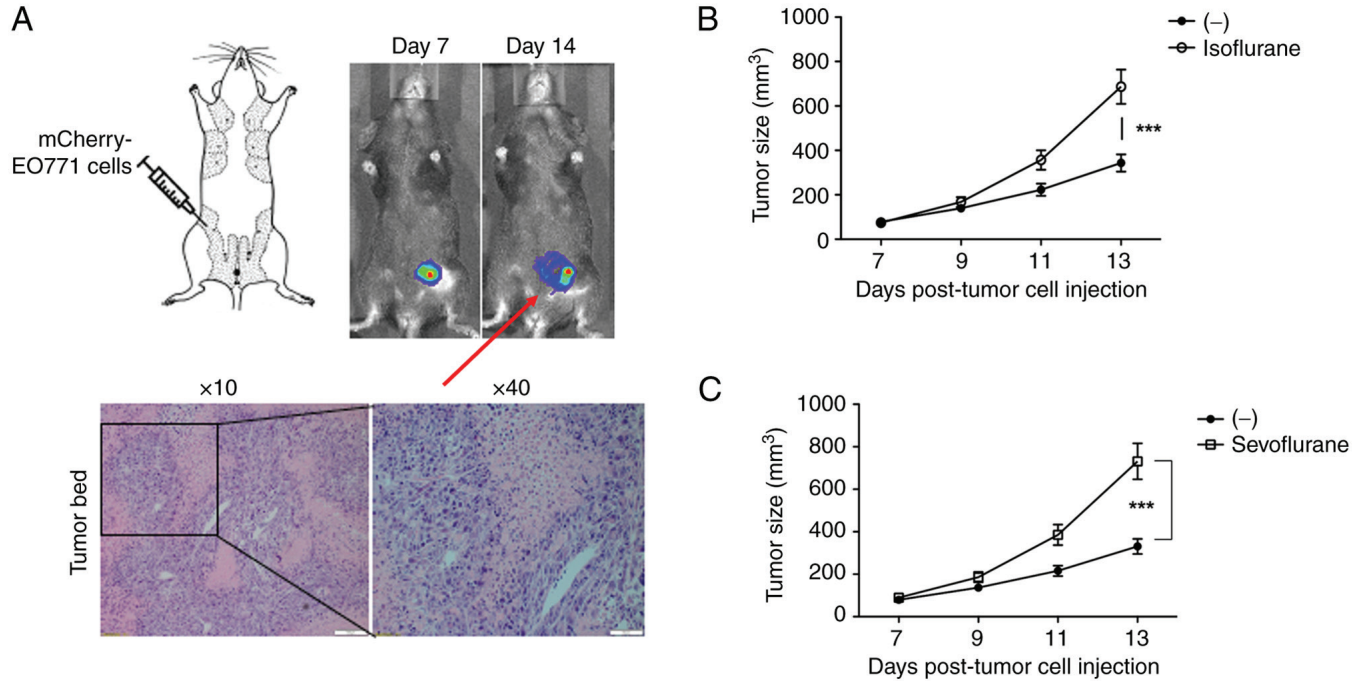

Figure 1. Effect of isoflurane on tumor growth of EO771 cells. (A) EO771 cell implantation model. The images of the tumor detected by the *in vivo* imaging system are shown along with the tumor bed histology. Magnification, x10 (left) or x40 (right). (B) WT mice (16 mice) were implanted with EO771 cells. Half of the mice (n=8) were exposed to isoflurane and half (n=8) were not. Tumor size was observed over a 2-week period. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. ***P<0.001. (C) WT mice (16 mice) were implanted with EO771 cells. Half of the mice (n=8) were exposed to sevoflurane and half (n=8) were not. Tumor size was observed over a 2-week period. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. ***P<0.001. WT, wild-type.

for isoflurane exposure) (Fig. 1B). We also tested another volatile anesthetic sevoflurane. Sevoflurane also significantly facilitated breast cancer growth (tumor size at day 13, 331.0 +/- 122.0 mm^3, maximum 614 mm^3 for no exposure and 731.4 +/- 292.6 mm^3, maximum 1,503 mm^3 for sevoflurane exposure) (Fig. 1C).

LFA-1 deficiency was associated with faster tumor growth, but Mac-1 deficiency was not. We previously showed that isoflurane directly bound to and inhibited adhesion molecules LFA-1 and Mac-1. Thus, we first examined the role of LFA-1 and Mac-1 in breast cancer growth. The deficiency of LFA-1 significantly facilitated the growth of EO771 cells as the tumor size at day 13 was 369.4 +/- 146.4 mm^3 (maximum 685 mm^3) for WT mice and 1,393.8 +/- 134.6 mm^3 (maximum 1,639 mm^3) for CD11a KO mice (Fig. 2A). Because KO mice could have compensatory changes, we also examined the effect of LFA-1 using CD11a monoclonal blocking antibody in both WT and CD11a KO mice. In line with the finding in Fig. 2A, CD11a mAb administration facilitated the growth of EO771 cells in WT mice (tumor size at day 13 1,467.2 +/- 372.6 mm^3, maximum 1,725 mm^3 for isotype antibody group and 2,697.0 +/- 109.2 mm^3, maximum 2,725 mm^3 for CD11a mAb group) (Fig. 2B). No difference was observed in CD11a KO mice (CD11a KO with isotype group, 2,510.9+/-350.0 mm^3, maximum 2,758 mm^3, and CD11a KO with CD11a mAb group, 3,088.0 +/- 405.4 mm^3, maximum 3,374 mm^3). In contrast, Mac-1 deficiency did not affect tumor growth (tumor size at day 21 1,769.3 +/- 545.4 mm^3, maximum 2,798 mm^3 for WT and 1,521.9 +/- 689.6 mm^3, maximum 2,343 mm^3 for CD11b KO mice) (Fig. 2C). Taken together, we found that both LFA-1 deficiency and inhibition significantly enhanced the growth of EO771 cells.

Breast cancer bed showed higher pro-tumor cytokine levels and PGE_2/LTD_2 levels. Because proinflammatory cytokines and a subset of lipid mediators have been shown associated with the growth of tumor, we examined their levels in CD11a KO mice. Pro-tumor cytokines IL-6, CXCR1 and G-CSF levels were significantly elevated in the tumor bed of CD11a KO mice (Fig. 3A). We also examined prostaglandin (PG) and leukotriene (LT) levels. We found that PGE_2 and LTD_4 levels were significantly elevated in CD11a KO mice (Fig. 3B). These data are in line with clinical data as PGE_2 mediated signal is important in propagating breast cancer (26) and LTD_4 level was elevated in patients with breast cancer (27).

Isoflurane did not further increase breast cancer size. The data so far indicated that LFA-1 would be isoflurane target to modulate tumor size. To test this hypothesis, we examined the effect of isoflurane in CD11a KO mice. Supportive of our hypothesis, isoflurane did not significantly affect the size of tumor in CD11a KO mice (tumor size at day 3, 1,115.0 +/- 1,07.7 mm^3, maximum 1,304 mm^3 for CD11a KO mice, and 1,210.6 +/- 115.0 mm^3, maximum 1,326 mm^3 for CD11a KO mice with isoflurane exposure) (Fig. 4A). LFA-1 is exclusively expressed on leukocytes. Thus, we also tested if isoflurane directly affected tumor size *in vitro*.

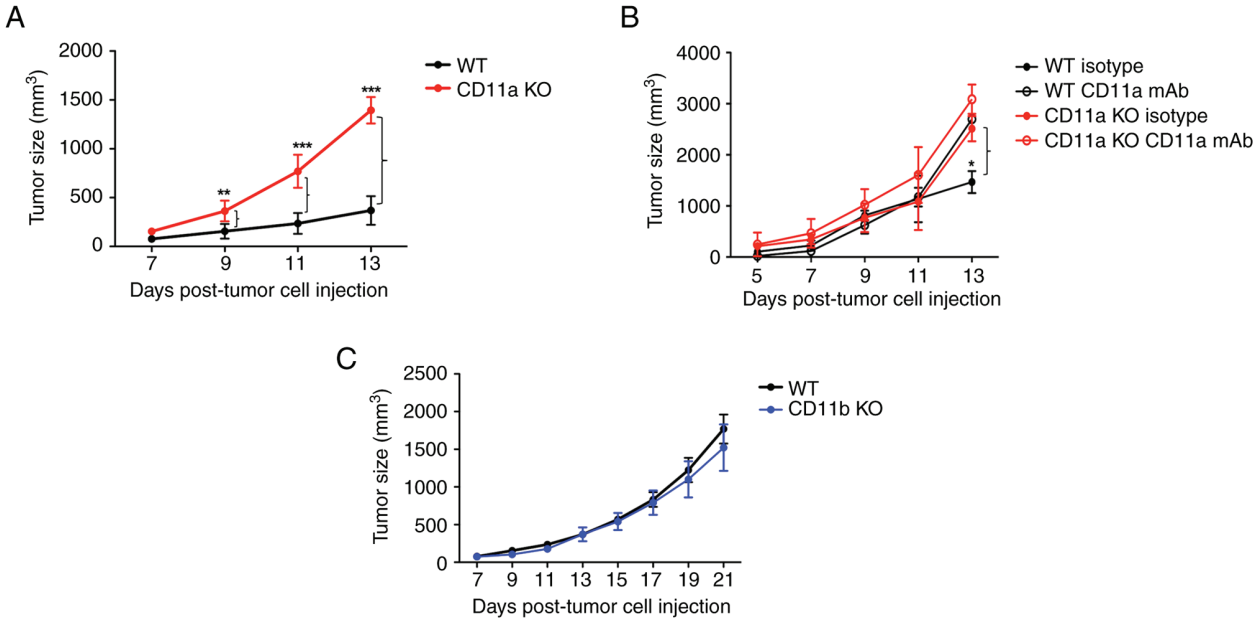

Figure 2. Role of CD11a in the growth of EO771 tumor cells. No anesthetic exposure was performed in the experiments presented here. (A) WT mice (n=8) and CD11a KO mice (n=8) were implanted with EO771 cells. Tumor size was observed over a 2-week period. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. **P<0.01 and ***P<0.001. (B) WT and CD11a KO mice were implanted with EO771 cells. A group of mice received isotype control on day 7 and day 10, and a group of mice received CD11a mAb. Each group consisted of 6 mice. Data are presented as the mean ± SD. Two-way mixed ANOVA with Bonferroni's post hoc analysis was used for this analysis. *P<0.05. (C) WT (n=8) and CD11b KO mice (n=8) were implanted with EO771 cells. Tumor size was observed over a 2-week period. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. No statistically significant difference was observed. KO, knockout; mAb, monoclonal antibody; WT, wild-type.

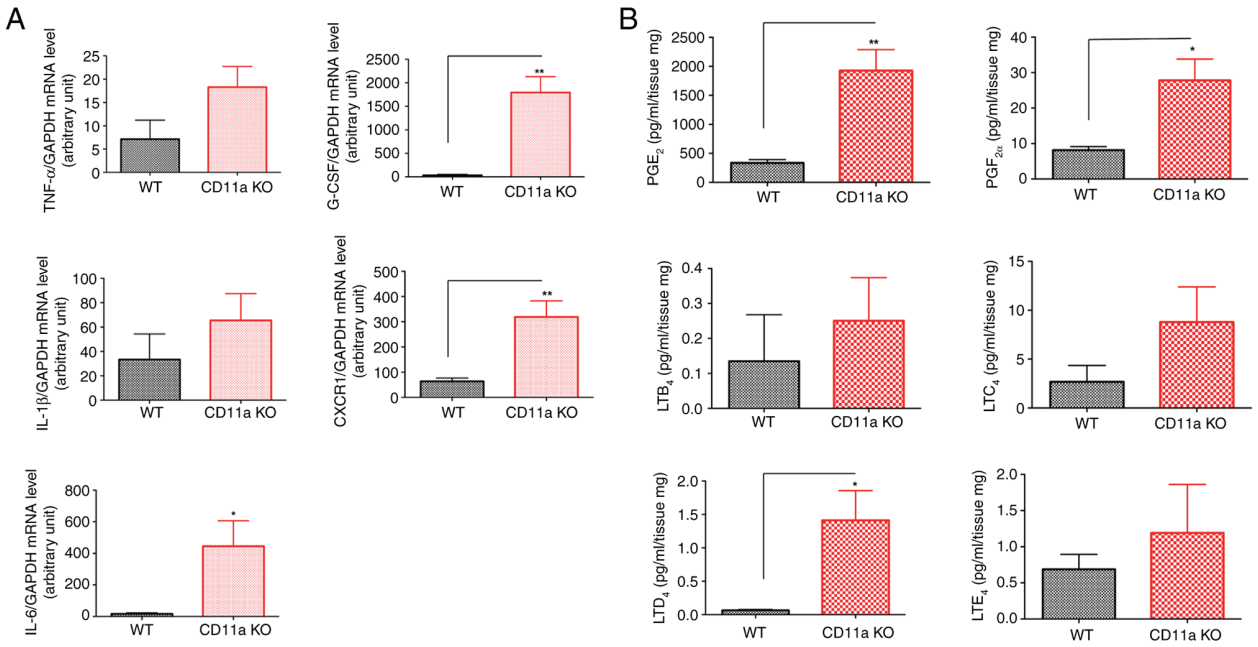

Figure 3. Cytokine and eicosanoid levels in the tumor bed tissues. No anesthetic exposure was performed in the experiments presented here. (A) Tumor bed tissues at 2 weeks after implantation were subjected to reverse transcription-quantitative PCR. GAPDH was used as the internal control housekeeping gene. Data are presented as the mean ± SD of quadruplicates. An unpaired Student's t-test was performed. *P<0.05 and **P<0.01. (B) Eicosanoid levels of tumor tissues were measured. Data are presented as the mean ± SD of quadruplicates. An unpaired Student's t-test was performed. *P<0.05 and **P<0.01. CXCR1, C-X-C motif chemokine receptor 1; G-CSF, granulocyte colony stimulating factor; KO, knockout; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; WT, wild-type.

As expected, isoflurane did not increase the EO771 cell number (Fig. 4B). This suggests that LFA-1 would be a major isoflurane target to modulate breast cancer growth *in vivo*.

Discussion

In this study, we showed that volatile anesthetic isoflurane and sevoflurane exposure significantly enhanced breast cancer

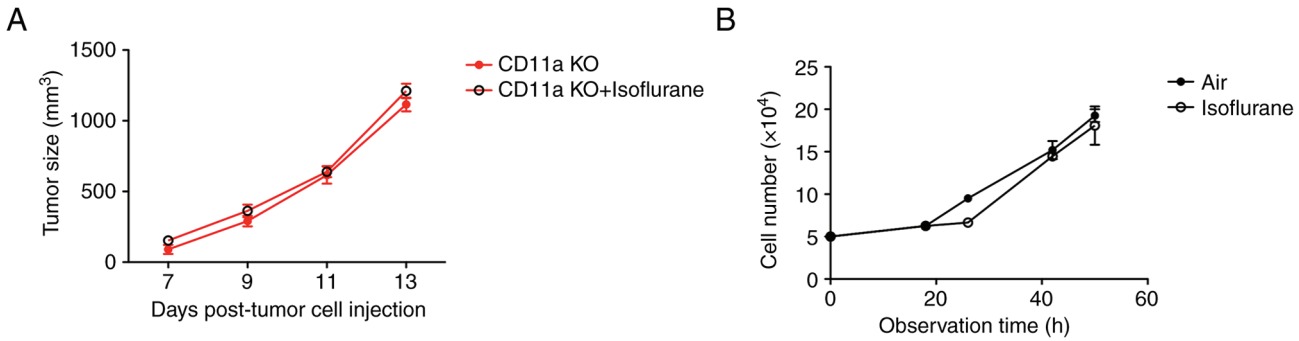

Figure 4. Effect of isoflurane on tumor growth of EO771 cells in CD11a KO mice. (A) CD11a KO mice (16 mice) were implanted with EO771 cells. Half of the mice (n=8) were exposed to isoflurane and half (n=8) were not. Tumor size was observed over a 2-week period. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. No statistically significant difference was observed. (B) EO771 cells were exposed to isoflurane for 3 h and their number was determined by manually counting using a hemocytometer after staining detached cells with trypsin. Cells were stained with trypan blue for counting. Data are presented as the mean ± SD. An unpaired Student's t-test was applied at each time point. No statistically significant difference was observed. KO, knockout.

growth. We also suggested that LFA-1 facilitate breast cancer growth via affecting LFA-1.

The role of anesthetic selection in cancer resection surgery has been a hot topic. Wigmore *et al* (1) reported that the use of intravenous anesthetics was significantly associated with better overall survival and less cancer recurrence than the use of volatile anesthetics. This landmark paper ignited the discussion of whether or not intravenous or volatile anesthetics should be used for general anesthesia for cancer resection surgery. A number of retrospective studies examined various type of cancer surgeries, largely favoring the use of intravenous anesthetics (28). In parallel, many investigators examined the effect of anesthetics using *in vitro* cell culture system and *in vivo* animal models. However, the mechanism of anesthetic-mediated tumor growth has been less studied *in vivo*. We previously demonstrated that commonly used volatile anesthetics isoflurane and sevoflurane directly bind to and inhibit LFA-1 (10-12), while an intravenous anesthetic propofol did not affect LFA-1 function at clinically relevant doses (29). We previously demonstrated the importance of LFA-1 as a volatile anesthetic target relevance in K562 cells, leukemia cells by showing that the inhibition of LFA-1 by isoflurane and sevoflurane attenuated natural killer (NK) cell- mediated cytotoxicity (30). In our data, we showed both isoflurane and sevoflurane facilitated breast cancer growth. While isoflurane also bound to and blocked Mac-1 (11,12), sevoflurane did not bind to and inhibit Mac-1 (10,13). Taken together, our data suggested that LFA-1 served as a target for both isoflurane and sevoflurane to facilitate breast cancer growth.

As LFA-1 is exclusively expressed on leukocytes, we expect that isoflurane significantly enhanced tumor growth via altering the phenotype of leukocytes. The analysis of tumor beds showed an increase in PGE_2 and LTD_4 levels in CD11a KO mice compared to WT mice. However, we still do not know what triggered this change. As LFA-1 is ubiquitously expressed on leukocytes, it is imperative to examine the role of LFA-1 in all leukocyte types. For example, LFA-1 is involved in NK cell-mediated tumor cytotoxicity (31,32). LFA-1 activation enriches tumor-specific T cells to improve anti-tumor responses (33). Both neutrophils and macrophages play a significant role in cancer immunity (34,35). However, how LFA-1 on neutrophils and macrophages affect cancer growth is not known. In the future, it would be critical to determine how LFA-1 affects cancer growth via leukocytes *in vivo*. In line with an increase in PGE_2 in CD11a KO mice, we previously showed that PGE_2 levels were significantly increased by isoflurane (21).

We need to note a few issues. While we measured the size of tumor to calculate the volume in the same way throughout the study, we did not use IVIG for additional confirmation. Although it is very common to study the effect of anesthetics on tumor growth as in our case, it is important to point out that anesthetics are usually given for tumor resection. To be completely in line with this scenario, it would be important to examine the effect of anesthetics using tumor resection model. However, a simple tumor resection and recurrence model has not been reported in breast cancer yet. In the model using 4T1 breast cancer cells, a nephrectomy has also been done to see tumor recurrence (36). In this study, we used the EO771 cell model, but it would be important to examine different types of cancer given each cancer is very different. LFA-1 binds to its ligand intercellular adhesion molecule-1 (ICAM-1). The expression of ICAM-1 can vary. For example, ICAM-1 is expressed more in triple negative breast cancer cells compared with other types (37). Although our data highly supported that both isoflurane and sevoflurane affected LFA-1 and facilitated tumor growth based on our previous structural studies (10-12), we did not show the direct binding of volatile anesthetics *in vivo*. Therefore, confirmatory experiment is needed to explicitly support the direct interaction between LFA-1 and isoflurane (sevoflurane) *in vivo*.

In summary, we showed that isoflurane significantly facilitated breast cancer growth via affecting LFA-1.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SK performed experiments and wrote the manuscript. WW performed experiments. LH performed experiments. TO performed experiments and edited the manuscript. KY designed the study, performed experiments and wrote the manuscript. SK, LH and KY confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

For animal experiments, Boston Children's Hospital IACUC approval (approved protocol nos. 16-03-3120 and 00001574; Boston, MA, USA) was obtained.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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