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TITLE: Molecular Cross-Talk: Bone Metastatic Prostate Cancer and Nociceptive Neurons

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14. ABSTRACT The goal of this project is to determine the roles of angiotensin II and its receptor in prostate cancer induced bone pain and bone metastatic growth. Aim 1 will provide the framework to identify the extent to which the interaction between cancer cells and nociceptive neurons through the angiotensin II and receptor axis affects cancer-induced bone pain. Aim 2 will determine the downstream molecular mechanisms whereby angiotensin II and its receptor axis affect bone pain. Aim 3 will define how nociceptive neuron influence tumor outgrowth. We believe that the insights derived from our investigations will lead to new strategies for reducing cancer-induced bone pain and also the outgrowth of bone metastasis. During this period, we further characterized a syngeneic murine prostate cancer (PCa)-induced bone pain model which allows us to further elucidate the roles of the crosstalk between bone metastatic PCa and sensory neurons in both bone metastatic progression and its resultant bone pain in an immunocompetent setting. We tested and refined methods for deep tissue clearing and 3D light sheet imaging of intact mouse bone for future investigation of cellular interactions between cancer cells, immune cells and sensory neurons. We also began to identify the roles of cancer-derived angiotensin II in the nerve growth in the bone metastatic setting.					
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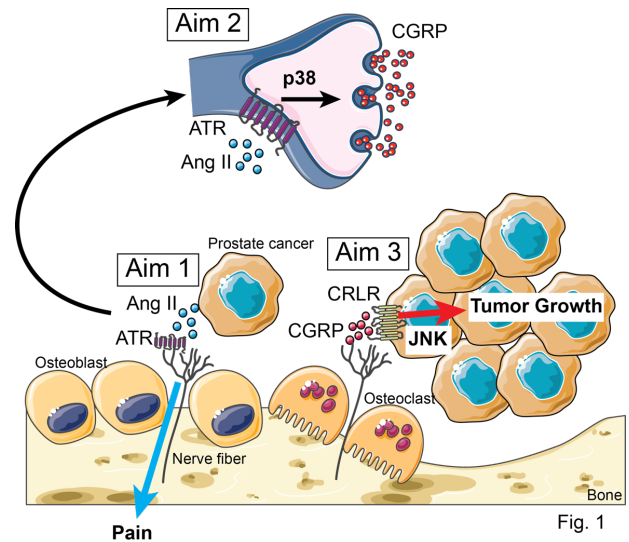
1. INTRODUCTION:

Cancer-related pain, both its causes and its management, poses a tremendous challenge to patients and their caregivers. In the quest for effective cancer therapy, maintaining quality of life can be as crucial as treating the tumor. Seventy-five percent of cancer patients experience pain throughout the course of the disease, and once cancer metastasizes to the bone, the first symptom is often acute bone pain. A full 80% of patients with bone metastasis suffer from cancer-induced bone pain (**CIBP**). Current analgesic strategies for CIBP using nonsteroidal anti-inflammatory drugs (**NSAIDs**) and opioids are effective, but side effects, abuse, and addiction are serious and growing concerns associated with their use. It is therefore essential to develop therapeutic agents that can eradicate bone metastatic diseases and relieve resultant pain.

Metastatic progression of disseminated tumor cells (**DTCs**) to bone may be environmentally directed by influences from the bone marrow microenvironment. Yet a full understanding of the more complex interactions that result in active bone marrow metastasis remains elusive. Our group recently discovered that early in the metastatic process, prostate cancer (**PCa**) cells target and commandeer the specific microenvironment for hematopoietic stem cells (**HSCs**), using mechanisms similar to those involved in HSC homing. Involvement of sympathetic and parasympathetic nerve fibers is crucial for both HSC maintenance and the dissemination of PCa to bone, suggesting that interactions between DTCs and nerves are at least in part responsible for controlling metastatic progression. However, the influence of sensory nerve fibers in the bone on PCa progression and metastasis has not been examined. Although it has been suggested that compression of the soft tissues or peripheral nerve fibers and interference with bone remodeling by bone tumors may be major causes of CIBP, little is known about the molecular interactions of DTCs with bone marrow nerve fibers, and how they affect CIBP.

Elucidating how these interactions influence CIBP is key. Angiotensin II (**Ang II**), a peptide that regulates blood pressure, is also involved in the progression of several types of cancer, including PCa. Ang II is pronociceptive in neuropathic and inflammatory pain models, and elicits sprouting of nociceptive neurons in the skin following inflammation through the angiotensin receptor (**ATR**). In a clinical study of several chronic musculoskeletal pain conditions, a small molecule inhibitor for ATR provided significant pain relief, suggesting that the Ang II/ATR axis controls pain signaling. Our preliminary data suggest that pain-associated neuropeptides can interact directly with PCa cells to cause enhanced proliferation. Therefore, it is critical to explore whether the Ang II/ATR axis is involved in the development of CIBP, and whether pain-associated neuropeptides regulate bone metastatic progression.

We hypothesize that **disseminated PCa stimulates sensory neurons that innervate bone through the Ang II/ATR axis; these cancer-associated sensory neurons then release neuropeptides, resulting in both CIBP and metastatic progression of PCa** (see Fig. 1). Ang II expressed by disseminated PCa induces CIBP (Aim 1) by stimulating sensory neurons to release calcitonin gene-related peptide (**CGRP**) through the ATR/p38 pathway (Aim 2). The nerve-derived CGRP enhances the growth of DTCs via its receptor calcitonin receptor-like receptor (**CRLR**) (Aim 3).



To address our hypothesis the following aims are proposed:

Aim 1: Determine how disseminated prostate cancer is involved in development of cancer-induced bone pain.

Aim 2: Define the intracellular signaling pathways in peripheral nerves stimulated by disseminated prostate cancer.

Aim 3: Determine the influence of nociceptive neurons on (a) tumor outgrowth in the bone and (b) cancer-induced bone pain.

The proposed studies will focus on fundamental mechanisms behind the novel concept that nerves in the bone marrow microenvironment have a crucial role in PCa bone metastasis. Aim 1 will provide the framework to identify the extent to which the interaction between DTCs and nociceptive neurons affects cancer-induced bone pain. Aim 2 will determine the molecular mechanisms of cancer-induced bone pain. Aim 3 will define how DTC/nociceptive neuron interactions influence tumor outgrowth. We believe that the insights derived from our investigations will lead to new strategies for reducing cancer-induced bone pain and also the outgrowth of DTCs. By promoting understanding of how the crosstalk between nociceptive neurons and DTCs participates in cancer-induced bone pain and tumor outgrowth, this study will lay the foundation for significant improvements in care of PCa patients, allowing development of pain targeting therapies that can also minimize bone metastatic progression. If successful, our study will not only shed new light on the fundamental mechanisms of PCa bone metastasis, but will quickly provide a gateway to novel treatment strategies to lower PCa-associated deaths and suffering, as some of the agents to be tested in this study have been in clinical trials for different diseases.

2. KEYWORDS:

Prostate Cancer; Bone metastasis; Disseminated tumor cells; Cancer-induced bone pain; Sensory nerves; Bone marrow microenvironment

3. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The goal of this project is to determine the roles of the interaction between bone metastatic prostate cancer and sensory nerves in cancer-induced bone pain and bone metastatic growth.

Task 1: Determine whether Ang II expressed by disseminated tumor cells affects cancer-induced bone pain using pharmacological interference of Ang II and ATR interactions.

Months 1-11.

- Submit documents for local IACUC review (**Months 1-3; Dr. Shiozawa, Dr. Peters**).
- Submit IACUC approval and necessary documents for ACURO review (**Months 4-6; Dr. Shiozawa**).
- Design and develop an animal model to measure the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 7-8; Dr. Shiozawa, Dr. Peters**).
- Test the effects of Ang II/ATR axis on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 9-11; Dr. Shiozawa, Dr. Peters**).

Task 2: Determine the effects of disseminated tumor cells on Ang II levels in the marrow and ATR expression in the dorsal root ganglia.

Months 12-14.

- Determine the kinetic pattern of Ang II levels in the tumor inoculated marrow and ATR expression in the tumor inoculated DRG (**Months 12-14; Dr. Shiozawa**).

Task 3: Determine the effects of Ang II expressed by disseminated tumor cells on cancer-induced bone pain by manipulating the tumor-nerve microenvironment.

Months 15-19.

- Design and develop nerve-specific ATR knockdown (KD) mice with adeno-associated viral vectors (**Months 15-16; Dr. Shiozawa, Dr. Peters**).
- Test the effects of Ang II/ATR axis on the tumor growth in the marrow, bone remodeling, and pain-related behavior within the same animal (ATR KD mice) (**Months 17-19; Dr. Shiozawa, Dr. Peters**).

Task 4: Determine whether the p38 pathway influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 20-22.

- Test the effects of p38 inhibitor on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 20-22; Dr. Shiozawa, Dr. Peters**).

Task 5: Determine the molecular mechanisms whereby Ang II expressed by disseminated tumor cells activates the bone marrow sensory nerves.

Months 23-24.

- Test the effects of ATR antagonist on the expression of pp38 and CGRP in DRG, spinal cord, and bone (**Months 23-24; Dr. Shiozawa, Dr. Peters**).

Task 6: Major Task 6: Define a link between Ang II expression by disseminated tumor cells with activation of the bone marrow sensory nerves in human subjects.

Months 1-34.

- Submit documents for local IRB review (**Months 1-3; Dr. Shiozawa**).
- Submit IRB approval and necessary documents for HRPO review (**Months 4-6; Dr. Shiozawa**).
- Clinical validation of results with bone biopsy samples of confirmed prostate cancer patients (**Months 7-34; Dr. Shiozawa, Dr. Peters**)
- Co-author manuscript on the effects of Ang II/ATR axis on the tumor growth, bone remodeling, and pain related behavior (**Months 23-26; Dr. Shiozawa, Dr. Peters**).

Task 7: Determine whether CGRP released from bone marrow sensory neurons influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 25-29.

- Design and develop nerve-specific CGRP knockdown (KD) mice with adeno-associated viral vectors (**Months 25-26; Dr. Shiozawa, Dr. Peters**).
- Establish CRLR KD PCa cells (**Months 25-26; Dr. Shiozawa**).

- Test the effects of CGRP/CRLR axis on the tumor growth in the marrow, bone remodeling, and pain-related behavior within the same animal (CGRP KD mice) (**Months 27-29; Dr. Shiozawa, Dr. Peters**).

Task 8: Determine whether CGRP promotes bone metastases through JNK pathway.

Months 30-31.

- Test the effects of CGRP antagonist on the expression of JNK in bone metastatic tumor (**Months 30-31; Dr. Shiozawa, Dr. Peters**).

Task 9: Determine whether JNK pathway influences tumor growth, skeletal remodeling, and cancer-induced bone pain.

Months 32-34.

- Test the effects of JNK inhibitor on the tumor growth in the marrow, bone remodeling, and pain related behavior within the same animal (**Months 32-34; Dr. Shiozawa, Dr. Peters**).
- Co-author manuscript on the effects of CGRP/CRLR axis on the tumor growth, bone remodeling, and pain related behavior (**Months 25-26; Dr. Shiozawa, Dr. Peters**).
- Co-author manuscript summarizing all the results (**Months 27-29; Dr. Shiozawa, Dr. Peters**).

What was accomplished under these goals?

(2017-2018)

Bone metastatic prostate cancer (PCa) causes enriches calcitonin gene-related peptide (CGRP)-expressing sensory nerves in the marrow, and that the CGRP/calcitonin receptor-like receptor (CRLR)/p38 pathway promotes PCa bone metastasis (Dr. Shiozawa).

CGRP downregulation in sensory nerves that innervate bone using adeno-associated virus serotype rh10 (AAVrh10) (Drs. Shiozawa and Peters).

(2018-2019)

Characterization of a syngeneic mouse model of prostate cancer-induced bone pain (Drs. Shiozawa and Peters).

Cancer-derived factors stimulated the neurite outgrowth of murine sensory neurons (Dr. Shiozawa).

(2019-2020)
Characterization of a syngeneic mouse model of prostate cancer-induced bone pain (Drs. Shiozawa and Peters).

During the previous period, we developed and characterized a syngeneic mouse model of PCa-induced bone pain which has the advantage of allowing studies in immunocompetent animals which better reflect the normal bone microenvironment. Briefly, male C57BL6 mice were inoculated into the femur with murine RM-1 PCa cells (1×10^3 cells/ $5 \mu\text{L}$) or Hank's buffered saline as a sham control. Prior to injection, the RM-1 PCa line was transfected with luciferase reporters and green fluorescent protein (GFP), or mCherry in order to visualize *in vivo* tumor growth longitudinally and quantify the extent of tumor burden at sacrifice. We observed a progressive increase in bioluminescence (BLI) in the ipsilateral hind limb of RM-1 but not sham mice between 7- and 21-days post-inoculation (Reported in the last year's progress report).

During this award period, we further characterized this syngeneic mouse model.

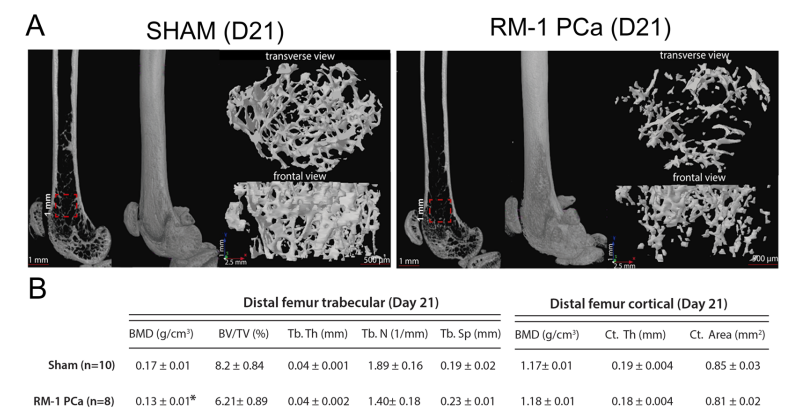


Figure 1. Assessment of bone morphology of mice inoculated into femur with PCa cells. μCT scans of femurs from sham and tumor bearing mice including 2D slice and 3D image of entire femur and distal metaphysis (insets, A). Quantification of key μCT outcomes (B) indicate loss of trabeculae due to osteolysis. Student's t-test * $p < 0.05$ versus Sham values. Sham $n = 10$, RM1-GFP $n = 8$

In this model, tumor induced bone remodeling, evident as cortical osteolytic lesions by μCT at Day 14 (Reported in the last year's progress report) and at Day 21 (**Fig. 1A&B**) and extra-periosteal aberrant bone formation by immunohistochemistry at Day 21 (Reported in the last year's progress report), was observed in the ipsilateral femur of RM-1 but not sham mice based on histological and radiographic analysis.

Tumor growth was accompanied by spontaneous guarding of the inoculated limb suggestive of ongoing pain and impairment of daily running wheel performance indicative of movement evoked pain (Reported in the last year's progress report and **Fig. 2**). Additionally, spinal cord tissue was collected and examined immunohistochemically for markers of central sensitization. We found that tumor-inoculated

mice had increased expression of dynorphin, pERK, and GFAP in the ipsilateral side of spinal cord (Reported in the last year's progress report). We also found the levels of CGRP and SP in terminal of sensory neurons within the spinal cord were significantly increased in tumor-inoculated mice, compared to sham mice (Reported in the last year's progress report). Immunohistochemical analyses demonstrated that the bone marrow of tumor-inoculated mice showed more vascularization in close proximity to prostate cancer cells, compared to sham mice (**Fig. 3**). The number of sensory (PGP9.5 +) nerve fibers was quantified in the periosteum, mineralized bone and bone marrow of tumor bearing and sham inoculated mice. Sensory neurons are observed in direct contact with tumor cells within the bone marrow at early time points (D11, **Fig. 4**) and at later time points predominantly within the periosteum in close proximity to prostate cancer cells and regions of remodeled bone (osteoclasts) (Reported in the last year's progress report and **Fig. 4**). These results are being prepared in a manuscript for submission during the next funding cycle. Future studies will use this model in combination with transgenic Cre/Flpo driver mice to better understand sensory neuron tumor interactions that drive PCa induced bone pain and disease progression.

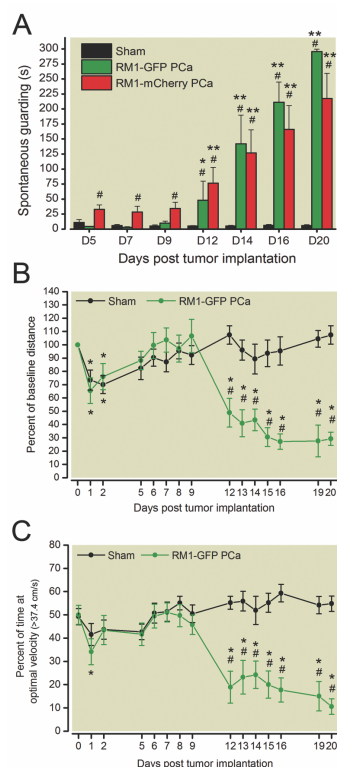


Figure 2. Assessment of pain behaviors of mice inoculated into femur with PCa cells.

Time course of ongoing or spontaneous pain in mice following intra-femoral-injection of RM1-GFP (green), RM1-mCherry (red) PCa cells or sham injections is evident as progressive increase in duration of guarding of the tumor bearing hindlimb during a 5 minute observation period (A). Two-way RM ANOVA with Bonferroni comparisons $\#P<0.05$ versus sham values within time point, $*P<0.05$ and $**P<0.001$ versus D5 within group. Sham $n=11$, RM1-GFP $n=8$, RM1-mCherry $n=8$. In a separate cohort of RM1-GFP and sham mice, we assessed the impact of bone cancer on running wheel performance as a measure of functional disability and potentially movement evoked pain. Mice were allowed access to a running wheel 5 days a week for 30-minute sessions. Prior to tumor inoculation, mice were exposed to the running wheel for three weeks to obtain baseline values. Tumor bearing mice displayed a progressive impairment in the time spent running at an optimal velocity during daily 30 minute sessions compared to sham mice beginning 12 days post injection (B). Similarly the distance traveled during a daily 30 minute session was reduced progressively compared to sham mice beginning 12 days post inoculation. There was a transient reduction in time spent at optimal velocity and distance traveled in both tumor injected and sham operated rats due to surgery. Two-way RM ANOVA with Bonferroni comparisons $\#P<0.05$ versus sham values within time point, $*P<0.05$ and $**P<0.001$ versus baseline D0 within group. Sham $n=12$, RM1-GFP $n=12$.

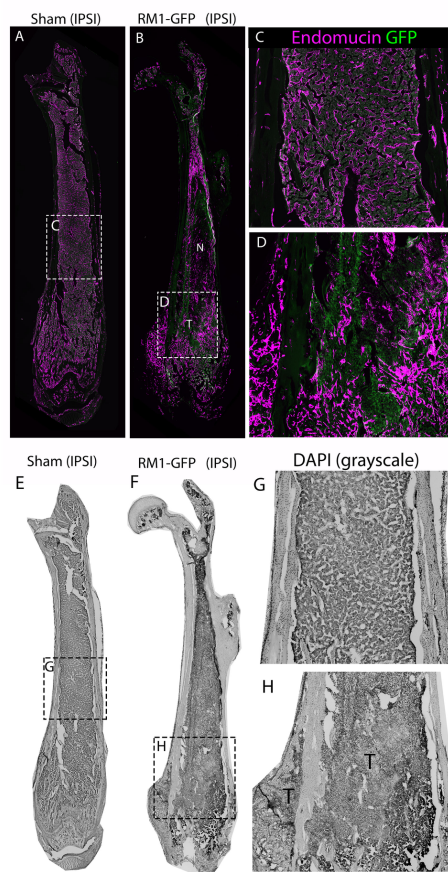


Figure 3. Relationship between blood vessels and PCa cells in tumor bearing mice.

Sections of the femur were collected from RM1-GFP and sham operated mice 21 days post-inoculation and labeled with an antibody against green fluorescent protein (GFP, green) and the endothelial and sinusoid marker endomucin (purple) and counterstained with DAPI to visualize total cellularity. Sham mice have uniform distribution of endomucin on blood vessels and sinusoids throughout the bone marrow (A, C). Tumor bearing mice showed reorganization of endomucin positive vessels near the leading edge of the tumor (T) marked by GFP+ cells (B, D) and loss of immunoreactivity at the mid-diaphysis due to necrosis (N). Based on DAPI staining approximately 80% of the femur was occupied by tumor cells 21 days post inoculation.

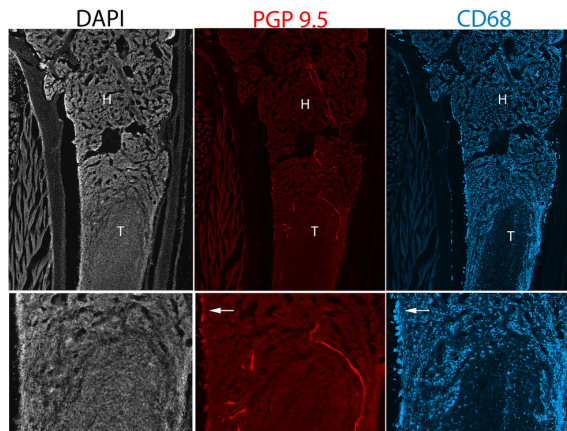


Figure 4. Relationship between nerve fibers and PCa cells in tumor bearing mice. Sections of the femur were collected from RM1-GFP and sham operated mice 11 days post-inoculation at a time point coinciding with the onset of cancer related pain behaviors. Femurs were counterstained with DAPI (A & D, white) and labeled with antibodies against the pan-neuronal marker PGP 9.5 (B & E, red) which labels sympathetic and sensory nerve fibers and CD68 (C & F, blue) which labels osteoclasts and activated M1 macrophage. Note the nerve fibers within the bone marrow are compressed by the invading cancer cells. Some CD68⁺ osteoclasts along the endosteal surface are also immunoreactive for PGP 9.5 (arrows).

Development of methods for deep tissue clearing and 3D light sheet imaging to investigate in more detail interactions between cancer cells, tumor associated macrophage and populations of nerve fibers within the bone microenvironment (Dr. Peters)

To further characterize the relationship between immune cells, nerve fibers and PCa cells in tumor bearing mice, we sought to evaluate several methods for clearing intact decalcified mouse bones from a 5-HT3R reporter mouse that exhibits GFP expression in a subpopulation of mostly myelinated high threshold mechanoreceptive sensory neurons (**Fig. 5**). We tested and refined previously published PACT-DeCal PEGASOS and BoneClear protocols and determined that a slightly modified PEGASOS protocol produced the most consistent results. During the next funding cycle, we will use these methods to examine the distribution and plasticity of CGRP + sensory neurons and RM1-mCherry PCa cells at various time points after tumor inoculation in the mouse femur. The results will be presented in a future publication.

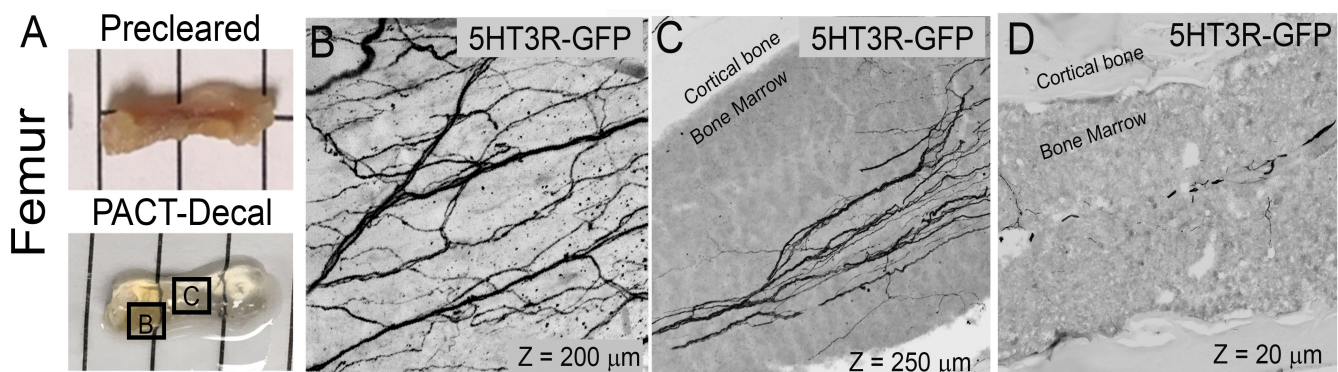
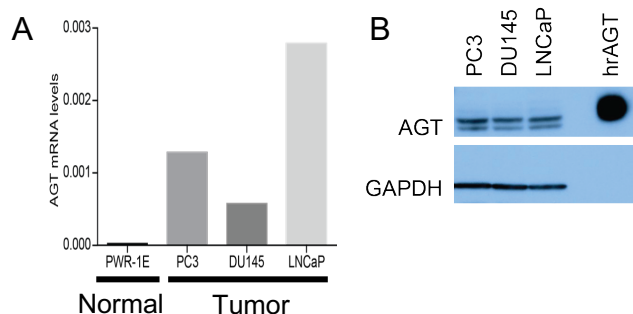


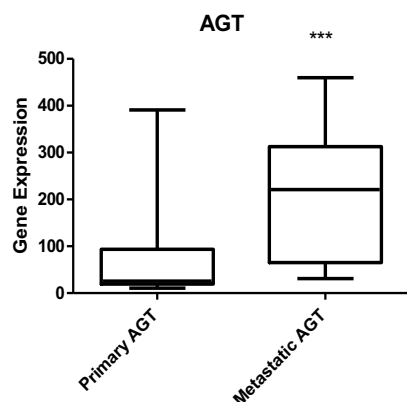
Figure 5. Image of whole mouse femur (A) prior to and following PACT-deCal procedure. (B) Maximum intensity projection of GFP-IR in the femur of a 5HT3R-GFP reporter mouse obtained from cleared femur corresponding to cortical region of the distal metaphysis including periosteum (B) and the bone marrow (C) obtained with confocal microscopy. For comparison, image of non-cleared 20 μ m thick section (D) shows only small fragments of nerve fibers. Color confocal images converted to gray scale and inverted to maximize contrast. Confocal images obtained using a 10x objective Zoom 1.4 from multiple optical scans between 200-250 microns in the Z plane.

Prostate cancer produces angiotensin II (Ang II) from AGT by ACE and secretes Ang II (Dr. Shiozawa).

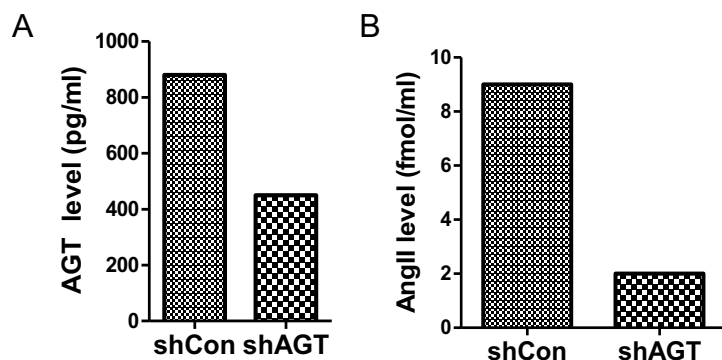
To test one of our hypothesis that bone metastatic prostate cancer-derived angiotensin II (Ang II) is involved in the induction of cancer-induced bone pain, we first determine whether prostate cancer cells express angiotensinogen (AGT), since Ang II is a byproduct of AGT. Prostate cancer cells express both AGT mRNA (**Fig. 6A**) and protein (**Fig. 6B**), whereas no or little expression of AGT was observed in normal prostate cells (**Fig. 6A**). Interestingly, prostate cancer cells obtained from metastatic organs express higher levels of AGT than those obtained from primary site (**Fig. 7**), suggesting that AGT expression in prostate cancer cells may correlate with the prostate cancer progression. Additionally, prostate cancer cells secrete both AGT (**Fig. 8A**) and Ang II (**Fig. 8B**). To further determine the mechanisms whereby prostate cancer produces Ang II, ACE (produces Ang II by cleaving Ang I) and ACE2 (produces Ang 1-7 by cleaving Ang II) enzymatic activities were measured. As you can see in **Fig. 9**, Ang I was converted into Ang II by ACE, but no conversion of Ang II by ACE2 was observed in prostate cancer cells. Together, these data suggest that prostate cancer produces Ang II from AGT by ACE and secretes Ang II. Future studies will investigate whether Ang II derived from prostate cancer causes cancer-induced bone pain by stimulating sensory neurons.

**Figure 6. Prostate cancer cells express AGT.**

(A) AGT mRNA expression of Normal prostate cells (RWR-1E) and three different PCa cells (PC3, DU145, and LNCaP) using GAPDH as reference gene. (B) Western blot analysis of AGT in three different PCa cell lines. Human recombinant AGT was used as a positive control. GAPDH served as a loading control.

**Figure 7. Prostate cancer cells obtained from metastatic organs express higher levels of AGT than those from primary tumors.**

Box plots of gene expression values for AGT in primary PCa (Primary, n=62) and metastatic PCa (Met, n=21) obtained from the GEO database (GSE 6919). Presented as mean $\pm$ SD, significance versus primary PCa (Student's *t*-test).

**Figure 8. Prostate cancer cells secrete AGT and Ang II.**

DU145 cells expressing either control or AGT shRNAs were plated on 10cm dishes. After 24 h serum starvation, conditioned media were collected to quantify the secretion of (A) AGT and (B) Ang II by a radioimmuno assay (RIA).

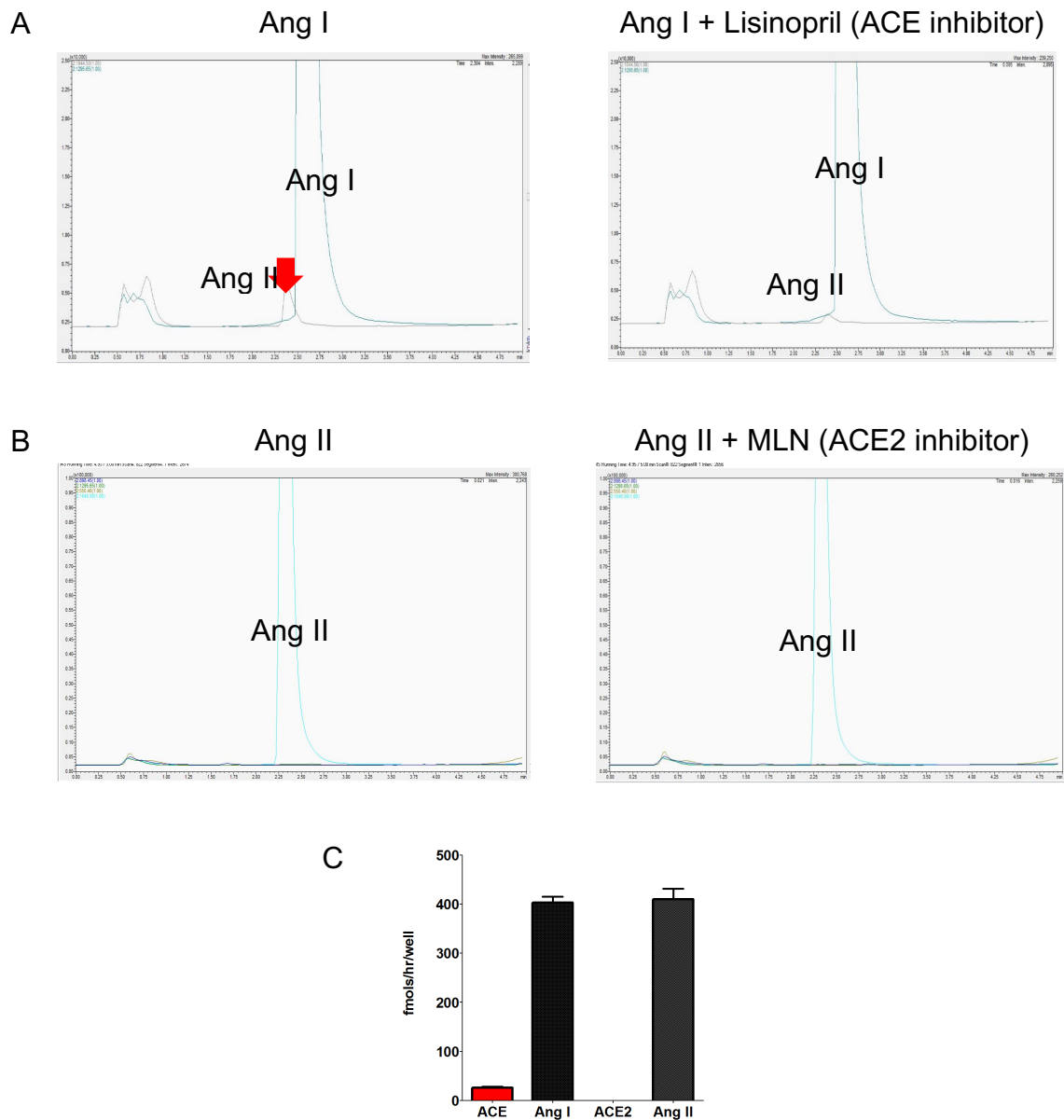


Figure 9. Ang I was converted into Ang II by ACE, but no conversion of AngII by ACE2 was observed in DU145 cells.

ACE and ACE2 activity in DU145 were measured by Ang I and Ang II peptide metabolism, respectively. (A) For ACE activity, cells were treated with Ang I and then followed by control or ACE inhibitor (Lisinopril). (B) For ACE2 activity, cells were treated with Ang II and then followed by control or ACE2 inhibitor (MLN-4760). Peptide products were determined by reverse-phase high-performance liquid chromatography (HPLC) separation. (C) Enzyme activities were expressed as fmol product of Ang-1 or Ang-II per hr per well (fmol/hr/well).

No cost extension on this award (Drs. Shiozawa and Peters).

One-year no cost extension on this award was approved due to the COVID-19 pandemic.

What opportunities for training and professional development did the project provide?

(2017-2018)

Dr. Shiozawa was invited to give a seminar at Jikei University School of Medicine (Tokyo, Japan) on October 11, 2017.

Dr. Peters organized a symposium at Wake Forest University Medical School entitled “*Translational Cancer Pain Research: Bridging the Gap Between Basic Science and Clinical Application* “. The symposium consisted of presentations from 3 clinicians (Dr. Francis Walker, Dr. Glenn Lesser, Dr. Roy Strowd) who specialize in clinical research focused on interventional studies for the treatment of cancer related pain and sensory dysfunction as well as 2 preclinical researchers (Dr. Chris Peters, Dr. Mario Danelo Boada) who are conducted mechanistic research studies in rodent models of cancer pain. The keynote speaker was Dr. Patrick Dougherty from MD Anderson Cancer Center who presented a lecture entitled “Inflammation and Ectopic Spontaneous Activity: New Findings of Chemotherapy Induced Peripheral Neuropathy in Humans & Animals”. This symposium came about largely as a result of the collaborative efforts driven by Dr. Shiozawa between clinicians within Wake Forest Comprehensive Cancer Center and faculty members within the Department of Anesthesiology at Wake Forest Baptist Medical Center.

(2018-2019)

Dr. Shiozawa was invited to give a talk at The U.S. Bone and Joint Initiative (USBJI) and Bone and Joint Canada (BJC) Young Investigator Initiative Workshop Spring 2019 (Rosemont, IL) on April 27, 2019.

Dr. Shiozawa was invited to give a seminar at University of North Carolina at Chapel hill, Adams School of Dentistry (Chapel hill, NC) on December 03, 2019.

Dr. Peters presented a poster at Neuroscience 2019 the Annual Society for Neuroscience Meeting in Chicago, Illinois October 19th-23, 2019.

(2019-2020)

Dr. Shiozawa serves as an Editor for HubLE Graphics Committee of The IFMRS (International Federation of Musculoskeletal Societies) HubLE.

Dr. Shiozawa presented an e-poster at American Society for Bone and Mineral Research (ASBMR) 2020 Annual Meeting, Virtual, September 11-14, 2020.

How were the results disseminated to communities of interest?

There is nothing to report at this time.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

In the next year of the award, we will continue to determine the roles of renin-angiotensin system in PCa-induced bone pain (Aim 1). We will also further elucidate the molecular mechanisms whereby bone metastatic PCa stimulates sensory nerves (Aim 2). In addition, we will continue to pursue studies examining the effects of sensory nerves on the progression of bone metastatic PCa (Aim 3).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

(2017-2018)

We found that PCa enhances the sprouting of CGRP-expressing sensory nerves both in vitro and in vivo.

We found that PCa patients with bone metastases have high levels of CGRP in their serum, that PCa in bone metastatic sites expresses high levels of CRLR, and that CRLC expression levels in PCa is associated with their recurrent free survival.

We found that CGRP induces PCa proliferation through CRLR/p38 pathway.

We developed the technique to manipulate gene expression in sensory nerves innervating bones using an AAVrh10 virus vector.

(2018-2019)

We developed a syngeneic murine cancer-induced bone pain model which allows us to further investigate the mechanisms how bone metastatic PCa influence bone pain behaviors in an immunocompetent setting. Future studies will use this model in combination with transgenic Cre/Flpo driver mice to better understand sensory neuron tumor interactions that drive PCa induced bone pain and disease progression.

We established an *in vitro* sensory neuron culture system to further study interaction between PCa and sensory neurons.

We developed a semi-automated quantification method to measure the *in vitro* neurite outgrowth of sensory neurons.

(2019-2020)

We further characterized a syngeneic murine prostate cancer (PCa)-induced bone pain model which allows us to further elucidate the roles of the crosstalk between bone metastatic PCa and sensory neurons in both bone metastatic progression and its resultant bone pain in an immunocompetent setting.

We developed and refined methods for deep tissue clearing and 3D light sheet imaging to investigate in more detail interactions between cancer cells, immune cells and populations of nerve fibers within the bone microenvironment.

We began to identify the roles of cancer-derived angiotensin II in the nerve growth in the bone metastatic setting.

We obtained the one-year no cost extension on this award due to the COVID-19 pandemic.

AWARD: PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Christopher Peters

What was the impact on other disciplines?

There is nothing to report at this time.

What was the impact on technology transfer?

There is nothing to report at this time.

What was the impact on society beyond science and technology?

There is nothing to report at this time.

AWARD: PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Christopher Peters

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that have a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal Publications

Peer reviewed journal

(2017-2018)

1. Park SH, Eber MR, Tsuzuki S, Booker ME, Sunil AG, Widner DB, Parker RA, **Peters CM**, **Shiozawa Y**. Adeno-associated virus serotype rh10 is a useful gene transfer vector for sensory nerves that innervate bone in immunodeficient mice. **Sci Rep**. 2017;7:17428. PMID: 29233995. PMCID: PMC5727257.

Status of Publication: Published

Acknowledgement of federal support: Yes

- Selected as Papers of the Week (9 Dec 2017- 15 Dec 2017) by Pain Research Forum

(2018-2019)

2. Jones JD, Sinder BP, Paige D, Soki FN, Koh AJ, Thieleb S, **Shiozawa Y**, Hofbauer LC, Daignault S, Roca H, McCauley LK. Trabectedin reduces skeletal prostate cancer tumor size in association with effects on M2 macrophages and efferocytosis. **Neoplasia**. 2018;21:172-184. PMID: 30591422. PMCID: PMC6314218.

Status of Publication: Published

Acknowledgement of federal support: Yes

(2019-2020)

3. Lycan TW, Hsu F, Ahn CS, Thomas A, Walker FO, Sanguenza OP, **Shiozawa Y**, Park SH, **Peters CM**, Romero-Sandoval EA, Melin SA, Sorscher S, Ansley K, Lesser GJ, Cartwright MS, Strowd RE. Neuromuscular ultrasound for taxane peripheral neuropathy in breast cancer. **Muscle and Nerve**. 2020;61:587-594. PMID: 32052458. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: Yes

4. Wu S-Y, Xing F, Sharma S, Wu K, Tyagi A, Liu Y, Zhao D, Deshpande RP, **Shiozawa Y**, Ahmed T, Zhang W, Chan M, Ruiz J, Lycan TW, Dothard A, Watabe K. Nicotine promotes brain metastasis by polarizing microglia and suppressing innate immune function. **JEM**. 2020;217:e20191131. PMID: 32496556. PMCID: PMC7398164.

Status of Publication: Published

Acknowledgement of federal support: No

Invited reviews

(2017-2018)

1. Park SH, Keller ET, **Shiozawa Y**. Bone marrow microenvironment as a regulator and therapeutic target for prostate cancer bone metastasis. **Calcif Tissue Int**. 2018;102:152-62. PMID: 29094177. PMCID: PMC5807175.
Status of Publication: Published
Acknowledgement of federal support: Yes
2. Widner DB, Files DC, Weaver KE, **Shiozawa Y**. Preclinical and clinical studies on cancer-associated cachexia. **Front Biol**. 2018;13:11-18. PMID: In Progress. PMCID: In Progress.
Status of Publication: Published
Acknowledgement of federal support: Yes
3. Park SH, Eber MR, Widner DB, **Shiozawa Y**. Role of the bone microenvironment in the development of painful complications of skeletal metastases. **Cancers (Basel)**. 2018;10:141. PMID: 29747461. PMCID: PMC5977114.
Status of Publication: Published
Acknowledgement of federal support: Yes

• **Selected as Featured Paper**

4. Widner DB, Park SH, Eber MR, **Shiozawa Y**. Interactions between disseminated tumor cells and bone marrow stromal cells regulate tumor dormancy. **Curr Osteoporos Rep**. 2018;16:596-602. PMID: 30128835. PMCID: In Progress.
Status of Publication: Published
Acknowledgement of federal support: Yes

(2018-2019)

Nothing to report.

(2019-2020)

Nothing to report.

Book

(2017-2018)

1. Park SH, Eber MR, **Shiozawa Y**. (2018) Tumor inoculation mouse models of prostate cancer bone metastasis. In Idris A (Ed.), *Bone Research Protocols - 3rd Edition*. In Press. London: Springer Nature.
Status of Publication: Accepted

Acknowledgement of federal support: Yes

2. Park SH, Eber MR, Taichman RS, **Shiozawa Y.** (2018) Determining Competitive Potential of Bone Metastatic Cancer Cells in the Murine Hematopoietic Stem Cell Niche. In Turksen K (Ed.), *Stem cells and Niche - 2nd Edition: Methods and Protocols*, Methods in Molecular Biology. In Press. New York: Humana Press. Methods Mol Biol. In Press. PMID: 30099699. PMCID: In Progress.

Status of Publication: Published

Acknowledgement of federal support: Yes

(2018-2019)

3. Park SH, **Shiozawa Y.** (2019) Genomic mutation as a potential driver of the development of bone-related cancers. In Zaidi M (Ed.), *Encyclopedia of Bone Biology*. In Press. Elsevier.

Status of Publication: In Press

Acknowledgement of federal support: Yes

4. **Shiozawa Y.** (2019) The roles of bone marrow-resident cells as a microenvironment for bone metastasis. In Birbrair A (Ed.), *Tumor Microenvironments in Different Organs – Part A*. In Press. Switzerland: Springer Nature.

Status of Publication: In Press

Acknowledgement of federal support: Yes

(2019-2020)

5. Eber MR, Jimenez-Andrade JM, **Peters CM, Shiozawa Y.** (2020) A method of bone-metastatic tumor progression assessment in mice using longitudinal radiography. In Deep G (Ed.), *Cancer Biomarkers: Methods and Protocols*, Methods in Molecular Biology, In Press. New York: Humana Press. Methods Mol Biol. In Press. PMID: In Progress. PMCID: N/A.

Status of Publication: In Press

Acknowledgement of federal support: Yes

Presentation

(2017-2018)

1. Williams SN, Eber MR, Tsuzuki S, Park SH, **Shiozawa Y.** Does histamine influence the osteoblastic activities that help regulate the progression of bone metastatic prostate cancer? The 2017 Annual Biomedical Research Conference for Minority Students (ABRCMS), Phoenix, AZ, USA, November 1-4, 2017. Poster.

(2018-2019)

2. Lycan T, Thomas A, Hsu FC, Cartwright MS, Walker FO, Ahn C, Sanguenza OP, **Shiozawa Y**, Park SH, **Peters CM**, Melin SA, Avery TP, Sorscher S, Lesser GJ, Strowd RE. Neuromuscular ultrasound for assessment of peripheral neuropathy in breast cancer patients receiving taxane therapy. *J Clin Oncol* 36, 2018 (suppl; abstr e22083). 2018 American Society Of Clinical Oncology (ASCO) Annual Meeting, Chicago, IL, USA, June 1-5, 2018. Poster.
3. Strowd RE, Lycan T, Thomas A, Hsu FC, Ahn C, Sanguenza O, **Shiozawa Y**, Park SH, **Peters CM**, EA Romero-Sandoval, Melin SA, Sorscher S, Lesser GJ, Walker FO, Cartwright MS. Neuromuscular ultrasound for the non-invasive assessment of breast cancer patients with peripheral neuropathy from taxanes. The American Academy of Neurology 71st Annual Meeting, Philadelphia, PA, USA, May 4-10, 2019. Poster.
4. Tsuzuki S, Park S, Eber M, Widner B, Kamata Y, Kimura T, Bianchi-Frias D, Coleman I, Nelson P, Hsu F-C, **Peters C**, **Shiozawa Y**. A pain-related neuropeptide calcitonin gene-related peptide promotes bone metastatic progression of prostate cancer through p38. American Society for Bone and Mineral Research (ASBMR) 2019 Annual Meeting, Orland, FL, USA, September 20-23, 2019. Poster.
5. Park S, Eber M, Tsuzuki S, Cain R, Widner B, Kamata Y, Kimura T, Bianchi-Frias D, Coleman I, Nelson P, Hsu F-C, **Peters C**, **Shiozawa Y**. The role of the SCF/c-kit pathway in cancer-induced bone pain. American Society for Bone and Mineral Research (ASBMR) 2019 Annual Meeting, Orland, FL, USA, September 20-23, 2019. Poster.
 - **Selected as a Plenary Poster**
6. Cain R, Park SH, Eber M, Martin TJ, Parker R, Jimenez-Andrade JM, **Shiozawa Y**, **Peters C**. Characterization of a syngeneic mouse model of prostate cancer induced bone pain. Neuroscience 2019, Chicago, IL, USA, October 19-23, 2019. Poster.

(2019-2020)

7. Ding H, Kiguchi N, Park SH, Kishioka S, **Shiozawa Y**, **Peters C**, Ko M-C. Functional roles of neuromedin B and gastrin-releasing peptide in regulating itch and pain in the spinal cord of primates. **FASEB J.** 2030;34 (S1):03569. Experimental Biology 2020 (Cancelled, but abstract was published).
8. Eber M, Park S, Hsu F-C, **Shiozawa Y**. Osteoblasts Derived from Mouse Mandible Enhance Tumor Growth of Prostate Cancer More Than Osteoblasts Derived from Long Bone. The Cancer and Bone Society (CABS) / American Society for Bone and Mineral Research (ASBMR) Pre- Meeting, The Seed and Soil: Therapeutic Targets for Cancer in Bone, Virtual, September 9-10, 2020. e-Poster.
9. Eber M, Park S, Hsu F-C, **Shiozawa Y**. Osteoblasts Derived from Mouse Mandible Enhance Tumor Growth of Prostate Cancer More Than Osteoblasts Derived from Long Bone. American Society for

AWARD: PC160455P1

TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Christopher Peters

Bone and Mineral Research (ASBMR) 2020 Annual Meeting, Virtual, September 11-14, 2020. e-Poster.

10. Widner DB, Eber M, Park S, **Shiozawa Y**. Skeletal Muscle Resident Mast Cells as a Potential Novel Biomarker for Cancer-Associated Cachexia. Cancer Cachexia 2020, Virtual, September 10-11, 2020. e-Poster.

Website(s) or other Internet site(s)

Nothing to report.

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Yusuke Shiozawa

Project Role: PI, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-9814-9230

Nearest person month worked: 3

Contribution to Project: Dr. Shiozawa is an Assistant Professor in the Department of Cancer Biology. He has extensive experience in the study of prostate cancer bone metastasis, and will provide oversight of the entire program including development and implementation of all policies, procedures, and processes. In this role, Dr. Shiozawa will be responsible for the completion of the project and for ensuring that systems are in place to guarantee institutional compliance with US laws, including biosafety and animal research guidelines, data collection and analyses, and facilities. Dr. Shiozawa will supervise other personnel on the project to ensure timely and effective studies.

Funding Support: Department of Defense, TEVA Pharmaceuticals, National Cancer Institute

Name: Christopher Peters

Project Role: Partnering PI, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 1.8

Contribution to Project: Dr. Peters is an Assistant Professor in the Department of Anesthesiology. Dr. Peters' research interests are in central and peripheral mechanisms of pain chronicity including postsurgical, bone cancer and chemotherapy induced pain and neuropathy. Dr. Peters will be responsible for supervising and training personnel in evoked and non-evoked bone cancer pain behavioral assays. As part of Aims 1 and 3, Dr. Peters will assist with *in vivo* validation of a viral vector approach for sensory neuron selective knockdown of ATR and CGRP. He will also assist with histological and immunohistochemical analysis of skeletal tissue as part of Aims 1-3. Dr. Peters has over 15 years of experience working with preclinical models of skeletal pain including rat and mouse models of bone cancer. He has extensive experience with the behavioral analysis, immunohistochemistry, biochemical analysis and fluorescent, brightfield, and confocal microscopic imaging required for this project. He will meet with Dr. Shiozawa on a weekly basis to discuss progress on projects related to this Partnering PI Prostate Cancer Research Idea Development Award.

Funding Support: National Institutes of Health, Department of Defense

Name: Fang-Chi Hsu

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Hsu is a Professor in the Department of Biostatistical Sciences. She will work closely with Dr. Shiozawa and the team to analyze the results of the project.

Funding Support: National Institutes of Health, Department of Defense

Name: Christopher Thomas

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.12

Contribution to Project: Dr. Thomas is an Associate Professor in the Department of Hematology/Oncology. On this project, he will assist Dr. Shiozawa's laboratory in tissue procurement, experimental design, and scientific strategy.

Funding Support: Department of Defense

Name: Debra Diz

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Diz is the Co-Director of the Cardiovascular Sciences Center and Professor, Department of General Surgery. Her research focuses on cardiovascular neuroscience and neuropharmacology related to the effects of angiotensin II on neuropeptide and release of other transmitters in the context of the renin-angiotensin-system in aging and metabolic regulation. On this project, she will assist Dr. Shiozawa's laboratory in radio binding assay.

Funding Support: National Institutes of Health, Department of Defense

Name: Mark Chappell

Project Role: Co-Investigator, W81XWH-17-1-0541

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Chappell is a Professor in the Department of General Surgery and member of the Cardiovascular Sciences Center. He is an expert in the assessment of the renin-angiotensin-system (RAS) components and is interested in the RAS in hypertension, diabetes, fetal programming and women's health issues. On this project, he will assist Dr. Shiozawa's laboratory in radioimmunoassay.

Funding Support: National Institutes of Health, Department of Defense

Name: Thomas Jeff Martin

Project Role: Co-Investigator, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 0.6

Contribution to Project: Dr. Martin is a Professor in the Department of Anesthesiology and Physiology & Pharmacology. Dr. Martin's research interests are on mechanisms of opioid abuse particularly in the setting of chronic pain. He has been instrumental in the development of operant based behavioral assays to monitor the effects of persistent pain on complex animal behaviors. Dr. Martin will assist with training, implementation and analysis of running wheel assays to assess progressive disability in mice inoculated with bone cancer as part of studies outlined in Aims 1-3.

Funding Support: National Institutes of Health, Department of Defense

Name: Juan Miguel Jimenez-Andrade

Project Role: Visiting scholar, W81XWH-17-1-0542

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3

Contribution to Project: Dr. Jimenez-Andrade is a Professor in the Autonomous University of Tamaulipas in Reynosa, Mexico. Dr. Jimenez-Andrade's laboratory research is focused on mechanisms of skeletal pain and osteoporosis. He assumed responsibility for performing microCT imaging and analysis as part of experiments in Aims 1-3. The subaward initially established with Ted Bateman at UNC was transferred to Dr. Jimenez-

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TITLE: Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PI: Christopher Peters

Andrade's institution effective September 2019. Additionally, he will assist with immunohistochemical analysis of decalcified bone sections as part of studies under this proposal.

Funding Support: Department of Defense, National Institutes of Health, UC MEXUS-CONACYT

Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations have been involved as partners?

The Autonomous University of Tamaulipas at Reynosa Mexico

8. SPECIAL REPORTING REQUIREMENTS:

QUAD CHARTS.

9. APPENDICES:

The original copies of manuscript are attached.

Molecular crosstalk: bone metastatic prostate cancer and nociceptive neurons

PC160455, PC160455P1

W81XWH-17-1-0541, W81XWH-17-1-0542

PI: Yusuke Shiozawa, Christopher Peters

Org: Wake Forest University Health Sciences

Award Amount: \$500,001 (Shiozawa)
\$496,403 (Peters)



Study/Product Aim(s)

- **Aim 1: Determine how disseminated prostate cancer is involved in development of cancer-induced bone pain.**
- **Aim 2: Define the intracellular signaling pathways in peripheral nerves stimulated by disseminated prostate cancer.**
- **Aim 3: Determine the influence of nociceptive neurons on (a) tumor outgrowth in the bone and (b) cancer-induced bone pain.**

Approach

The goal of this project is to determine the roles of the interaction between bone metastatic prostate cancer and sensory nerves in cancer-induced bone pain and bone metastatic growth using our innovative mouse models that enable us to measure cancer bone pain, tumor growth, and bone dynamics within the same animals. In addition, using biopsy samples from patients, we will verify the results from the animal experiments.

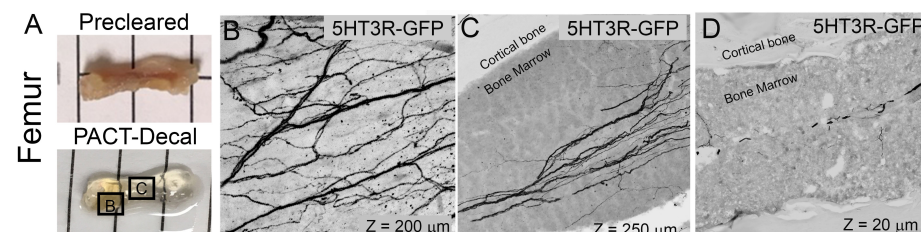


Image of whole mouse femur (A) prior to and following PACT-deCal procedure. (B) Maximum intensity projection of GFP-IR in the femur of a 5HT3R-GFP reporter mouse obtained from cleared femur corresponding to cortical region of the distal metaphysis including periosteum (B) and the bone marrow (C) obtained with confocal microscopy. For comparison, image of non-cleared 20 um thick section (D) shows only small fragments of nerve fibers. Color confocal images converted to gray scale and inverted to maximize contrast. Confocal images obtained using a 10x objective Zoom 1.4 from multiple optical scans between 200-250 microns in the Z plane. 5-HT3R-GFP labels a population of myelinated nociceptive sensory neurons

- We refined methods for clearing and deep tissue and 3D imaging of intact mouse bone

Timeline and Cost

Activities	CY	17	18	19	20
Aim 1 (Animal Studies)					
Aim 2 (Animal Studies)					
Aim 2 (Bone sample analyses)					
Aim 3 (Animal Studies)					
Estimated Budget (\$K)		\$100	\$325	\$325	\$246

Updated: 10/18/2018

Goals/Milestones (Example)

CY17 Goal – Team development

- ☑ Set up and organize the monthly meeting

CY18 Goals – Animal studies

- ☑ Develop the technique to manipulate gene in nerves with vector
- ☑ Determine the roles of nerves in cancer progression

CY19 Goal – Bone biopsy sample analyses/Animal studies

- ☑ Develop the system to assess bone biopsy samples
- ☑ Determine the roles of nerves in cancer progression
- ☑ Determine the roles of cancer in cancer-induced bone pain

CY20 Goal – Bone biopsy sample analyses/Animal studies

- ☑ Determine the roles of cancer in cancer-induced bone pain
- ☐ Assess the nerve/cancer interaction in bone biopsy samples

Comments/Challenges/Issues/Concerns

- One-year no cost extension on this award was approved.

Budget Expenditure to Date

Projected Expenditure: \$996K

Actual Expenditure: \$900K

Neuromuscular ultrasound for taxane peripheral neuropathy in breast cancer

Thomas W. Lycan DO, MHS¹  | Fang-Chi Hsu PhD² | Christine S. Ahn MD³ | Alexandra Thomas MD¹ | Francis O. Walker MD⁴ | Omar P. Sanguenza MD⁵ | Yusuke Shiozawa MD, PhD⁶ | Sun Hee Park PhD⁶ | Christopher M. Peters PhD⁶ | Edgar Alfonso Romero-Sandoval MD⁷ | Susan A. Melin MD¹ | Steven Sorscher MD¹ | Katherine Ansley MD¹ | Glenn J. Lesser MD¹ | Michael S. Cartwright MD⁴ | Roy E. Strowd MD⁴

¹Internal Medicine: Hematology and Oncology, Wake Forest School of Medicine, North Carolina

²Biostatistics and Data Science, Wake Forest School of Medicine, North Carolina

³Dermatology, Wake Forest School of Medicine, North Carolina

⁴Neurology, Wake Forest School of Medicine, North Carolina

⁵Pathology, Wake Forest School of Medicine, North Carolina

⁶Cancer Biology, Wake Forest School of Medicine, North Carolina

⁷Anesthesiology, Wake Forest School of Medicine, North Carolina

Correspondence

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Email: tlycan@wakehealth.edu

Funding information

Department of Defense, Grant/Award Numbers: W81XWH-19-1-0045, W81XWH-17-1-0541, W81XWH-14-1-0403

Abstract

Background: Our study aim was to evaluate neuromuscular ultrasound (NMUS) for the assessment of taxane chemotherapy-induced peripheral neuropathy (CIPN), the dose-limiting toxicity of this agent.

Methods: This cross-sectional study of breast cancer patients with taxane CIPN measured nerve cross-sectional area (CSA) by NMUS and compared with healthy historical controls. Correlations were determined between CSA and symptom scale, nerve conduction studies, and intraepidermal nerve fiber density (IENFD).

Results: A total of 20 participants reported moderate CIPN symptoms at a median of 3.8 months following the last taxane dose. Sural nerve CSA was 1.2 mm² smaller than healthy controls ($P \leq .01$). Older age and time since taxane were associated with smaller sural nerve CSA. For each 1 mm² decrease in sural nerve CSA, distal IENFD decreased by 2.1 nerve/mm (R^2 0.30; $P = .04$).

Conclusions: These data support a sensory predominant taxane neuropathy or neuronopathy and warrant future research on longitudinal NMUS assessment of CIPN.

KEYWORDS

breast cancer, chemotherapy, neuropathy, taxane, ultrasound

1 | INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is a common toxicity of many classes of chemotherapy, including taxanes, platinum, vinca alkaloids, and others. Taxanes, such as paclitaxel and docetaxel,

have had an increasing role in the treatment of breast cancer and are now used in the majority of patients receiving perioperative chemotherapy.¹ When chemotherapy cannot be given as intended due to dose reductions, it is associated with worse progression-free and overall survival.² Although spontaneous recovery is possible, in roughly half

Abbreviations: AANEM, American Association of Neuromuscular & Electrodagnostic Medicine; ALS, amyotrophic lateral sclerosis; BMI, body mass index; CIPN, chemotherapy-induced peripheral neuropathy; CSA, cross-sectional area; DRG, dorsal root ganglion; DWG, distal wrist crease; H&E, hematoxylin and eosin; IENFD, intraepidermal nerve fiber density; LMN, lower motor neuron; NCS, nerve conduction study; NMUS, neuromuscular ultrasound; PGP, protein gene product; QLQ, Quality of Life Questionnaire; SNAP, sural sensory nerve action potential.

of cases functional disability and risk of falls from CIPN persists for years.^{3,4} Currently, there is no predictive method to identify patients who are at high risk for developing permanent, debilitating CIPN.

There are few clinically available point-of-care tests to objectively predict, diagnose, or monitor CIPN. Self-reported symptom scales such as the European Organization for Research and Treatment of Cancer CIPN 20 Quality of Life Questionnaire (QLQ)⁵ are potentially predictive of CIPN but have inter-scale and inter-observer variability.^{6,7} Quantitative sensory testing fails to predict the severity of CIPN.^{8,9} Nerve conduction studies (NCSs) are insensitive to small fiber changes; typical findings include mild conduction velocity slowing at sites of entrapment or reduction of sural sensory responses.¹⁰⁻¹² Skin biopsy for measuring intraepidermal nerve fiber density (IENFD) is the gold standard for the diagnosis of sensory-predominant small fiber neuropathy but is not an ideal test due to its invasive nature.^{3,6}

Studies have found that neuromuscular ultrasound (NMUS) can detect peripheral nerve lesions generally with enlargement of CSA in patients with demyelinating hereditary,^{13,14} inflammatory,^{15,16} entrapment,¹⁷ and diabetic neuropathies¹⁸ though reduction of CSA has also been reported (eg, amyotrophic lateral sclerosis [ALS],¹⁹ spinocerebellar ataxia syndrome²⁰⁻²³). CSA is generally larger in demyelinating than axonal polyneuropathies²⁴ and more commonly reported in large fiber neuropathies, though one study of patients with small fiber neuropathies demonstrated enlargement of CSA compared with matched-healthy controls.²⁵

Less is known about the usefulness of NMUS for assessing the effect of chemotherapy upon peripheral nerves. Four studies have used NMUS for the detection of CIPN among gastrointestinal cancer patients who received oxaliplatin. Compared with historical reference values, three studies found no consistent change in CSA,^{24,26} although one did note an increase in peripheral nerve CSA at compression sites (median nerve at wrist, ulnar nerve at elbow).²⁷ The fourth study compared neurological measures before and after chemotherapy and found increased CSA of the tibial nerve and at one compression site of the fibular nerve (fibular head), but did not find changes in CSA in other nerves (sural, median, ulnar, radial).²⁸ Importantly, the mechanism and symptoms of neurotoxicity are markedly different between taxanes (eg, microtubular stabilization, hypoesthesia) and oxaliplatin (eg, inhibition of sodium channels; cold-induced paresthesias). Therefore, while prior studies of oxaliplatin CIPN are certainly informative, taxane-specific studies are needed to further characterize this syndrome.

In this study, our aim was to test NMUS as a noninvasive, point-of-care test to measure nerve CSA in patients with active taxane CIPN symptoms with the hypothesis that nerve CSA would be altered.

2 | METHODS

2.1 | Eligibility characteristics

Our single-arm, prospective pilot study enrolled breast cancer patients with self-reported symptomatic CIPN for comprehensive neurological assessment, including NMUS. The trial was conducted in accordance

with the International Conference on Good Clinical Practice Standards and the Declaration of Helsinki. The protocol was approved by institutional review board and ethics committees of our institution and the trial was registered on ClinicalTrials.gov (NCT03139435). All patients provided informed consent. Eligible patients were ≥ 18 -year-old women with breast cancer (any stage) and clinical symptoms of peripheral neuropathy due to either previous or current taxane exposure, as clinically attributed by the patient and their medical oncologist. We noted the presence of comorbidities (such as diabetes), which can predispose toward peripheral neuropathy with nerve enlargement.¹⁸ We excluded patients with a self-reported or documented history of pre-existing peripheral neuropathy before initiation of the taxane. Patients were followed, either by telephone or in person, for 30 days to ensure the biopsy site healed.

2.2 | Assessments

Participants underwent a cross-sectional assessment that included focused history, neurological exam, and diagnostic testing by a neurologist at a single visit. NMUS with a GE S8 system and a routine 15-MHz linear transducer (GE Healthcare, Chicago, IL) was used to measure CSA at nerve sites as per previously described methodology and institutional practice.²⁹ These sites were at the posterior distal leg (sural nerve), the ankle (tibial nerve), and the forearm and distal wrist crease (DWC; median nerve). The median nerve was assessed at two places to control for enlargement that can occur just proximal to entrapment at the carpal tunnel (at the DWC) due to subclinical carpal tunnel syndrome. Reference values for NMUS were from a published historical dataset.²⁹ The 20-item self-reported neuropathy scale (QLQ-CIPN20) was administered as previously described with the exception of the hearing loss item; this item was excluded from data collection because taxanes are not associated with ototoxicity.³⁰

NCS assessed the tibial motor nerve, median motor nerve, and the sural sensory nerve (antidromic, taken at two sites on the ankle) as per AANEM standards³¹ and compared with reference values from healthy adults.³² IENFD was measured with two skin biopsies (4–5 mm), one at the upper thigh and the second on the ipsilateral distal leg in sural nerve territory (10 cm above lateral malleolus). As previously described, biopsied tissues were cut into 50- μ m sections for H&E staining and immunostaining with an antibody to protein gene product (PGP) 9.5, a neuron- and neuroendocrine cell-specific ubiquitin carboxy-terminal hydrolase expressed throughout the peripheral nervous system.³³

Slides were processed and read by two collaborators in dermatopathology with experience in reading skin biopsy samples for IENFD. The same two dermatopathologists reviewed all cases. IENFD was measured as the linear density (per millimeter) of PGP 9.5-positive fibers that crossed the dermal-epidermal junction. Serum and additional unstained biopsy slides were stored and transferred for exploratory analyses. Although not blinded to the diagnosis of CIPN, the examiner performing NMUS did not review the patient's symptoms before performing the study and was not aware of the cumulative dose,

timing since taxane therapy, severity of clinical symptoms, or IENFD results at the time of testing. Dermatopathologists were not blinded to the patient but did not have access and were blinded to the NCS and NMUS data at the time of their analysis.

2.3 | Study endpoints

The primary outcome was tibial nerve CSA by NMUS compared with historical controls. Secondary outcomes included sural nerve CSA by NMUS and determination of whether tibial or sural nerve CSA correlated with QLQ-CIPN20, NCS, or IENFD. Exploratory outcomes included measures of median nerve CSA.

2.4 | Statistical analysis

The tibial nerve CSA, sural nerve CSA, and sural nerve amplitude were compared with historical data^{29,34} from healthy patients using two-sample t-test; primary historical data were used for the analysis of CSA measures. Phase of treatment was defined as active (≤ 30 days since last taxane administration), recent (>30 and ≤ 180 days), and remote (>180 days). Comparison of CSA, QLQ scales, and IENFD

measures between different phases of taxane exposure were performed using the Kruskal-Wallis tests. Spearman's rank correlation coefficients were used to study the associations between two measures (eg, distal and proximal IENFD). Linear regression models were used to examine the associations between the dependent variable (nerve CSA) and covariates of interest (NCS measures, self-reported neuropathy scale, and IENFD). Clinical factors (eg, age) that were thought to be potentially associated with nerve CSA were fitted in the model one at a time.

3 | RESULTS

Between May 4, 2017, and November 13, 2018, 25 patients were screened for eligibility; 3 did not meet inclusion criteria, and 2 declined to participate as summarized in CONSORT flow diagram (Figure 1). Recruitment was by provider referral and by paper fliers posted in both patient and provider areas of the oncology clinic. All patients completed their planned evaluation and follow-up. One patient was excluded from median nerve analysis due to symptoms and NCS findings that were diagnostic of carpal tunnel syndrome.

A total of 20 patients underwent evaluation with their baseline clinical characteristics summarized in Table 1. Average cumulative

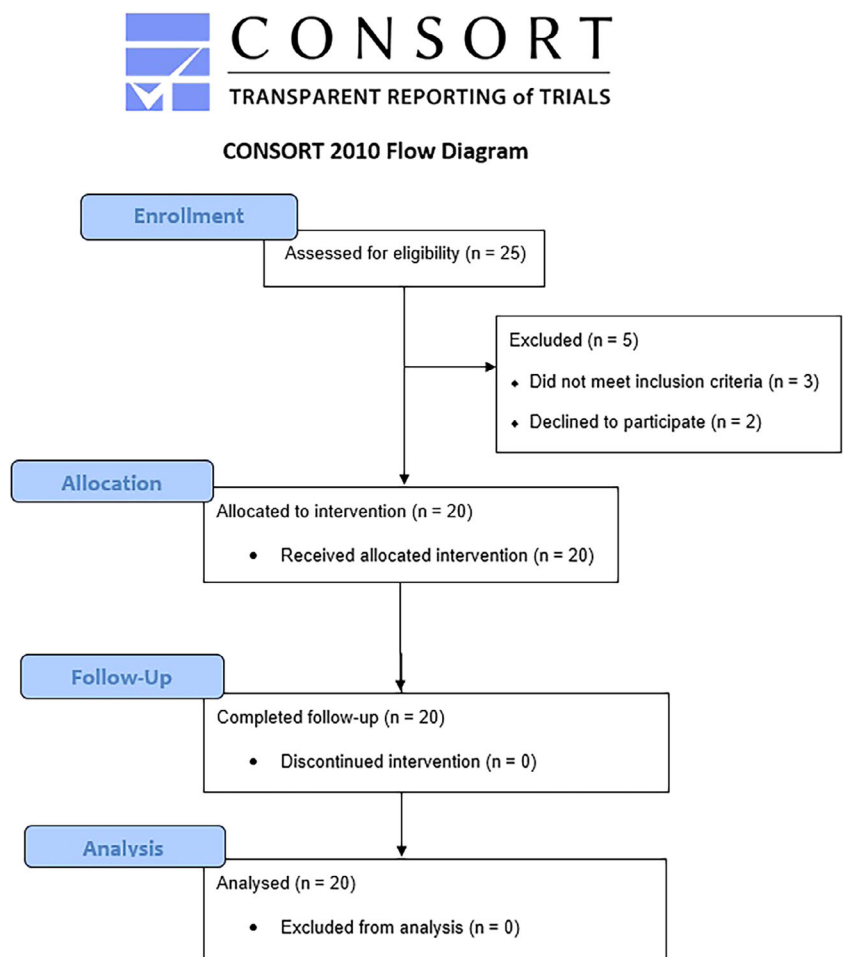


FIGURE 1 CONSORT flow diagram [Color figure can be viewed at wileyonlinelibrary.com]

taxane dose was 817 mg/m²; for reference, the typical dosing regimen for adjuvant paclitaxel in breast cancer is 80 mg/m²/week for 12 weeks (total cumulative dose of 960 mg/m²). CIPN patients reported moderate-to-severe CIPN symptoms by QLQ-CIPN20, which were predominantly sensory (19.1 ± 4.9; max 32) as opposed to motor (15.6 ± 5.8; max 32) or autonomic (3.3 ± 1.6; max 8). All patients had normal strength (5/5) in foot dorsiflexion; deep tendon reflexes at the ankles were absent in 13/20 (65%), reduced in 4/20 (20%).

Sural nerve amplitude was significantly lower (mean 10.9 ± 5.8 μV) than healthy historical controls (mean 17.2 ± 10.1 μV; $P < .01$) with 8 patients below the normal range (<7.1 μV).³⁴ There were no associations between tibial nerve CSA and tibial compound muscle action potential amplitude (Spearman's $r = 0.12$), distal latency (−0.24), or conduction velocity (0.21). There were no associations between sural nerve CSA and sural sensory nerve action potential (SNAP) amplitude (Spearman's $r = 0.20$), latency (−0.26), or conduction velocity (0.27). Sural SNAP amplitude was not associated with symptom severity by QLQ-CIPN20 (Spearman $r_s [18] = -0.23$).

Distal IENFD had mean 6.6 ± 3.8 nerves/mm (range, 2.4–15 nerves/mm) with 30% of participants below the normal range, defined as the 5th

percentile among historical healthy controls (3.8 nerves/mm). Proximal IENFD had mean 11.0 ± 6.0 nerves/mm (range, 9–24 nerves/mm) with 15% of participants below the normal range, defined as the 5th percentile among historical healthy controls (5.2 nerves/mm).³⁵ Distal and proximal IENFD were associated with each other (Spearman $r_s [19] = 0.49$; $P = .03$). Neither distal nor proximal IENFD was associated with symptom severity by QLQ-CIPN20 (Spearman $r_s [19] = -0.16$; 0.12, respectively) or with cumulative dose (Spearman $r_s [20] = 0.42$; 0.19, respectively). Proximal IENFD was negatively associated with tibial amplitude (Spearman $r = -0.46$; $P = .04$); otherwise, there was no association between IENFD (either proximal or distal) and any other NCS measures.

CSA was evaluated by NMUS at 74 nerve sites among the 20 patients with no repeated measurements. The average sural nerve CSA was significantly smaller than healthy historical controls (Table 2). Older age was associated with significantly smaller sural nerve CSA (Spearman $r_s [20] = -0.65$, $P \leq .01$). More days since taxane chemotherapy was also associated with smaller sural nerve CSA ($r_s [20] = -0.46$; $P = .04$). Sural nerve CSA was not significantly associated with either body mass index (BMI) (Spearman $r_s [20] = 0.25$) or cumulative taxane dose (Spearman $r_s [20] = 0.40$); neither were the CSA of other nerves. Unadjusted sural nerve CSA did not have an association with distal IENFD, but when controlled for age and days from last taxane administration, distal IENFD reduced by 2.1 nerve/mm for each 1 mm² decrease in sural nerve CSA (Table 3).

Descriptive data of nerve findings by phase of treatment (active, recent, remote) are summarized in Table 4. When compared by phase of treatment and independently from each other, tibial nerve CSA ($P = .44$), sural nerve CSA ($P = .17$), proximal IENFD ($P = 0.43$), distal IENFD ($P = .32$), QLQ sensory scale ($P = .46$), and sural amplitude ($P = .45$) did not meet statistical significance by Kruskal-Wallis one-way analysis of variance.

Median nerve CSA at the compressive site (DWC) was larger than among healthy historical controls, whereas at the noncompressive site (forearm), there was no significant difference. These results were the same even when including the participant with carpal tunnel syndrome ($n = 20$), with median nerve CSA at the DWC larger than healthy historical controls ($P = .02$) and no significant difference at the

TABLE 1 Demographic and disease characteristics of the participants

Parameter		Mean (SD) or n (%)
Age, mean (SD)		55.4 (10.5)
BMI, mean (SD)		29.8 (6.9)
Stage of breast cancer, n (%)	Stage I	8 (40%)
	Stage II	5 (25%)
	Stage III	2 (10%)
	Stage IV	5 (25%)
Prior radiation therapy, n (%)		10 (50%)
Prior surgery for breast cancer, n (%)		19 (95%)
Prior hormone therapy for breast cancer, n (%)		8 (40%)
Prior lines of systemic cancer therapy, median (IQR)		1 (1–3)
Most recent taxane, n (%)	Paclitaxel	10 (50%)
	Docetaxel	8 (40%)
	Nab-paclitaxel	2 (10%)
Cumulative taxane dose, mg/m ² , mean (SD)		817.3 (414.9)
Months since last taxane dose, median (IQR)		3.8 (1.0–6.1)
Relevant comorbidities, n (%)	Diabetes, no end-organ damage	4 (20%)
	Hypothyroidism	3 (15%)
	Depression	3 (15%)
	Alcoholism	1 (5%)
	Cerebrovascular disease	1 (5%)
	Vitamin B12 deficiency	1 (5%)

IQR, interquartile range; Nab, nanoparticle albumin-bound.

TABLE 2 Comparison of nerve CSA by NMUS between CIPN patients and healthy historical controls

	CIPN patients, mm ² , mean (SD)	Healthy controls, mm ² , mean (SD)	t value	P value
Tibial nerve CSA	13.0 (3.6)	14.0 (4.3)	0.94	.35
Sural nerve CSA	4.1 (1.3)	5.3 (1.8)	2.85	<.01*
Median nerve CSA (forearm)	7.3 (1.4)	7.7 (1.7)	0.89	.37
Median nerve CSA (DWC)	12.5 (3.9)	10.1 (2.7)	−2.39	.03**

*Statistically significant at $\alpha \leq .05$. Df = 138.

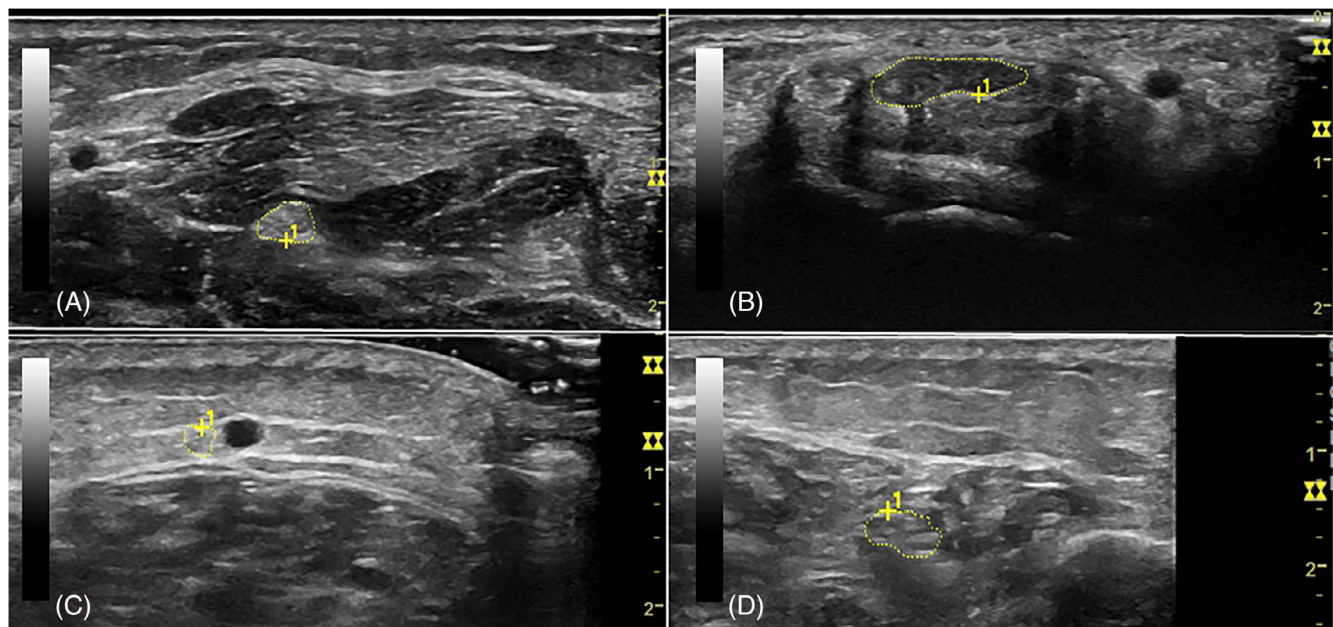
**Statistically significant at $\alpha \leq .05$. Satterthwaite unequal variances; Df = 16.

TABLE 3 Multiple regression analysis of factors to predict distal IENFD

Factors	Univariate		Multivariate model 1*		Multivariate model 2†	
	Beta	P value	Beta	P value	Beta	P value
Sural CSA	0.35	.13	0.99	.15	2.10	.04‡
Days since last taxane	−0.29	.21	−0.01	.25	0.19	.13
Age	−0.12	.62			−0.01	.09

*Model 1: $R^2 = 0.19$; $F(2,19) = 2.03$; $P = .16$.†Model 2: $R^2 = 0.30$; $F(3,19) = 2.32$; $P = .11$.‡Statistically significant at $\alpha \leq .05$.**TABLE 4** Comparison of findings between different phases of taxane exposure

	Active taxane, mean (SD)	Recent taxane, mean (SD)	Remote taxane, mean (SD)
No. of participants	6	9	5
Cumulative dose (taxane mg/m ²)	995.8 (95.8)	713.9 (440.7)	789.0 (587.1)
Tibial nerve CSA (mm ²)	14.2 (4.8)	13.2 (3.2)	11.2 (2.3)
Sural nerve CSA (mm ²)	4.8 (1.6)	3.9 (0.6)	3.6 (1.5)
Forearm median nerve CSA (mm ²)	7.3 (1.9)	8 (1.0)	6.2 (0.4)
DWC median nerve CSA* (mm ²)	13.8 (6.5)	13.6 (3.0)	11.0 (2.4)
QLQ sensory scale	17.2 (4.3)	20.4 (5.2)	19.2 (5.4)
QLQ motor scale	12.3 (3.4)	18.6 (6.6)	14.2 (4.4)
QLQ autonomic scale	2.5 (0.5)	3.9 (1.8)	3.2 (1.6)
Proximal IENFD (nerves/mm)	13.1 (6.7)	8.9 (3.6)	12.2 (8.5)
Distal IENFD (nerves/mm)	8.9 (4.9)	5.5 (2.9)	5.9 (3.0)

*P value significant to $<.05$ (.0219) by Kruskal–Wallis one-way analysis of variance.**FIGURE 2** Representative imaging by NMUS. A, Median nerve at forearm in participant 3 (CSA, 9 mm²). B, Median nerve at wrist in participant 3 (CSA, 25 mm²). C, Sural nerve in participant 6 (CSA, 4 mm²). D, tibial nerve in participant 6 (CSA, 19 mm²) [Color figure can be viewed at wileyonlinelibrary.com]

forearm ($P = .41$). Representative NMUS imaging is shown in Figure 2. Descriptive data from all participants is shown in Supporting Information Table S1, which is available online.

The study was well-tolerated without any adverse effects and with a high uptake among participants with just 2 of 25 participants declining to complete the study protocol after registration. Skin

biopsy led to one adverse event in a patient who had redness and irritation attributed to an inflammatory local reaction and not an infection.

4 | DISCUSSION

The key findings of this study are that, compared with healthy historical controls, patients with a small fiber neuropathy caused by taxane exposure had (1) decreased sural nerve size, (2) increased median nerve size but only at a compression site, and (3) NMUS findings that correlated well with IENFD loss on skin biopsies. Reduced CSA was specific to the sural sensory nerve and was not seen in the tibial nerve. This is likely reflective of the predominantly sensory manifestation of taxane CIPN, which was confirmed in our sample using a validated symptom scale.

NMUS assessment of the median nerve at its compression site (DWC) found that CIPN patients had increased CSA compared with healthy controls. When moved proximally to assess the median nerve at a noncompressive site (forearm), there was no difference in CSA between CIPN patients and healthy controls. This discrepancy is often seen with NMUS evaluation of symptomatic carpal tunnel syndrome and other polyneuropathies.³⁶ These findings suggest that, at compression sites, CIPN may aggravate nerve entrapment and cause an increase in CSA of peripheral nerves. This discrepancy in the effects of CIPN between compression and noncompression anatomic sites has been described in NCS¹¹ and was suggested in a prior study of oxaliplatin CIPN.²⁷

The finding of decreased peripheral nerve CSA is uncommon and has been reported in ALS¹⁹ and spinocerebellar ataxia syndrome.²⁰⁻²³ In vitro³⁷ and animal studies³⁸ have shown that the neurotoxicity of microtubule-targeting agents (eg, taxanes) is predominantly due damage to the dorsal root ganglion (DRG).^{39,40} This leads to axonal loss in the longest sensory nerves and contributes to the clinical symptoms consistent with a ganglionopathy. In ALS, the reduction in nerve CSA is thought to reflect nerve atrophy from lower motor neuron (LMN) dropout⁴¹ and may have utility as a marker of LMN involvement.⁴² We hypothesize that the decreased CSA observed in our study may reflect the sensory ganglionopathy of taxane CIPN with sensory nerve atrophy. Because this study was limited to taxane CIPN, these results are not broadly applicable to other classes of chemotherapies whose neurotoxic mechanisms vary.

Multivariable analysis was notable for two confounding factors between CIPN and CSA, that is, age and time since taxane exposure. Age has previously been reported as having a very small negative correlation with peripheral nerve CSA.²⁹ The association with time is a novel finding; we hypothesize that this may be due to the gradual atrophy of damaged peripheral nerves following the initial insult. This is supported by the finding of decreased distal IENFD among patients with more time since taxane. Future studies will need to further account for these confounding factors.

There were several limitations of this pilot study, most of which were inherent to its design and small sample size. This study was designed to provide a broader evaluation of the pathophysiology of CIPN by including participants at all timepoints during and after

taxane (active, recent, remote). However, this heterogeneity decreased the statistical power to detect changes in CSA, which may only occur in a time-specific manner. Because there is no intervention available for CIPN once the patient has completed taxane chemotherapy, there is little clinical utility for improved evaluation of chronic CIPN. Therefore, future studies of the clinical use of NMUS, including one that is currently enrolling patients,⁴³ will focus on patients with CIPN who are being considered for additional taxane chemotherapy so that dosing may be adjusted accordingly. This study was limited to female participants; reassuringly, in the prior study of healthy controls, there was no significant difference in nerve CSA by sex.²⁹ Prior studies⁴⁴ have reported variability among healthy controls with sural nerve CSA approximately 2–6 mm², our healthy control data had a mean of around 5 mm². Our study was not funded to support collection of another set of matched control values, which is why we used our previously published set.²⁹ A newer high-resolution probe could also be considered for future studies, as ultrahigh-frequency NMUS has been found to be useful for assessing changes at the fascicular level.⁴⁵

CIPN is a common complication of breast cancer treatment with a critical need for a better diagnostic imaging modality. NMUS was found to be a rapid, noninvasive assessment of CIPN, which was well-tolerated and available at the point-of-care using a routine 15-Hz linear probe. This study's findings warrant future research on the longitudinal assessment of sural nerve CSA in CIPN.

CONFLICT OF INTEREST

Michael Cartwright has intellectual property with Elsevier for a textbook on Neuromuscular Ultrasound. Francis Walker has received honoraria and support for expenses from Grifols and AskBio; research funding from Vaccinex, TEVA Pharmaceuticals, and Pfizer; intellectual property with Elsevier and UpToDate; and support with interest-free equipment loans from Natus, Monarch Medical, and Terason. Yusuke Shiozawa has received research funding from TEVA Pharmaceuticals. The remaining authors have no conflicts of interest.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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REFERENCES

1. Giordano SH, Lin YL, Kuo YF, Hortobagyi GN, Goodwin JS. Decline in the use of anthracyclines for breast cancer. *J Clin Oncol*. 2012;30(18):2232-2239.
2. Budman DR, Berry DA, Cirincione CT, et al. Dose and dose intensity as determinants of outcome in the adjuvant treatment of breast cancer. The Cancer and Leukemia Group B. *J Natl Cancer Inst*. 1998;90(16):1205-1211.
3. Osmani K, Vignes S, Aissi M, et al. Taxane-induced peripheral neuropathy has good long-term prognosis: a 1- to 13-year evaluation. *J Neurol*. 2012;259(9):1936-1943.

4. Winters-Stone KM, Horak F, Jacobs PG, et al. Falls, Functioning, and disability among women with persistent symptoms of chemotherapy-induced peripheral neuropathy. *J Clin Oncol*. 2017;35(23):2604-2612.
5. Lavoie Smith EM, Barton DL, Qin R, Steen PD, Aaronson NK, Loprinzi CL. Assessing patient-reported peripheral neuropathy: the reliability and validity of the European Organization for Research and Treatment of Cancer QLQ-CIPN20 Questionnaire. *Qual Life Res*. 2013;22(10):2787-2799.
6. Park SB, Goldstein D, Krishnan AV, et al. Chemotherapy-induced peripheral neurotoxicity: a critical analysis. *CA Cancer J Clin*. 2013;63(6):419-437.
7. Reeves BN, Dakhil SR, Sloan JA, et al. Further data supporting that paclitaxel-associated acute pain syndrome is associated with development of peripheral neuropathy: North Central Cancer Treatment Group trial N08C1. *Cancer*. 2012;118(20):5171-5178.
8. Forsyth PA, Balmaceda C, Peterson K, Seidman AD, Brasher P, DeAngelis LM. Prospective study of paclitaxel-induced peripheral neuropathy with quantitative sensory testing. *J Neurooncol*. 1997;35(1):47-53.
9. Hershman DL, Weimer LH, Wang A, et al. Association between patient reported outcomes and quantitative sensory tests for measuring long-term neurotoxicity in breast cancer survivors treated with adjuvant paclitaxel chemotherapy. *Breast Cancer Res Treat*. 2011;125(3):767-774.
10. Chaudhry V, Rowinsky EK, Sartorius SE, Donehower RC, Cornblath DR. Peripheral neuropathy from taxol and cisplatin combination chemotherapy: clinical and electrophysiological studies. *Ann Neurol*. 1994;35(3):304-311.
11. Chen X, Stubblefield MD, Custodio CM, Hudis CA, Seidman AD, DeAngelis LM. Electrophysiological features of taxane-induced polyneuropathy in patients with breast cancer. *J Clin Neurophysiol*. 2013;30(2):199-203.
12. Kroigard T, Schroder HD, Qvortrup C, et al. Characterization and diagnostic evaluation of chronic polyneuropathies induced by oxaliplatin and docetaxel comparing skin biopsy to quantitative sensory testing and nerve conduction studies. *Eur J Neurol*. 2014;21(4):623-629.
13. Schreiber S, Oldag A, Kornblum C, et al. Sonography of the median nerve in CMT1A, CMT2A, CMTX, and HNPP. *Muscle Nerve*. 2013;47(3):385-395.
14. Cartwright MS, Brown ME, Eulitt P, Walker FO, Lawson VH, Caress JB. Diagnostic nerve ultrasound in Charcot-Marie-Tooth disease type 1B. *Muscle Nerve*. 2009;40(1):98-102.
15. Beekman R, van den Berg LH, Franssen H, Visser LH, van Asseldonk JT, Wokke JH. Ultrasonography shows extensive nerve enlargements in multifocal motor neuropathy. *Neurology*. 2005;65(2):305-307.
16. Zaidman CM, Pestronk A. Nerve size in chronic inflammatory demyelinating neuropathy varies with disease activity and therapy response over time: a retrospective ultrasound study. *Muscle Nerve*. 2014;50(5):733-738.
17. Cartwright MS, Walker FO. Neuromuscular ultrasound in common entrapment neuropathies. *Muscle Nerve*. 2013;48(5):696-704.
18. Breiner A, Qrimli M, Ebadi H, et al. Peripheral nerve high-resolution ultrasound in diabetes. *Muscle Nerve*. 2017;55(2):171-178.
19. Cartwright MS, Walker FO, Griffin LP, Caress JB. Peripheral nerve and muscle ultrasound in amyotrophic lateral sclerosis. *Muscle Nerve*. 2011;44(3):346-351.
20. Leadbetter R, Weatherall M, Pelosi L. Nerve ultrasound as a diagnostic tool for sensory neuronopathy in spinocerebellar ataxia syndrome. *Clin Neurophysiol*. 2019;130(4):568-572.
21. Pelosi L, Leadbetter R, Mulroy E, Chancellor AM, Mossman S, Roxburgh R. Peripheral nerve ultrasound in cerebellar ataxia neuropathy vestibular areflexia syndrome (CANVAS). *Muscle Nerve*. 2017;56(1):160-162.
22. Pelosi L, Mulroy E, Rodrigues MJ, Roxburgh RH. Neuronopathy and neuropathy in autosomal dominant spino-cerebellar ataxia (SCA): a preliminary peripheral nerve ultrasound study. *Clin Neurophysiol*. 2017;128(12):2436-2437.
23. Pelosi L, Mulroy E, Leadbetter R, et al. Peripheral nerves are pathologically small in cerebellar ataxia neuropathy vestibular areflexia syndrome: a controlled ultrasound study. *Eur J Neurol*. 2018;25(4):659-665.
24. Zaidman CM, Al-Lozi M, Pestronk A. Peripheral nerve size in normals and patients with polyneuropathy: an ultrasound study. *Muscle Nerve*. 2009;40(6):960-966.
25. Ebadi H, Siddiqui H, Ebadi S, Ngo M, Breiner A, Bril V. Peripheral nerve ultrasound in small fiber polyneuropathy. *Ultrasound Med Biol*. 2015;41(11):2820-2826.
26. Grimm A, Decard BF, Axer H, Fuhr P. The Ultrasound pattern sum score - UPSS. A new method to differentiate acute and subacute neuropathies using ultrasound of the peripheral nerves. *Clin Neurophysiol*. 2015;126(11):2216-2225.
27. Briani C, Campagnolo M, Lucchetta M, et al. Ultrasound assessment of oxaliplatin-induced neuropathy and correlations with neurophysiologic findings. *Eur J Neurol*. 2013;20(1):188-192.
28. Pitarokoli K, Hoffken N, Lonneker N, et al. Prospective study of the clinical, electrophysiologic, and sonographic characteristics of oxaliplatin-induced neuropathy. *J Neuroimaging*. 2019;29(1):133-139.
29. Cartwright MS, Passmore LV, Yoon JS, Brown ME, Caress JB, Walker FO. Cross-sectional area reference values for nerve ultrasonography. *Muscle Nerve*. 2008;37(5):566-571.
30. Eckhoff L, Knoop A, Jensen MB, Ewertz M. Persistence of docetaxel-induced neuropathy and impact on quality of life among breast cancer survivors. *Eur J Cancer*. 2015;51(3):292-300.
31. American Association of Neuromuscular & Electrodagnostic Medicine (AANEM). Proper performance and interpretation of electrodiagnostic studies. [Corrected]. *Muscle Nerve*. 2015;51(3):468-471.
32. Chen S, Andary M, Buschbacher R, et al. Electrodiagnostic reference values for upper and lower limb nerve conduction studies in adult populations. *Muscle Nerve*. 2016;54(3):371-377.
33. Lauria G, Devigili G. Skin biopsy as a diagnostic tool in peripheral neuropathy. *Nat Clin Pract Neurol*. 2007;3(10):546-557.
34. Benatar M, Wu J, Peng L. Reference data for commonly used sensory and motor nerve conduction studies. *Muscle Nerve*. 2009;40(5):772-794.
35. McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. *Arch Neurol*. 1998;55(12):1513-1520.
36. Shen J, Cartwright MS. Neuromuscular ultrasound in the assessment of polyneuropathies and motor neuron disease. *J Clin Neurophysiol*. 2016;33(2):86-93.
37. Scuteri A, Nicolini G, Miloso M, et al. Paclitaxel toxicity in post-mitotic dorsal root ganglion (DRG) cells. *Anticancer Res*. 2006;26(2A):1065-1070.
38. Zhang H, Li Y, de Carvalho-Barbosa M, et al. Dorsal root ganglion infiltration by macrophages contributes to paclitaxel chemotherapy-induced peripheral neuropathy. *J Pain*. 2016;17(7):775-786.
39. Nicolini G, Monfrini M, Scuteri A. Axonal transport impairment in chemotherapy-induced peripheral neuropathy. *Toxics*. 2015;3(3):322-441.
40. Malacrida A, Meregalli C, Rodriguez-Menendez V, Nicolini G. Chemotherapy-induced peripheral neuropathy and changes in cytoskeleton. *Int J Mol Sci*. 2019;20(9):pii E2287.
41. Barnes SL, Simon NG. Clinical and research applications of neuromuscular ultrasound in amyotrophic lateral sclerosis. *Degener Neurol Neuromuscul Dis*. 2019;9:89-102.
42. Schreiber S, Abdulla S, Debska-Vielhaber G, et al. Peripheral nerve ultrasound in amyotrophic lateral sclerosis phenotypes. *Muscle Nerve*. 2015;51(5):669-675.

43. U.S. National Library of Medicine. Acupuncture in reducing chemotherapy-induced peripheral neuropathy in participants with stage I-III breast cancer. <https://ClinicalTrials.gov/show/NCT03505671>. Accessed February 17, 2020.
44. Rbia N, Nijhuis THJ, Roukema GR, Selles RW, van der Vlies CH, Hovius SER. Ultrasound assessment of the sural nerve in patients with neuropathic pain after ankle surgery. *Muscle Nerve*. 2018;57(3):407-413.
45. Cartwright MS, Baute V, Caress JB, Walker FO. Ultrahigh-frequency ultrasound of fascicles in the median nerve at the wrist. *Muscle Nerve*. 2017;56(4):819-822.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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ARTICLE

Nicotine promotes brain metastasis by polarizing microglia and suppressing innate immune function

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Up to 40% of lung cancer patients develop brain metastasis, and the median survival of these patients remains less than 6 months. Smoking is associated with lung cancer. However, how smoking impacts the development of brain metastasis remains elusive. We examined 281 lung cancer patients with distant metastasis and found that smokers exhibited a significantly high incidence of brain metastasis. We found that nicotine enhanced brain metastasis, while a depletion of microglia suppressed this effect in vivo. Nicotine skewed the polarity of microglia to the M2 phenotype, thereby increasing the secretion of IGF-1 and CCL20, which promoted tumor progression and stemness. Importantly, nicotine enhanced the expression of SIRPα in microglia and restricted their phagocytic ability. We also identified a compound, parthenolide, that suppressed brain metastasis by blocking M2 polarization. Our results indicate that nicotine promotes brain metastasis by skewing the polarity of M2 microglia, which enhances metastatic tumor growth. Our results also highlight a potential risk of using nicotine for tobacco cessation.

Introduction

Approximately 170,000 patients in the United States are diagnosed with brain metastasis every year (Ellis et al., 2012). Lung, breast, and skin cancers are the most common tumor types that metastasize to the brain (Bacha et al., 2018; Huang et al., 2018; Redmer, 2018; Xing et al., 2018). Patients with metastatic lung cancer have a very poor prognosis (Mujoomdar et al., 2007), and those with brain metastasis have a 1-yr survival rate of 4–20% (Gaspar et al., 1997; Sperduto et al., 2008; Verger et al., 2005). Despite significant improvements in the treatment of metastatic cancer, the therapeutic options for brain metastasis are still limited mainly due to inefficient drug delivery to brain as well as limited surgical approaches to multiple metastases. Radiation therapies, such as stereotactic radio surgery and fractionated whole brain radiation, are standard therapeutic techniques employed for brain metastasis; however, they provide only limited local tumor control and, more often than not, intracranial failure leading to neurological death. The development of lung cancer brain metastasis is a complex process, requiring the invasion of primary tumor cells into surrounding tissue and vessels, trafficking through the circulatory system, and colonizing into the brain parenchyma (Fidler, 2015). Therefore, understanding the underlying mechanisms that drive brain

metastasis and developing more effective therapies are urgently needed.

Cigarette smoking is one of the major risk factors for lung cancer (Lee et al., 2012). Our previous study have shown that smoking is correlated to rapid progression of lung cancer brain metastasis (Shenker et al., 2017). Tobacco smoke contains a toxic mix of >7,000 chemicals. Among them, nicotine is a major cigarette component associated with human tobacco consumption and addiction (Braverman, 1999; Heishman, 1999). Nicotine is not a carcinogen; however, several studies have reported the tumor-promoting effects of nicotine via activation of nicotinic acetylcholine (nACh) receptor in the tumor cells. The nACh receptor is comprised of combination of multiple subunits, and they are expressed in a variety of brain cells including neuron, astrocytes, endothelial cells, and microglia (Mazzaferro et al., 2017). The α4β2 is the most abundant class of nACh receptor in the brain and is the principal mediator of nicotine dependence (Hawkins et al., 2004; Mazzaferro et al., 2017). This suggests that nicotine may be capable of reprogramming the brain tumor microenvironment (TME) via activation of its receptor to promote tumor progression.

Several studies have shown that the interaction of neoplastic cells with microglial cells, brain-residing macrophages, profoundly

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affects tumor progression in glioblastoma (da Fonseca and Badie, 2013; Roesch et al., 2018; Wei et al., 2013). In brain metastatic lesions of lung cancer patients, abundant activated microglia were found to be infiltrated (He et al., 2006). Importantly, nACh receptor has been shown to be expressed on microglia (Morioka et al., 2015; Sadigh-Eteghad et al., 2016), raising the possibility that nicotine may stimulate microglia's immunomodulatory pathways, which affects their immune function. Microglia are known to polarize into classical (M1) or alternative (M2) phenotypes by various microenvironment stimuli (Wei et al., 2013; Wu and Watabe, 2017). M1 cells suppress tumors by releasing cytotoxic factors and also by phagocytosis. In contrast, M2 cells have a pro-tumor response by activating immunosuppressive factors. It is virtually unknown how metastatic cells escape immune attack of microglia and then colonize in the brain. In this study, we showed that nicotine promoted lung cancer brain metastasis by enhancing both tumor progression and stemness by skewing microglial polarization and inhibiting its anti-tumor phagocytic ability. We also found that a natural compound, parthenolide (PTL), effectively suppressed tumor progression and stemness by blocking nicotine-induced polarization of the microglia in the brain.

Results

Smoking increases brain metastasis of lung cancer

To examine the effect of tobacco smoking on brain metastasis, we analyzed the incidence and mortality rate of 281 patients with lung cancer brain metastasis (Fig. S1) with or without a history of smoking. All patients were diagnosed with stage IV disease. Among them, 79 patients were diagnosed with brain metastasis. 59 patients were current smokers at the time of diagnosis and treatment, while 222 of them had either a previous or no smoking history. We found that the incidence of brain metastasis was significantly higher in current smokers compared with the never or the former smokers group (Fig. 1 A). The results of Kaplan–Meier analyses indicate that the current smokers were associated with worse brain metastasis progression free (Fig. 1 B) and overall survival (Fig. 1 C) compared with those who were not currently smoking. Since activation of microglia was previously reported to be associated with brain tumor progression (da Fonseca and Badie, 2013; He et al., 2006; Roesch et al., 2018; Wei et al., 2013), we examined the status of microglia activation in brain metastatic lesions and found that current smokers exhibited a significantly increased number of Iba1⁺ (pan-microglial marker) cells in the brain metastatic lesions (Fig. 1 D) compared with noncurrent smokers. Importantly, the majority of infiltrated microglia in current smokers was identified to be pro-tumor M2 microglia (CD206⁺ cells; Fig. 1 D). These results suggest a role of smoking-mediated M2 microglial polarization in brain metastatic tumor growth.

Nicotine promotes brain metastasis through activation of microglia

Because M2 microglia are abundantly infiltrated in brain metastatic lesions of current smokers, we examined the possibility that nicotine promotes brain metastasis by polarizing microglia to M2 microglia. We first examined the expression of nicotine receptors in microglia cells and in tumor cells and found that

microglia expressed high levels of $\alpha 4\beta 2$, which is the most sensitive receptor of nicotine in the brain (Fig. 2 A). We also found that microglia expressed a significantly higher level of $\alpha 4\beta 2$ receptor than macrophage (Fig. S2 A). Moreover, the expression of $\alpha 4\beta 2$ receptor in microglia dramatically increased upon nicotine treatment (Fig. S2 A). We then examined the effect of nicotine on microglia polarization and tumor progression using the LL/2 mouse Lewis lung carcinoma model. The LL/2 cell is originated from the C57BL/6 mouse; however, this cell line can grow tumors in both C57BL/6 and BALB/c mice as reported previously (Janker et al., 2018; Kamei et al., 2009; Scholar et al., 1989). Therefore, we tested the effect of nicotine in both models. We intracardially inoculated luciferase-labeled LL/2 cells into both wild-type mice (C57BL/6 or BALB/c) followed by injecting nicotine (1 mg/kg) every 3 d until the end point. We found that nicotine significantly increased the incidence and growth of brain metastasis in both models (Fig. S2, B–D, for C57BL/6 model; Fig. 2 B and Fig. S2, E and F, for BALB/c model). On the other hand, nicotine did not promote bone metastasis (Fig. 2 B). The result of our immunohistochemical analysis for brain metastatic lesions revealed that high levels of activated microglial cells accumulated in the tumor mass in the nicotine-treated group (Fig. 2 C). Importantly, the majority of these microglia in the nicotine-treated mice were found to be M2 microglia (CD206⁺/Iba1⁺), and fewer M1 microglia (Iba1⁺/F4/80⁺) were identified in the brain lesions (Fig. 2 D and Fig. S2, G and H), suggesting that nicotine promoted microglia polarization to the M2 phenotype in the brain metastatic lesions. Infiltrated peripheral macrophages/microglia are known to modulate brain tumor progression (Bacha et al., 2018; Ellis et al., 2012). To define the exact role of macrophage and microglia on brain metastasis, we stained brain metastatic tumors with antibody against the macrophage marker CD45. We also examined the ratio of the number of microglia (CD45⁺/Iba1⁺) and peripheral macrophages (CD45⁺/Iba1⁺) in the brain metastatic lesions and found that macrophages comprised only 12–14% of CD45⁺/Iba1⁺ cells, and the number of macrophages was not altered between nicotine-treated and -untreated groups (Fig. S2 I). These results strongly suggest that nicotine promotes brain metastasis by skewing microglia to the M2 phenotype. To further validate this hypothesis, we examined the effect of depletion of “original” microglia by treating animals with the inhibitor for CSF1 receptor (CSF1R), PLX3397. CSF1 signaling is necessary for microglia viability, and treating mice with PLX3397 has been previously shown to eliminate ~99% of all microglia in the brain (Elmore et al., 2014). We therefore treated immune-competent wild-type mice with PLX3397 and transplanted them with LL/2 mouse lung cancer cells followed by nicotine treatment. To avoid a whole-body effect of PLX3397, the PLX3397 was locally injected into brain through intracranial injection 1 wk after the transplantation of LL/2 cells followed by nicotine treatment (Fig. S2 J). Strikingly, blocking microglia by PLX3397 significantly suppressed nicotine-related brain metastasis of lung cancer (Fig. 2, E–G), prolonged brain metastasis-free survival (Fig. 2 H), and repressed the nicotine-related M2 microglial polarization (Fig. 2, I and J) in the BALB/c mouse model. Similarly, PLX3397 suppressed nicotine-related brain metastasis and inhibited M2

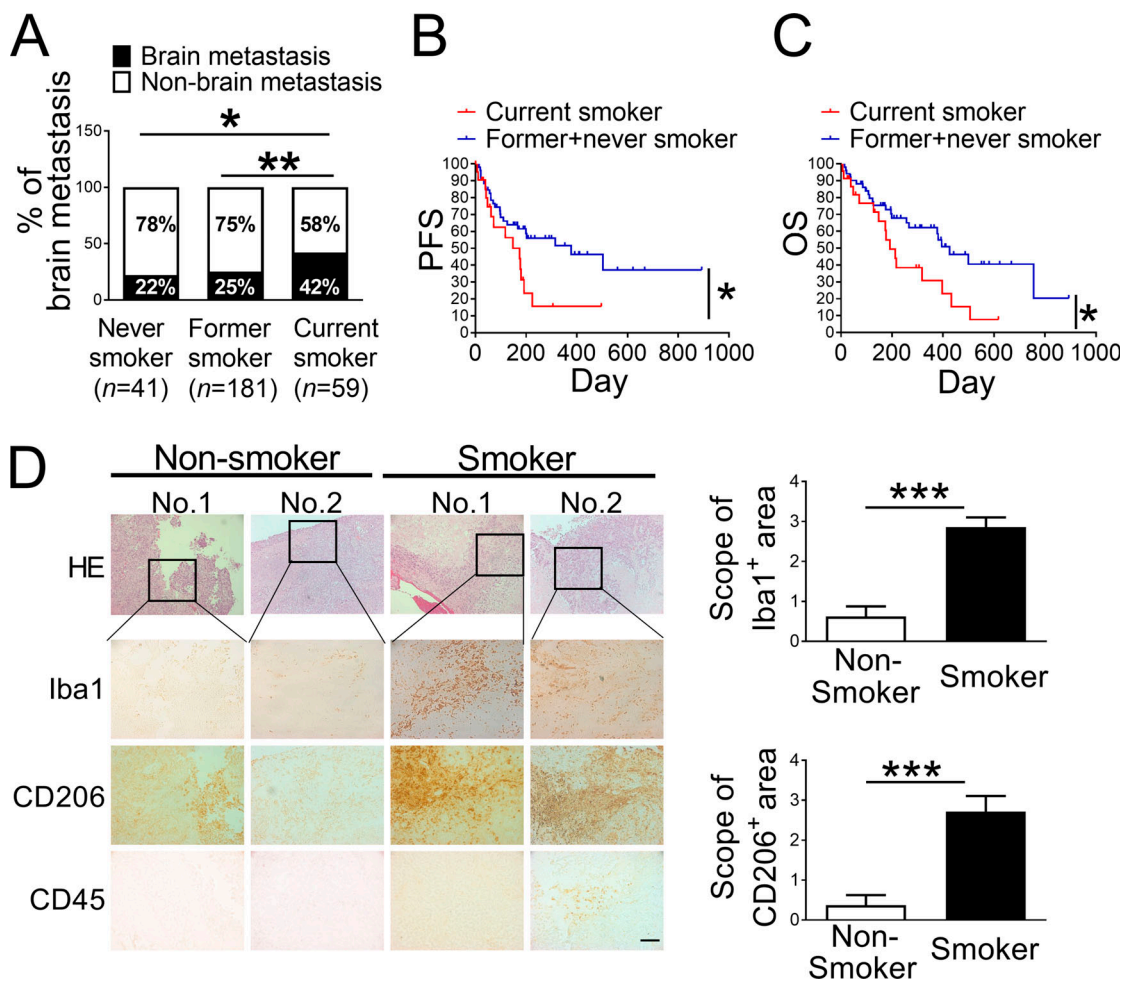


Figure 1. Cigarette smoking increases incidence and mortality rate of lung cancer brain metastasis. (A) Incidence of brain metastasis among 281 stage IV lung cancer patients composed of both current and noncurrent smokers at the time of diagnosis. All patients were admitted to Wake Forest Baptist Hospital. (B and C) Progression-free survival (PFS; B) and overall survival (OS; C) of patients with brain metastasis (n = 79) and with or without smoking history were examined by Kaplan–Meier analysis. (D) Representative images of immunohistochemical analysis for Iba1⁺, CD206⁺, and CD45⁺ cells in the H&E-stained brain metastatic lesions of lung cancer patients who were current smokers (n = 7) and noncurrent smokers (n = 4). Scale bar, 100 μ m. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

microglial polarization in the C57BL/6 mouse model (Fig. S2, K–N). We also treated nude mice with PLX3397 and transplanted them with H2030BrM cells via an intracardiac injection followed by nicotine treatment (Fig. S2, O–R). Nicotine-induced brain metastasis was significantly suppressed after PLX3397 treatment (Fig. S2, O–Q). The brain metastasis-free survival was also significantly prolonged by PLX3397 treatment (Fig. S2 R). PLX3397 treatment showed no detectable liver toxicity (Fig. S2 S). These results suggest that the nicotine-induced brain metastasis is indeed mediated by the effect of nicotine on microglia.

Nicotine skewed M2 microglial polarization

As shown in Fig. 2 D, the number of M2 microglia was increased after nicotine treatment, suggesting that nicotine promotes microglial polarization. To further investigate the effect of nicotine on microglia polarization, the microglial cells were treated with nicotine followed by measurement of expression of M1 and M2 markers. Our results showed that nicotine did not affect tumor cell proliferation (Fig. S2 T) at 1 μ M concentration, but the

same dose of nicotine significantly increased the mRNA expression of M2-related genes, *arginase-1* (*Arg1*), *arginase-2* (*Arg2*), and *CD204*, in both human and mouse microglial cells lines (Fig. 3, A and B) and mouse primary microglial cells (Fig. 3 C). Since STAT3 pathway is involved in M2 microglia (Qin et al., 2017), we examined the status of phospho-STAT3 and JAK expression in microglia with or without nicotine treatment, and found that nicotine treatment indeed enhanced the expressions of JAK/STAT3 (Fig. 3 D). We then treated the microglia with nicotine in the presence of STAT3 inhibitor (STATITC) and found that inhibiting the STAT3 by STATITC significantly suppressed the nicotine-induced STAT3 activation (Fig. 3 E). The *Arg1* gene is an M2 marker and is known to be regulated by STAT3 (Yi and Kim, 2017). We found that nicotine-induced up-regulation of the *Arg1* gene promoter was significantly suppressed by STATITC treatment (Fig. 3 F). Moreover, inhibition of STAT3 reversed the nicotine-mediated suppression of Iba1⁺/F4/80⁺ (M1) and promotion of Iba1⁺/CD206⁺ cells (M2; Fig. 3, G and H). These results suggest that the nicotine promotes M2 microglial polarization via activation of the STAT3 pathway.

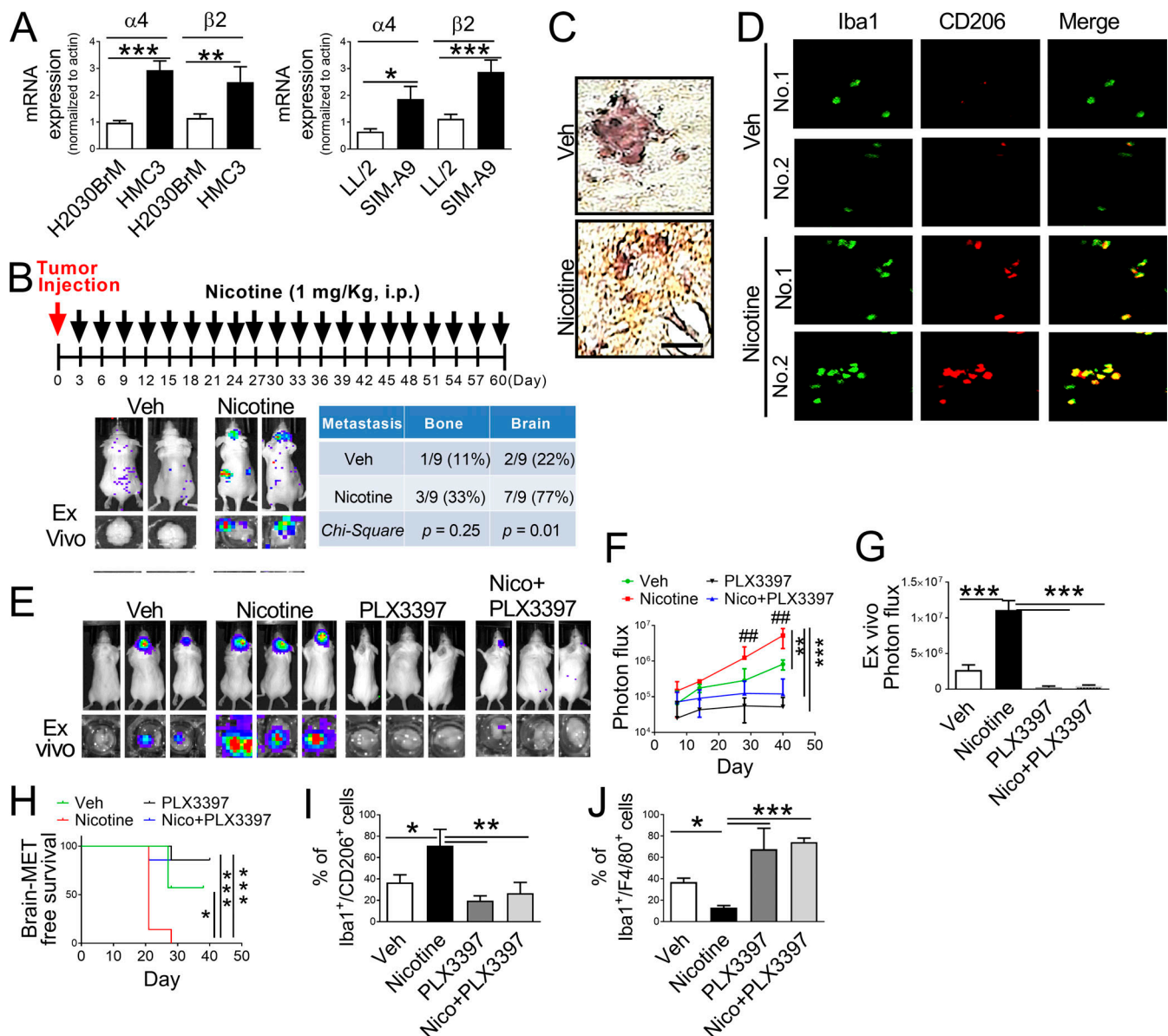


Figure 2. Nicotine promotes brain metastasis by activating microglia in vivo. (A) Expression of $\alpha 4 \beta 2$ nicotine receptor on human microglia (HMC3), mouse microglia (SIM-A9), human lung cancer cells (H2030BrM), and mouse lung cancer cells (LL/2) were evaluated by qRT-PCR ($n = 3$ /group). (B) LL/2 (5×10^4 cells) were intracardially injected into immune-competent BALB/c mice ($n = 9$). After 3 d of intracardiac transplantation of LL/2 cells, mice received nicotine treatment (1 mg/kg) by intraperitoneal injection every 3 d for 60 d. Upper panel: BLI images of brain metastatic lesions of representative mice from each experimental group (vehicle alone or nicotine treatment). Lower panel: total photon flux of ex vivo image of brain metastatic lesions was measured by BLI at the end point (day 60). Quantitative data of bone and brain metastasis of lung cancer are shown in the right panel. (C) At the end point, the brain sections from mice with or without treatment with nicotine were examined for Iba1⁺ signal (brown) on microglia. Scale bar, 20 μ m. (D) Representative images of immunohistochemical analysis for CD206⁺ and Iba1⁺ microglia in the metastatic brain lesions of mice that were treated with or without nicotine ($n = 9$ /group). Scale bar, 100 μ m. (E) Mouse lung cancer LL/2 cells were intracranially injected into wild-type BALB/c mice ($n = 9$ /group) followed by administering PLX3397 by intracranial injection. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the end point (day 40). (F) Quantitative data of in vivo brain metastasis of lung cancer ($n = 9$ /group). (G) Ex vivo BLI signals in the brain at the end point ($n = 9$ /group). (H) Kaplan-Meier analysis of brain metastasis-free survival was performed ($n = 9$ /group). (I and J) Metastatic brain tumors in E were isolated from the brain and were examined by FACS for M2 (I) and M1 (J) microglial polarization ($n = 4$ or 5/group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus respective nicotine group. ##, $P < 0.01$ versus respective PLX3397 or Nico+PLX3397 group.

Nicotine-treated microglial cells promote tumor growth and stemness

To examine the effect of nicotine-induced M2 microglia on tumor progression, we treated lung cancer brain metastatic cells, H2030BrM and PC9BrM, with conditioned medium (CM) generated from human microglia that were treated with or without

nicotine. We found that CM derived from nicotine-treated microglia significantly increased sphere formation (Fig. 4 A) and population of ESA⁺/CD44⁺ or aldehyde dehydrogenase (ALDH⁺) cancer stem cells (CSCs; Fig. 4 B). The expression of stemness-inducing genes, SOX2 and NANOG, was also significantly up-regulated when the brain metastatic cells were treated with

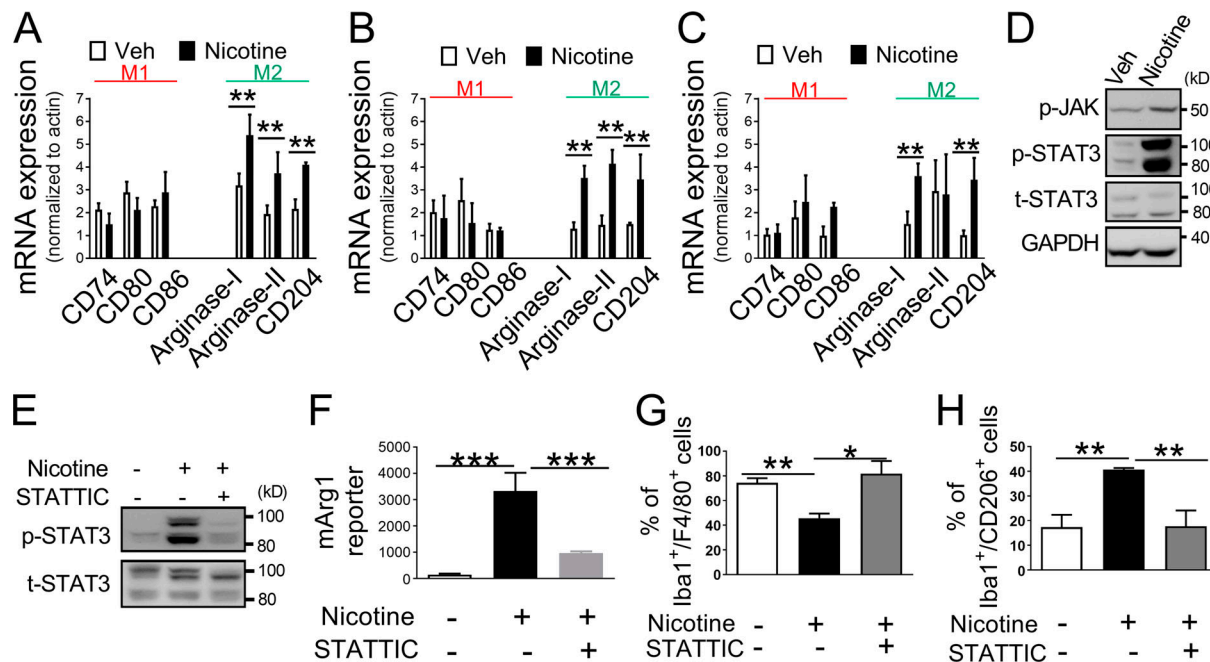


Figure 3. Nicotine promotes M2 microglial polarization via STAT3 activation. (A–C) Expressions of M1/M2 microglia markers were examined after the treatment of human microglia (HMC3) (A), mouse microglia (SIM-A9; B), and primary mouse microglia (C) with either vehicle or nicotine (1 μ M). The value of qRT-PCR in each figure was normalized using actin as a control. The y axis indicates arbitrary unit ($n = 3$ /group). (D) Human microglia (HMC3) were treated with or without nicotine (1 μ M), and the expressions of JNK and STAT3 were examined by Western blot. (E) Human microglia (HMC3) were treated with or without nicotine and/or STATTC (0.5 μ M) for 24 h, and the expression of STAT3 was examined by Western blot. (F) Mouse microglia cells (SIM-A9) were treated with or without nicotine and/or STATTC (0.5 μ M) for 24 h followed by examining the luciferase-promoter activity of *Arg1* (M2; $n = 4$ /group). (G and H) Mouse microglia (SIM-A9) were treated with or without nicotine (1 μ M) and/or the STAT3 inhibitor (0.5 μ M) for 24 h. Cells were then subjected to FACS analysis for analysis of Iba1⁺/F4/80⁺ (M1 cell; G) and Iba1⁺/CD206⁺ (M2 cell; H; $n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

the CM of nicotine-treated microglia (Fig. 4, C and D). Furthermore, nicotine-treated microglial CM also promoted the colony-forming ability of both H2030BrM and PC9BrM cells (Fig. 4 E). We then sought to identify the factor(s) in CM that are responsible for the increase in stemness. We performed the cytokine array analysis (consisting of 99 factors) and found that nicotine significantly augmented the secretion of CCL20 and IGF-1 in the microglial CM (Fig. S3 A). The results of screening were verified using human microglial cells (Fig. S3 B). Furthermore, blocking STAT3 by STATTC significantly suppressed nicotine-mediated up-regulation of CCL20 and IGF-1 (Fig. S3 B). We observed that microglia produced higher levels of IGF-1 and CCL20 than macrophage (Fig. S3 C). The expression of receptors of IGF-1 and CCL20 were significantly increased in H2030BrM cells after the treatment with CM of microglia that were exposed to nicotine (Fig. 4 F). We then treated lung cancer cells with recombinant CCL20 and IGF-1 and examined their effect on CSC properties. We found that both cytokines significantly enhanced the sphere formation (Fig. 4 G), ESA⁺/CD44⁺ CSC population (Fig. 4 H), and mRNA expression of CSC markers SOX2 and NANOG (Fig. 4, I and J). In addition, IGF-1 and CCL20 significantly increased the colony-forming ability of brain metastatic lung cancer cells (Fig. 4 K). To understand the clinical relevance of these results, we used The Cancer Genome Atlas (TCGA) dataset and found that high expression of CCL20 was associated with poor overall survival in lung cancer patients (Fig. S3 D);

however, no such correlation was observed for the IGF-1 gene (Fig. S3 E). Furthermore, expressions of both CCL20 and its receptor, CCR6, were up-regulated in the primary tumor site of lung cancer patients compared with normal lung tissue in the same dataset (Fig. S3, F and G). Moreover, microglia expressed a higher level of CCL20 mRNA than lung cancer cells, indicating that microglia are potentially major sources of CCL20 in brain TME (Fig. S3, H and I). To examine whether CCL20 is involved in the growth-promoting effect of CM derived from nicotine-treated microglia, CCL20 was depleted in the CM using CCL20 neutralizing antibody. Our result indicates that depletion of CCL20 significantly suppressed the generation of CSC by nicotine-treated CM (Fig. 4 L). To further validate our results, we examined the levels of CCL20 in brain and serum of the nicotine-treated mice represented in Fig. 2 E and found that CCL20 indeed increased in the brain and serum of nicotine-treated animals (Fig. 4, M and N). In contrast, depletion of microglia by PLX3397 significantly suppressed the levels of nicotine-induced CCL20 in brain and serum of nicotine-treated animals (Fig. 4, M and N). Furthermore, consistent with our in vivo results, we found that the serum level of CCL20 was significantly higher in lung cancer patients who were current smokers compared with noncurrent smokers (Fig. 4 O).

We also examined the direct effect of nicotine on cell growth by treating the human lung cancer brain metastatic cells, H2030BrM, with nicotine alone or with nicotine-treated

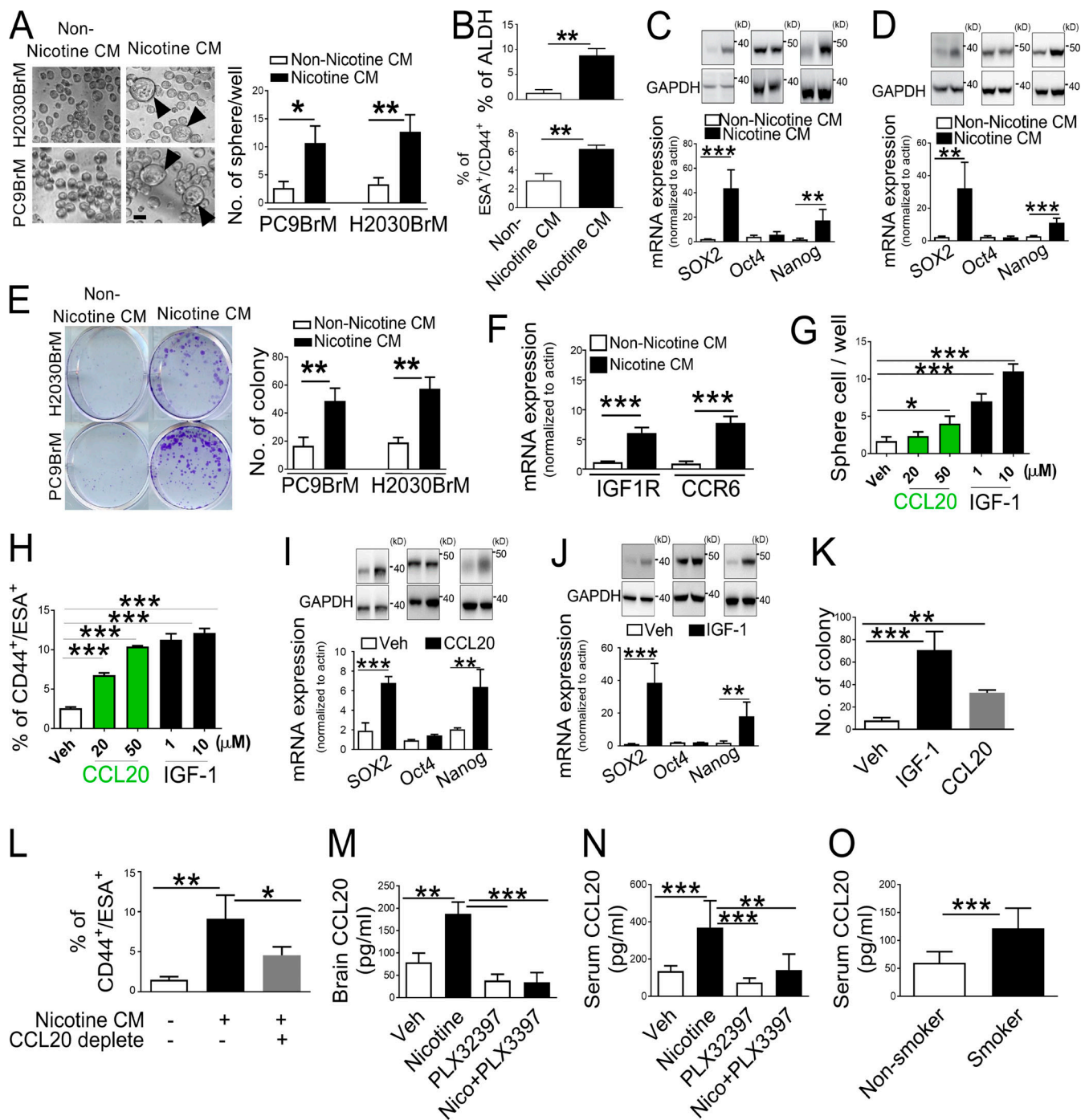


Figure 4. Nicotine-mediated microglial polarization promotes tumor stemness. (A) Effect of human microglial cells (HMC3) treated with or without nicotine (1 μM), and collection of CM. Brain metastatic lung cancer cells (H2030BrM and PC9BrM) were treated with the non-nicotine and nicotine CM for 24 h, and their sphere-forming ability was measured. Left panel: representative images. Arrowheads indicate sphere formation. Right panel: quantification of number of spheres. Non-nicotine or nicotine CM: microglia cells were treated with PBS or nicotine (1 μM) for 24 h, and they were washed with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 h (*n* = 4/group). Scale bar, 10 μm. (B) Effect of the human microglial CM on CSC markers, ESA⁺/CD44⁺, and ALDH activity were examined using FACS (*n* = 4/group). (C and D) Expressions of stemness genes in H2030BrM (C) and in PC9BrM cells (D) were examined after the treatment with or without nicotine-derived human microglial CM by qRT-PCR (*n* = 6/group). (E) Colony-forming abilities of H2030BrM and PC9BrM cells were measured in the presence or absence of the CM derived from nicotine-treated human microglia. Left panel: representative images. Right panel: quantification of number of colonies (*n* = 4/group). (F) Expressions of IGF-1 and CCL20 receptors in H2030BrM cells were examined after the treatment of cells with or without nicotine-derived human microglial CM by qRT-PCR (*n* = 4/group). (G) Effect of recombinant proteins, CCL20 and IGF-1, on sphere formation ability of H2030BrM was measured (*n* = 3/group). (H) H2030BrM cells were treated with CCL20 or IGF-1 for 24 h followed by measuring the ESA⁺/CD44⁺ stem cell population by FACS (*n* = 4/group). (I and J) H2030BrM cells were treated with or without 20 μM of CCL20 (I) or 1 μM of IGF-1 (J) for 24 h, and the expressions of stemness genes were examined by qRT-PCR and Western blot (*n* = 4/group). (K) Colony-forming assay was performed for the same set of samples as in G and H (*n* = 4/group). (L) Human microglia were treated with or without nicotine (1 μM) for 24 h, and their CM was prepared. The CM was then

added with anti-CCL20 antibody to deplete CCL20. H2030BrM cells were then treated with the CCL20-depleted CM followed by assaying the ESA⁺/CD44⁺ stem cell population by FACS ($n = 4/\text{group}$). **(M and N)** Metastatic lesions in the brain and serum were prepared from the mice used in Fig. 2 E. The protein expression of CCL20 in the brain (M) and in the serum (N) in each group was measured by ELISA ($n = 6/\text{group}$). **(O)** The protein expression of CCL20 in the serum of lung cancer patients with or without smoking history were measured by ELISA ($n = 10/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

microglial CM. Although colony-forming ability increased in both groups (Fig. S3 J), the CSC population and expression of stem cell markers were significantly elevated only in cells treated with the nicotine-treated microglial CM (Fig. S3, K and L). Consistent with these results, treating the mouse lung cancer cell, LL/2, with CM derived from nicotine-treated mouse microglia significantly increased the levels of CSCs (Fig. S3 M) and promoted the colony-forming ability (Fig. S3 N) and tumor cell viability (Fig. S3 O). We also examined whether nicotine-induced brain metastasis is mediated via M2 microglial polarization by STAT3 activation. We blocked the M2 polarization of microglia by STAT3IC and examined the expression of *IGF-1* and *CCL20* by quantitative real-time PCR (qRT-PCR). We found that the nicotine treatment significantly up-regulated the *IGF-1* and *CCL20*, while STAT3IC reversed the effect (Fig. S3, P and Q). The CM was then added to the culture of LL/2 cells. We found that CM derived from microglia that were treated with both nicotine and STAT3IC significantly suppressed CSC population (Fig. S3 R) and tumor cell viability (Fig. S3 S).

Nicotine suppresses innate immune function of microglia

Microglia are known to exhibit phagocytic activity against brain tumor cells, like macrophages (Hutter et al., 2019). However, to escape from microglia-mediated phagocytosis, tumor cells express CD47 on their cell surface, which activates so-called “do not eat me” signaling. To investigate if nicotine affects this immune function of microglia, we measured the expression of signal-regulatory protein α (SIRP α) on microglia and CD47 on tumor cells. We found that the mRNA and protein expression of SIRP α were up-regulated in human microglia after nicotine treatment (Fig. 5 A). We also found that nicotine significantly increased the mRNA and protein expression of CD47 in both H2030BrM and PC9BrM cells (Fig. 5 B). Importantly, the expression of CD47 is up-regulated in brain metastasis of lung cancer patients with smoking history (Fig. 5 C). We then examined the effect of nicotine on phagocytic ability of microglia. We prelabeled H2030BrM cells with the fluorochrome-PKH26, a red-fluorescent vital dye that binds tightly to cell membranes (Munn and Cheung, 1990). The labeled cells were incubated with microglia that were treated with or without nicotine. We found that the size of PKH26⁺ tumor cells and microglia cells was similar at 0 h in the co-culture (Fig. 5 D). However, after 24 h of incubation, the small and irregular shape of PKH26⁺ cells was accumulated in the microglia. This result suggests that tumor cells were rapidly degraded by nicotine-treated microglia through phagocytosis. As shown in Fig. 5, D and F, both human and mouse microglial cells showed strong phagocytic activities toward the H2030BrM and LL/2 cells. Importantly, this phagocytic ability was greatly compromised in the presence of nicotine (Fig. 5, E and G). Blocking STAT3 signaling by STAT3IC

treatment suppressed nicotine-mediated down-regulation of phagocytic ability of microglia (Fig. 5 E). We also examined the expression of SIRP α in microglia of the nicotine-treated mouse brain represented in Fig. 2 E and found that the CD47 and SIRP α expression were strongly elevated in the brain of mice treated with nicotine (Fig. 5, H–J). These results suggest that nicotine promotes M1/M2 conversion and suppresses the innate immune function of microglia, which promotes brain metastasis progression.

PTL suppresses nicotine-induced M2 microglia polarization and enhances their phagocytic ability

Our results strongly suggest that blocking M2 polarization of microglia may be an effective therapeutic approach for brain metastasis. Therefore, we screened a natural compound library to identify compounds that block M2 macrophage polarization using an Arg1 promoter reporter system. We screened a total of 103 compounds that are known to be blood–brain barrier (BBB) permeable. As shown in Fig. S4 and Fig. 6 A, we identified three compounds (>15-fold change, $P < 0.001$) that effectively suppressed the nicotine-induced activation of Arg1 promoter at a concentration of 1 μM . We selected the top compound, PTL, which suppressed the Arg1 promoter activity by >20-fold, for further study (Fig. 6 A). PTL is an abundant sesquiterpene lactone found in the medicinal herb Feverfew (*Tanacetum parthenium*). It has been used to treat inflammation and headache for centuries in Europe (Liu et al., 2018). To confirm the suppressive effect of PTL on nicotine-induced M2 microglial polarization, microglia were treated with nicotine in the presence or absence of PTL (Fig. S5 A), and expressions of M1 and M2 markers were examined. We found that PTL significantly decreased the nicotine-mediated M2 polarization of microglia (Fig. 6 B). Furthermore, the nicotine-mediated reduction of Iba1⁺/CD11b⁺ cells (M1) and elevation of Iba1⁺/CD206⁺ cells (M2) were significantly reversed by PTL (Fig. 6 C). Previous studies have shown that PTL modulated the JAK2/STAT3 signaling in response to cytokine stimulations (Liu et al., 2018; Sobota et al., 2000). We indeed found that PTL suppressed the nicotine-induced JAK2/STAT3 activation (Fig. 6 D), suggesting that PTL blocks the nicotine-induced M2 microglial polarization by targeting STAT3. Furthermore, the nicotine-induced suppression of phagocytic ability of microglia was restored when cells were treated with PTL (Fig. 6 E). The H2030BrM cells were treated with CM of microglia that were exposed to nicotine only or nicotine plus PTL. We found that the ESA⁺/CD44⁺ CSCs (Fig. 6 F) and colony-forming ability (Fig. 6 G) were significantly suppressed by CM of microglia treated with nicotine plus PTL. We also examined the mRNA expression of *CCL20* and found that the nicotine-mediated secretion of *CCL20* was down-regulated by the PTL treatment (Fig. 6 H). Next, to investigate the effect of PTL on

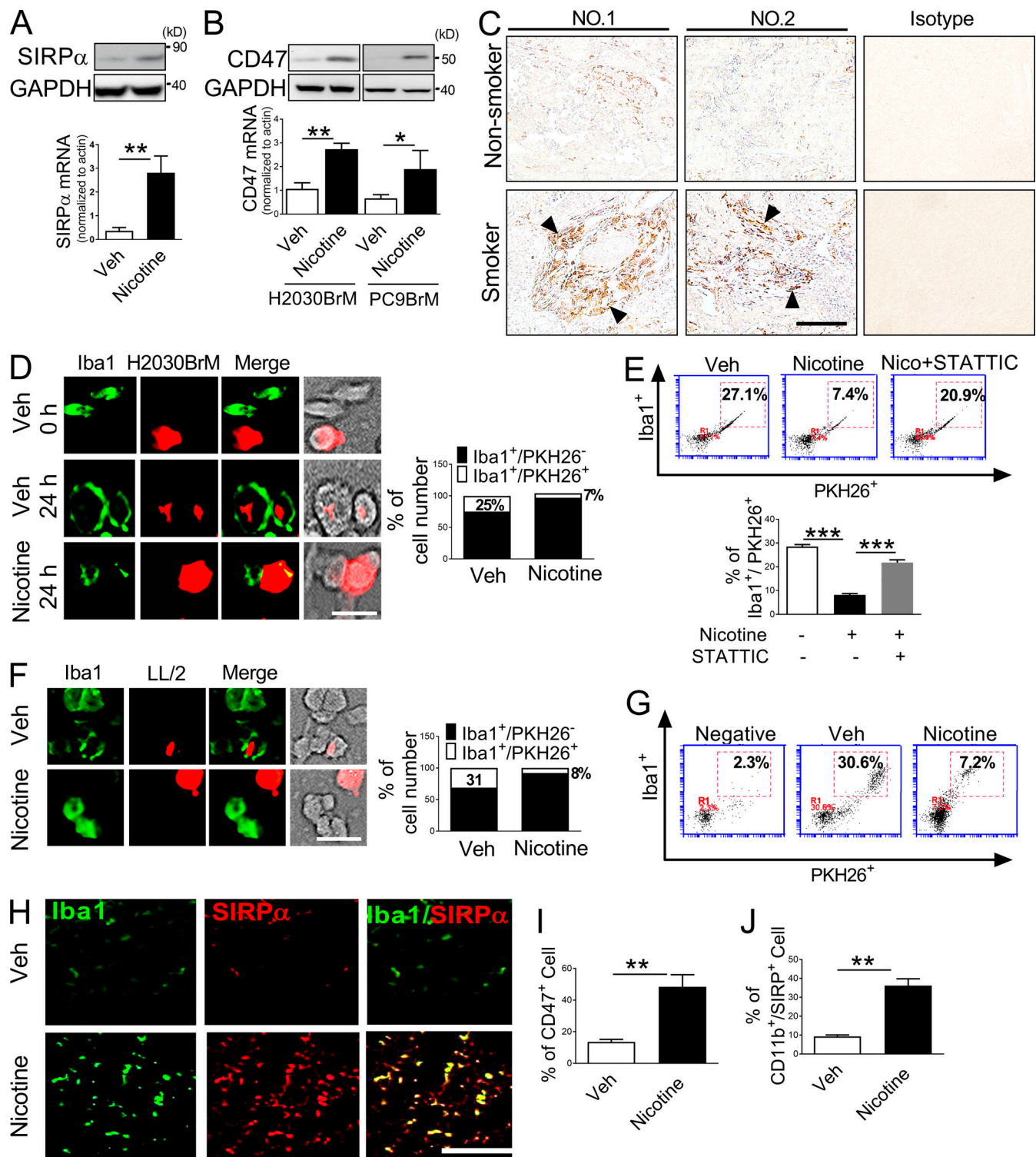


Figure 5. Nicotine suppresses innate anti-tumor immune function of microglia. (A) Human microglia cells (HMC3) were treated with or without nicotine (1 μ M) for 24 h, and the expression of SIRP α was examined by qRT-PCR and Western blot ($n = 4$ /group). (B) H2030BrM and PC9BrM cells were treated with or without nicotine (1 μ M) for 24 h, and the expression of CD47 was examined by qRT-PCR and Western blot ($n = 4$ /group). (C) Representative images of immunohistochemical analysis for CD47 $^{+}$ cells in the metastatic brain lesions of lung cancer patients with or without smoking history. Arrows indicate CD47 $^{+}$ cells. Non-smoker ($n = 4$) and smoker ($n = 7$). Scale bar, 50 μ m. (D) Phagocytic activities of HMC3 human microglial cells (green) with or without nicotine treatment were examined using PKH26-labeled H2030BrM cells (red). Both cells were incubated together for 0 or 24 h and photographed (left panels), and the number of microglia that phagocytosed tumor cells was counted to quantify microglial phagocytic activity as shown in the right panel ($n = 4$ /group). Scale bar, 10 μ m. (E) A parallel set of cells in D was analyzed by FACS to quantify phagocytic abilities of microglia after nicotine or STATTC treatment. Upper panels: representative FACS data. Lower panel: statistical analysis of the FACS data ($n = 4$ /group). (F) Mouse microglia (SIM-A9; green) were incubated with PKH26-labeled LL/2 mouse lung cancer cells (red) for 24 h. They were then photographed under a fluorescent microscope ($n = 4$ /group). Scale bar, 100 μ m.

(G) Phagocytic abilities of microglia with or without nicotine treatment were examined by incubating mouse microglia with PKH26-labeled LL/2 mouse lung cancer cells for 24 h, followed by analysis of the cells by FACS ($n = 4$). (H) The metastatic lesions in the brain of mice in Fig. 2 E were sectioned, and they were subjected to immunohistochemical analysis for SIRPα and Iba1. Representative immunohistochemistry images of the microglia are shown ($n = 9$ /group). Scale bar, 100 μ m. (I and J) The metastatic lesions in the brain of mice in Fig. 2 E were subjected to FACS analysis for CD47⁺ tumor cell (I) and SIRPα/CD11b⁺ (J) microglia cells ($n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

nicotine-related brain metastasis in vivo, wild-type mice (C57BL/6 and BALB/c) were transplanted with LL/2 mouse lung cancer cells followed by administering nicotine (1 mg/kg) only or nicotine plus PTL (1 mg/kg) via an intraperitoneal injection. As shown in Fig. 6, I–K (BALB/c) and Fig. S5, B and C (C57BL/6), PTL treatment significantly suppressed nicotine-mediated brain metastasis and prolonged the brain metastasis-free survival (Fig. 6 L). To examine whether the PTL treatment also reverses microglia polarization in the brains of these mice, we performed flow cytometry (FACS) analysis of mouse brain and stained brain sections for M1 and M2 markers. A significant increase in Iba1⁺/F4/80⁺ (M1 marker) and decrease in Iba1⁺/CD206⁺ (M2 marker) were observed in brain microglia of PTL-treated mice (Fig. 6, M and N; and Fig. S5, D and E). To validate our results in human cell line, we used the xenograft model of H2030BrM cell and tested the efficacy of PTL. As shown in Fig. S5, F–K, PTL significantly suppressed the nicotine-induced brain metastasis and M2 microglia polarization. PTL treatment did not affect body weight of mice and showed no detectable liver toxicity (Fig. S5 L). We also examined the direct effect of PTL on cell viability and CSC population in vitro (Fig. S5 M). Our results showed that PTL suppressed the abundance of CD44⁺/ESA⁺ CSC and cell viability at the concentration of 10–20 μ M, but not at 1 μ M, which is the dose used throughout our in vitro experiments (Fig. S5, N and O), indicating that the anti-tumor effect of PTL is not mediated by direct cytotoxic effect, and it is rather mediated by the effect on microglia. Furthermore, PTL significantly suppressed stemness promoting effect of nicotine on microglia (Fig. S5 P) and the expression of stemness-related genes (Fig. S5, Q and R). These results suggest that PTL suppresses nicotine-related brain metastasis of lung cancer by skewing polarity of M1 microglia and up-regulating their phagocytic ability.

Discussion

The incidence of brain metastasis among lung cancer patients is 10–36% (Bacha et al., 2018; Barnholtz-Sloan et al., 2004; Schouten et al., 2002), although these data are considered to be underestimated (Bacha et al., 2018; Schouten et al., 2002). The majority of the lung cancer patients are diagnosed with advanced stage disease, most with stage IV cancer. More than 90% of lung cancer deaths are attributed directly to the metastatic disease and include those brain metastases (Ali et al., 2013; Kakusa et al., 2018). Cigarette smoking is a major risk factor for lung cancer (Lee et al., 2012), and our previous study showed a negative impact of smoking on lung cancer brain metastasis (Shenker et al., 2017). In the present study, we have found a significantly higher incidence of brain metastasis among current smokers with stage IV lung cancer.

Nicotine is not a carcinogen, but it causes addiction by activating nACh receptor, which is abundantly expressed by various

brain cells including microglia. Direct tumor promoting effects of nicotine have been previously reported (Schaal and Chellappan, 2014; Sun et al., 2017; Wang et al., 2017; Yoneyama et al., 2016); however, how nicotine promotes tumor by activating TME is still poorly understood. The current report is the first to show the critical impact of nicotine on brain TME to promote brain metastasis by skewing microglia to M2 phenotype and suppressing their role in innate immunity. Our results indicate that the phenotypic conversion of microglia by nicotine is mediated through the nACh receptor–STAT3 pathway, and that inhibition of STAT3 blocked the M2 conversion, suppressed the secretion of pro-tumor M2 cytokines, and reactivated their phagocytic activity toward tumor cells (Fig. 6 O).

Previous studies showed that microglial cells and macrophages in the brain are derived from two different sources, parenchymal resident microglia and monocytes/macrophages that enter the brain from the bone marrow (Wu and Watabe, 2017). Although microglia and macrophage cells show similarity in their surface markers and physiological functions, recent studies have demonstrated that microglia and macrophages are two distinct myeloid populations with different developmental origins (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). Microglial cells originate from erythromyeloid progenitors that begin on embryonic day (E) 7.5–E8.0 in the blood islands of the yolk sac. Until E9.5, erythromyeloid progenitors migrate to the developing central nervous system and mature into microglia (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). These early microglial cells reside in the brain throughout life and are thought to sustain the local microglial population. In contrast, macrophages originate from the hematopoietic stem cells that start in the aorta–gonad–mesonephros region at E10.5, and then in the fetal liver at E12.5. After the postnatal stage, macrophages are produced from monocytes in the bone marrow (Gomez Perdiguero et al., 2015; Prinz and Priller, 2014; Schulz et al., 2012). Furthermore, recent studies demonstrated that microglial cells and macrophage have distinct and specific surface antigens (Feng et al., 2015). Studies on CX3CR1⁺/GFP/CCR2⁺/RFP knock-in fluorescent protein reporter mice revealed that microglial cells are CX3CR1⁺/CCR2[−]/CD45[−], while the macrophages are CX3CR1[−]/CCR2⁺/CD45⁺ (Feng et al., 2015), strongly supporting the notion that microglial cells and macrophages are from different populations and they can be distinguished. Badie and Scharfner performed flow cytometric analysis and characterized the distribution of microglial cells and macrophages in experimental gliomas and found that microglial cells (CD11b/c^{high}, CD45^{low}), mainly present at the site of tumor or tumor periphery, accounted for 13–34% of the tumor mass. In contrast, macrophages (CD11b/c^{high}, CD45^{high}) were less prominent within the tumors or the tumor periphery and accounted for just

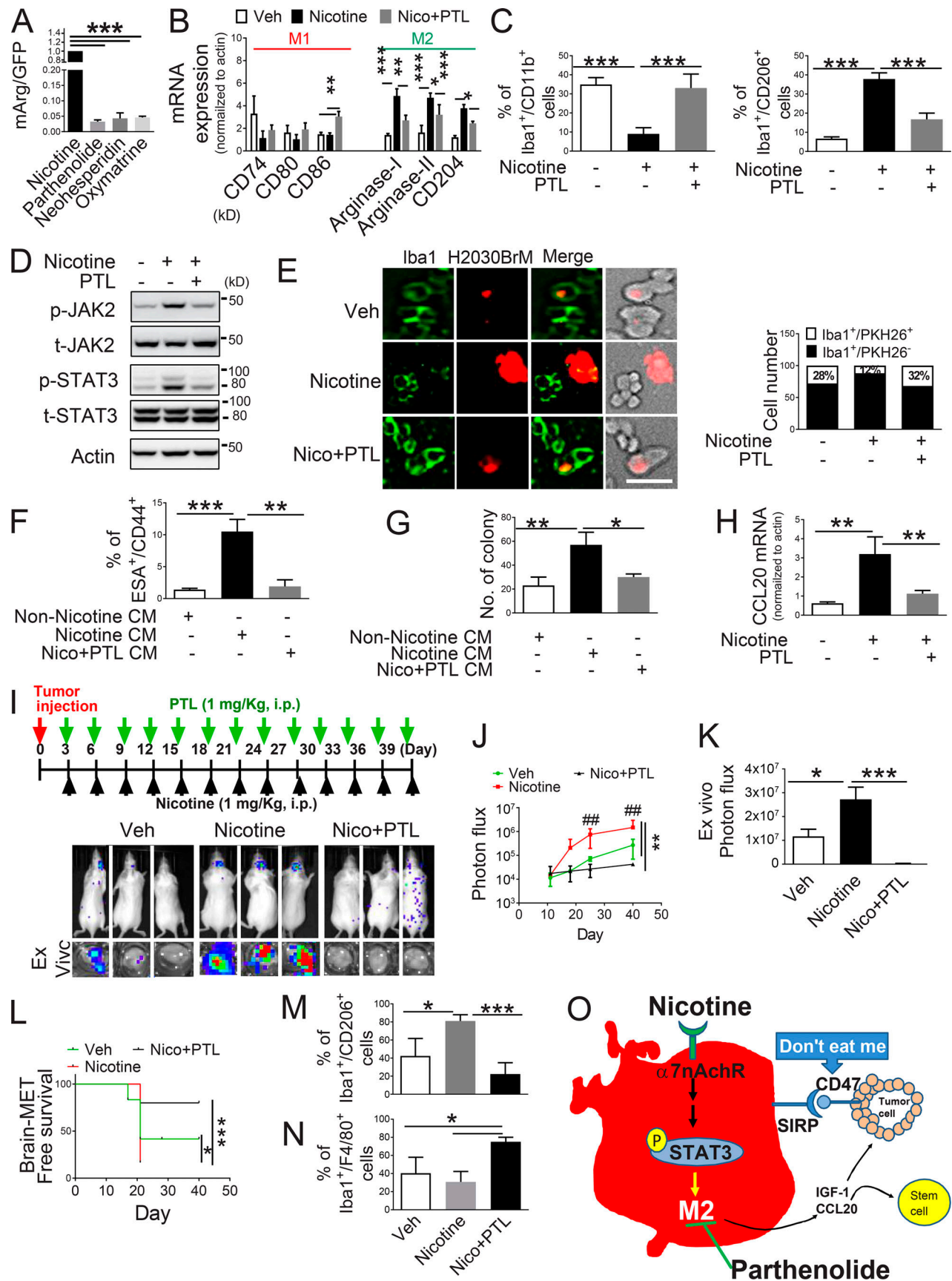


Figure 6. **PTL suppresses brain tumor progression by blocking nicotine-induced M2 microglia polarization.** (A) Human microglia cells (HMC3) with the Arg1 reporter plasmid were cultured in the presence or absence of compounds that were identified as the top three most effective inhibitors for Arg1 during our

initial screening (see Materials and methods). After 48 h of incubation, luciferase reporter activity was measured ($n = 4/\text{group}$). **(B)** Expression of surface markers of M1/M2 microglia was examined by qRT-PCR after microglial cells were treated with or without nicotine plus PTL ($n = 4/\text{group}$). **(C)** The same set of samples in B was evaluated for quantification of Iba1⁺/CD11b⁺ (M1) cells and Iba1⁺/CD206⁺ (M2) cells by FACS ($n = 4/\text{group}$). **(D)** The same set of samples in C was examined for quantification of the protein expression of JAK2 and STAT3 by Western blot. **(E)** Human microglial (HMC3) cells (green) with or without nicotine treatment (1 μM) in the presence or absence of PTL (1 μM) were incubated with PKH26-labeled H2030BrM cells (red) for 24 h and photographed (left panels), followed by measurement of the microglial phagocytic activity (right panel; $n = 4/\text{group}$). Scale bar, 10 μm . **(F)** CM was prepared from human microglia (HMC3) treated with or without nicotine and PTL. The CM was added to the culture of H2030BrM, and cells were incubated for 48 h followed by evaluation of CSC population by FACS. Non-nicotine, nicotine, or Nico+PTL CM: microglia were treated with PBS, nicotine, or nicotine plus PTL for 24 h. They were then washed twice with PBS and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for a further 24 h ($n = 4/\text{group}$). **(G)** For the same set of samples as F, colony-forming ability was also measured ($n = 4/\text{group}$). **(H)** Human microglia were treated with or without nicotine (1 μM) in the presence or absence of PTL for 24 h, followed by assessment of the expression of CCL20 by qRT-PCR ($n = 4/\text{group}$). **(I)** The mouse lung cancer cells, LL/2, were intracardially injected into wild-type BALB/c mice. After 3 d of intracranial transplantation of LL/2 cells, mice received nicotine (1 mg/kg) plus PTL (1 mg/kg) by an intraperitoneal injection every 3 d for 40 d. Upper panel: BLI images of representative mice from each experimental group at day 40. Lower panel: total photon flux of ex vivo brain metastatic lesions was measured by BLI at the endpoint (day 40; $n = 9/\text{group}$). **(J)** Quantitative data of BLI in the brain regions are shown ($n = 9/\text{group}$). **(K)** Ex vivo signals in the whole brains at the end point were quantified. **(L)** The Kaplan–Meier analysis of brain metastasis-free survival was performed ($n = 9/\text{group}$). **(M and N)** Metastatic brain tumors in I were isolated from the brain and were examined by FACS for M2 (M) and M1 (N) microglial polarization ($n = 9/\text{group}$). **(O)** A proposed model illustrating a nicotine-induced brain metastasis ($n = 9/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

4.2–12% of the tumor mass (Badie and Schartner, 2000). These results also suggest that microglial cells play a key role in mediating brain tumor progression.

We have also found that nicotine dramatically increased the mRNA expression of $\alpha\beta 2$ receptor on microglia cell compared with macrophage. These results suggest that microglia is more responsive to nicotine compared with macrophage, resulting in increase in the incidence of brain metastasis. Furthermore, it was reported that the numbers of macrophage/microglia were significantly higher in the central nervous system than in the peripheral system after injury (Leskovar et al., 2000). In addition, the peak concentrations of three cytokines, TNF α , IL-1, and IL-6, appeared earlier and were significantly higher in the injured central nervous system than peripheral system (Leskovar et al., 2000), suggesting differences in the reparative responses of macrophage/microglia in the PNS and central nervous system to injury.

In this study, we showed that blocking microglia CSF1R by PLX3397 significantly suppressed nicotine-mediated brain metastasis, suggesting that cancer cells lose growth support function from M2 microglia. Previous studies showed that PLX3397 reprogrammed tumor-infiltrating macrophages, enhanced antigen presentation (Zhu et al., 2014), and suppressed TAM infiltration in the tumor (Butowski et al., 2016; Cannarile et al., 2017; Cuccarese et al., 2017). We also found that PLX3397 skewed the population of microglia/macrophage to M1 phenotype. Therefore, while blocking the proliferation of microglia/macrophage, PLX3397 also promotes the residual microglia's innate immune function to tumor cells. Our results indicate that nicotine increased the secretion of IGF-1 and CCL20 in the CM of microglial cells. IGF-1 is known to enhance tumor cell survival and proliferation via activation of the PI3K-Akt signaling (Park et al., 2016; Zhu et al., 2011). However, the role of CCL20 on tumor progression has not yet been well defined. CCL20 is a critical chemoattractant responsible for the recruitment of inflammatory immune cells (Nandi et al., 2014). The expression of CCL20 has been shown to be overexpressed in a variety of tumors including lung cancer (Liu et al., 2016; Wang et al., 2015, 2016). In pancreatic cancer, CCL20 has been found to be

overexpressed in metastatic tumor tissues (Liu et al., 2016). It was reported to promote epithelial-mesenchymal transition and invasive ability of tumor cells by activating the MAPK and PI3K pathways and matrix metalloproteinase signaling (Marsigliante et al., 2016; Wang et al., 2016). Previous studies showed that CCL20 promotes cell proliferation through activation of MAPK pathway (Lu et al., 2017) and up-regulating transcription of c-Fos and c-Myc. Others have shown that CCL20 enhanced the chemotherapy resistance of triple-negative breast cancer by promoting ALDH⁺ population of breast cancer cells through PKC ζ - and p38 MAPK-mediated NF- κ B activation (Chen et al., 2018). We found that stem cell genes NANOG and SOX2 were up-regulated in CCL20-treated cells, suggesting that CCL20 promoted the stemness of lung cancer brain metastatic cells via NANOG/SOX2. One of the major sources of CCL20 is a tumor-associated macrophage, especially M2 macrophage (Liu et al., 2016). In this study, we found that nicotine strongly promoted phenotypic conversion of microglia to M2 and enhanced secretion of CCL20 to stimulate tumor cell growth and cancer cell stemness. The depletion of microglia in our animal model significantly reversed the effect of nicotine on brain metastasis. Furthermore, we showed that the effect of CCL20 on tumor stem cell was mediated by up-regulation of SOX2 and NANOG genes. These results suggest that CCL20 may serve as a novel therapeutic target and biomarker for brain metastasis of lung cancer.

SIRP α is expressed predominantly in myeloid and neuronal cells (Adams et al., 1998; Kharitononkov et al., 1997). Normal cells express CD47 that interacts with SIRP α on macrophage/microglia to suppress their phagocytic activity. Several studies have shown that the expression of SIRP α is also associated with polarization of microglia/macrophage (Pan et al., 2013). Furthermore, knockdown of SIRP α in macrophages increased the production of M1 cytokines in the tumor environment (Pan et al., 2013). Blocking SIRP α also triggered the Akt survival signal in macrophages and enhanced macrophage migratory activities toward tumors (Pan et al., 2013), suggesting that SIRP α also affects microglia's function. In this study, we found that the nicotine increased the expression of SIRP α on microglia, while nicotine also promoted the expression of CD47 on the tumor

cells, resulting in suppression of phagocytic ability of microglia. There are two possible mechanisms that are involved in the anti-tumor effect of microglia. One is direct internalization of tumor cells into the lysosome of microglia by phagocytosis. The other possibility is that tumor cells are first killed by cytotoxic factors released by microglia followed by phagocytosis of the apoptotic debris by microglia. In addition, it is noteworthy that the CD47-SIRP α signal was previously found to modulate T cell differentiation and regulate T cell priming to the tumor site (Liu et al., 2015b; Zhang and Petro, 1996). Therefore, the CD47-SIRP α signal that was activated by nicotine plays a pivotal role in modulating anti-tumor innate as well as adaptive immune responses in TME.

By screening a BBB permeable natural compound library, we identified PTL as a potent inhibitor for nicotine-induced M2 conversion of microglia. PTL is an abundant sesquiterpene lactone found in the medicinal herb Feverfew (*T. parthenium*). It has been used for centuries as a traditional medicine to treat arthritis, fever, and headache (Liu et al., 2015a; Rummel et al., 2011). PTL was also previously shown to exhibit direct tumor suppressive activity (Talib and Al Kury, 2018) by modulating multiple pathways, including p53, vascular endothelial growth factor (Talib and Al Kury, 2018), B-Raf/MAPK/Erk (Lin et al., 2017), and STAT3 pathways (Carlisi et al., 2011; Skoumal et al., 2011; Song et al., 2014). However, these direct tumor cytotoxic effects mediated by PTL require a relatively high dose, typically >10 μ M. In this study, we found that sub-micromolar dose (<1 μ M) of PTL effectively suppressed nicotine-induced polarization of microglia to M2 phenotype and enhanced its M1 phenotype. On the other hand, the low concentration (1 μ M) of PTL did not affect the population of CSC or tumor cell growth, which strongly suggests that the anti-tumor effect of PTL on nicotine-promoted brain metastasis shown in our study is mediated via modulation of microglial function and not by direct effect of PTL on tumor cells. Importantly, PTL was shown to be able to cross the BBB due to high membrane permeability (Könczöl et al., 2013). Considering the inhibitory effect of PTL on nicotine-induced microglial activation and their minimum toxicity at the effective dose, PTL could be used for the prevention and treatment of brain metastasis, particularly for patients with past and current smoking history.

Nicotine is highly addictive and considered as the major obstacle for smoking cessation (Henningfield et al., 1991). Unfortunately, many cancer patients have difficulty in quitting smoking even after their diagnosis due to nicotine addiction. E-cigarette, nicotine patch, and nicotine gum are nicotine replacement therapies for smoking cessation used by these patients (Hartmann-Boyce et al., 2018; Stead et al., 2012). Although nicotine, per se, is not a carcinogen, many reports document its tumor-promoting effects (Li et al., 2017; Schaal and Chellappan, 2014; Schaal et al., 2018) as well as its pro-inflammatory effect, which suppresses the phagocytosis ability of the alveolar macrophages (Scott et al., 2018). Considering our results that clearly demonstrated profound and long-term effects of nicotine on brain metastasis progression, extra caution should be paid in the use of nicotine for smoking cessation.

Materials and methods

Human subjects

We used a cohort of patients with a diagnosis of nonsmall cell lung cancer with or without brain metastases treated at Wake Forest Baptist Comprehensive Cancer Center between May 2015 and April 2018. We collected a total of 281 stage IV nonsmall cell lung cancer patients with or without a history of smoking. Among them, 79 patients were diagnosed with brain metastasis. 59 patients were current smokers at the time of diagnosis and treatment. We defined the “never smokers” ($n = 44$) as those who have no smoking history and those who had smoked <10 pack/year but stopped smoking. The “former smokers” ($n = 181$) are defined as those who smoked >10 pack/year but quit smoking in the past. Patient characteristics included histology status, smoking history, pack/years, progression status, and date of death. Patient information about age, gender, cancer type, and smoking status are described in Fig. S1. All samples used in this study were obtained under the approval of the institutional review boards of Wake Forest School of Medicine Institutional and Wake Forest Baptist Comprehensive Cancer Center. Written informed consent was obtained from all participants.

Cell culture and reagents

Human lung cancer cell lines, H2030BrM and PC9BrM, were kind gifts from J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). H2030BrM and PC9BrM cell lines were authenticated by using GenePrint 10 STR System (Promega, B9510). SIM-A9 was obtained from Kumi Nagamoto-Combs, through Kerafast.com. HMC3 and LL/2 cells were purchased from American Type Culture Collection (ATCC) and were authenticated by ATCC. The H2030BrM and PC9BrM were cultured in RPMI supplemented with 10% FBS, streptomycin (100 mg/ml), and penicillin (100 U/ml). LL/2 cells were cultured in DMEM medium supplemented with 10% FBS. SIM-A9 and HMC3 were cultured in DMEM/F12 medium supplemented with 5% FBS. All cells were grown at 37°C under 5% CO₂. All cell lines were obtained between 2010 and 2016, and they were ensured to be negative of mycoplasma. The HMC3 and SIM-A9 cells were seeded in a 10-cm dish. After reaching 70% confluence, cells were incubated with or without 1 μ M nicotine in DMEM/F12 medium supplemented with 2% FBS or in medium containing nicotine plus the STAT3 inhibitor STATTIC (0.5 μ M). After 24 h, cells were washed twice with PBS and then incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for 24 h. The CM harvested from the cell culture were centrifuged at 300 g for 10 min to remove the cells and stored at -80°C. To investigate the effect of nicotine-derived CM on tumor progression, we harvested CM from microglial cells that were treated with or without nicotine, followed by treatment of tumor cells with the CM. All cell lines were tested by using universal mycoplasma detection kit (ATCC, 30-1012k, lot 70008746).

Animal models

Animals were treated in accordance with the U.S. National Institutes of Health Animal Protection Guidelines, and the protocol was approved by the Wake Forest Baptist Health Institutional

Animal Care and Use Committee. Female nude, BALB/c, and C57BL/6 mice of 5–6 wk of age were used. The mice were housed (four to five per cage) at a stable temperature ($24 \pm 1^\circ\text{C}$), in a 12-h light/dark cycle, and with unrestricted access to food and water. Several studies showed that human lung adenocarcinomas cell H2030BrM and mouse Lewis lung carcinoma cell LL/2 exhibited their highly metastatic abilities in the nude, C57BL/6, and BALB/c mice, respectively (Chen et al., 2016; Janker et al., 2018; Kamei et al., 2009; Malladi et al., 2016; Nguyen et al., 2009; Scholar et al., 1989). Actively growing luciferase-labeled H2030BrM and LL/2 cells were intracardially injected at a concentration of 2×10^5 H2030BrM cells or 5×10^4 LL/2 cells in 100 μl PBS into the left cardiac ventricle. To confirm a successful injection, the photon flux from whole body of the mice was immediately measured using IVIS Xenogen bioimager (Caliper). To examine the effect of nicotine, the mice received nicotine (1 mg/kg) by intraperitoneal injection every 3 d. To examine the role of microglia on nicotine-related brain metastasis, the mice were administered PLX3397 (20 mg in 7 μl , Selleck Chemicals USA), a selective inhibitor of CSF1R (also known as macrophage colony-stimulating factor receptor), which reduces viability of microglial cells. To avoid whole-body effect, the PLX3397 was directly injected into the right mouse brain after 1 wk of transplantation of mouse lung cancer cell LL/2. Mice were divided into four groups: (1) tumor transplant only, (2) tumor transplant plus nicotine, (3) tumor transplant plus PLX3397, and (4) tumor transplant plus PLX3397 and nicotine. To examine the effect of natural compound (PTL) on modulation of microglial cell polarization, the mice were administered PTL (1 mg/kg) plus nicotine by intraperitoneal injection. Mice were divided into three groups: (1) tumor transplant only, (2) tumor transplant plus nicotine, and (3) tumor transplant plus PTL and nicotine. Tumor growth was monitored by bioluminescence until day 60. For bioluminescent imaging, the mice were injected with D-luciferin intraperitoneally (100 mg/kg), followed by capturing images every week using IVIS Xenogen bioimager. The brain metastasis was monitored, and the luminescence was quantified once per week. Finally, whole brain was removed and incubated in PBS with 0.6 mg/ml luciferin for 5 min, and photon flux was measured by an in vivo image system (IVIS).

Intracranial implantation

Mice were anesthetized with a ketamine/xylazine (90–120/7–10 mg/kg) mixture and fixed into a stereotactic head frame (Kopf stereotactic frame). A 1.5-mm bur hole was drilled 1 mm anterior to the coronal suture on the right hemisphere and 2 mm lateral from the midline. A Hamilton syringe fixed onto the head frame was used to inject LL/2 (2×10^5 cells/5 μl) into the right frontal lobe of the brain. The skin incision was then closed with 4–0 silk thread. The ketoprofen (2 mg/kg) was given to the mice to reduce pain. After 1 wk of tumor transplantation, 36 mice were randomly divided into four groups: (1) tumor transplant only (control group), (2) tumor transplant plus nicotine, (3) tumor transplant plus PLX3397, and (4) tumor transplant plus PLX3397 and nicotine. The PLX3397 and PLX3397 plus nicotine groups received PLX3397 (20 mg in 7 μl) treatment by intracranial injection every 2 wk at the same area of tumor transplantation. The control group received the same volume of PBS.

Arg1 gene promoter reporter assay and natural compound screening

To examine the nicotine-induced M2 microglial polarization and examine the effect of natural compounds on microglial polarization, SIM-A9 cells were first infected with lentivirus containing GFP gene, and GFP⁺ cells were sorted by flow cytometry (FACS). The GFP⁺ SIM-A9 cells were seeded in 96-well plates for 1 d and were transfected with the Arg1 promoter reporter plasmid (Addgene) using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were treated with only nicotine or nicotine plus natural compound in presence of 2% FBS and cultured for another 24 h. Cells were washed twice and were treated with D-luciferin for 5 min. The expression of luciferase was detected by using an IVIS Xenogen bioimager. For Arg1 promoter luciferase normalization, the photon flux was divided by GFP signal, which was measured by a Multi-Mode Reader (Biocompare). Each experiment was conducted a minimum of three times.

qRT-PCR

For qRT-PCR, the treated cells were washed with PBS and lysed using the TRIzol reagent. The isolated total RNA was quantified by a NanoDrop 2000 (Thermo Fisher Scientific). Then, RNA was reverse transcribed to cDNA using a Reverse Transcription Superscript kit (Bio-Rad). The cDNA was then amplified with a pair of forward and reverse primers for the following genes: hCD74-F 5'-GCGACCTTATCTCCAACAATG-3', hCD74-R 5'-GTCCTCCAGTTCCAGTGACT-3', hCD80-F 5'-CTCTTGGTGCTGGCTGGTCTTT-3', hCD80-R 5'-GCCAGTAGATGCGAGTTTGTGC-3', hCD86-F 5'-CCATCAGCTTGTCTGTTTCATTCC-3', hCD86-R 5'-GCTGTAATCCAAGGAATGTGGTC-3', hArginase-1-F 5'-GCGGGCGGACGC TGGCG-3', hArginase-1-R 5'-GCAGCGGGACCATGTCCACTC-3', hArginase-2-F 5'-AGACGCGAGTGCATTCCATC-3', hArginase-2-R 5'-CACTAATGGTACCGATTGCCA-3', hCD204-F 5'-GGTTTCAATTGTAAGAGAGAGAAG-3', hCD204-R 5'-CTGAGCAATTCTTCGTTTCCC-3', mCD74-F 5'-AGAGCCAGAAAGGTGCAGC-3', mCD74-R 5'-GATGCATCACATGGTCTCTGG-3', mCD80-F 5'-ACGTATTGGAAGGAGATTACAGCT-3', mCD80-R 5'-TCTGTACAGC GTTACTATCCCGC-3', mCD86-F 5'-TTACGGAAGCACCCACGATG-3', mCD86-R 5'-CCTGTTACATTCTGAGCCAGT-3', hCHR NA7-F 5'-CGTGGATGAGAAGAACCAAG-3', hCHR NA7-R 5'-CCATTGGGGATATAGCCACT-3', hSOX2-F 5'-GGGAAATGGGAG GGGTGCAAAAGAGG-3', hSOX2-R 5'-TTGCGTGAGTGTGGATGG GATTGGTG-3', hOCT4-F 5'-GCTGGAGAAGGAGAAGCTGGAGC-3', hOCT4-R 5'-GCTAAGCTGCAGAGCCTCAAAGC-3', hNanog-F 5'-CTTGGAAGCTGCTGGGGAAG-3', hNanog-R 5'-GATGGGAGG AGGGGAGAGGA-3', hCD47-F 5'-GCTAAAATATCGTGTGTTTTC ATGGTTTTC-3', hCD47-R 5'-GCTGTACTAAACACATAGTAGTG AAGT-3', hSIRP-F 5'-GCCTTAGTCGTTCCGCCGCG-3', hSIRP-R 5'-GCCGCTTGCCCTACTCCT-3', hIGF-1-F 5'-GCTGTGCCTGC TCACCTTCACCAG-3', hIGF-1-R 5'-GCCTCCTTAGATCACAGCTC CGGAAG-3', hCCL20-F 5'-TCTCGCTCCAGCATGAAAG-3', and hCCL20-R 5'-CAGATCTCCTTGGCCACAATG-3'.

PCR reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and CFX Connect (Bio-Rad). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of PCR using the following profile: 94°C , 30 s; 60°C , 30 s; and 72°C , 30 s.

Immunocytochemistry and immunofluorescence

The human brain metastasis tissues of lung cancer were obtained from Wake Forest Baptist Comprehensive Cancer Center Tumor Tissue and Pathology Shared Resource. Tissue sections were stained using anti-CD206 (1:200, R&D Systems) for M2 microglia and anti-ionized calcium-binding adapter molecule-1 (Iba1; 1:1,000, Abcam) for microglia and CD45 (1:500, Abcam) for macrophage. Brain sections were then incubated with appropriate biotin-conjugated secondary antibodies and diaminobenzidine as the substrate. As a control, isotype antibodies were used. To examine the expression of SIRP α on microglia, 10- μ m-thick frozen mouse brain sections were stained using rabbit anti-Iba1 (1:1,000; Abcam) for microglia and anti-SIRP α (1:200) (Cell Signaling). Brain sections were incubated for 1 h in a PBS solution containing 0.1% Triton X-100 and 3% bovine serum albumin at room temperature, and then transferred to a solution containing anti-SIRP α antibody overnight at 4°C. The secondary antibody conjugated with fluorescent dye Alexa Fluor 594 (1:1,000, Invitrogen) was used to detect the expression of SIRP α . Sections were then incubated overnight at 4°C in a solution containing anti-Iba1 antibodies. Antibodies against rabbit IgG conjugated with fluorescent dye Alexa Fluor 488 (1:1,000, Invitrogen) were used to detect the microglial cells. Fluorescence images were acquired using a microscope (BZ-X710, Keyence) connected to a computer equipped with imaging software (BZ-X Viewer). Appropriate isotype antibodies were used as controls. To determine the area of Iba1⁺ and CD206⁺ cells in brain metastasis, we chose three randomly selected fields in each tumor and measured staining intensity after taking images followed by using the Image-Pro software. We also chose three nontumor areas in the corresponding slide and measured intensity as background, and this background was subtracted from the intensity in the tumor area. The average intensity of three fields was used as the score of that patient. Therefore, each patient has a single score. We then divided the score range by setting the lowest score as 0 and the highest to be +3.

Western blot

Cells were homogenized in the radioimmunoprecipitation assay buffer and then centrifuged at 17,000 *g* for 30 min at 4°C. The protein concentrations of the supernatants were determined by NanoDrop 2000. Supernatants (30 μ g of total protein) were mixed with sample buffer containing 0.5 M dithiothreitol, heated to 80°C for 10 min, loaded into each well of 10% polyacrylamide gel, and resolved at 120 V for 2 h. The separated proteins bands were transferred to a polyvinylidene fluoride membrane, blocked with 5% milk, and probed with respective primary antibodies: total-STAT3 (1:1,000, Cell Signaling), phospho-STAT3 (1:1,000, Cell Signaling), GAPDH (1:10,000, Cell Signaling), SOX2 (1:500, Cell Signaling), OCT4 (1:1,000, Cell Signaling), NANOG (1:500, Cell Signaling), and SIRP α (1:1,000, Cell Signaling). The bound antibodies were detected using an enhanced chemiluminescence detection kit (GE Healthcare Lifescience). The band intensities were measured using an imaging system (Amersham Imager 600; UVP) and analyzed using ImageJ (1.43u; <http://rsb.info.nih.gov/ij/>). For gel loading control, membranes were incubated with monoclonal GAPDH antibody.

CCL20 ELISA

Mouse brain specimens were homogenized in radioimmuno-precipitation assay lysis buffer and centrifuged at 13,000 *g* for 30 min at 4°C. The protein concentrations of the supernatant were measured and adjusted to 1 mg/ml. The human serum of lung cancer patients was obtained from FOX CHASE Cancer Center. The serum was further diluted fourfold in assay buffer. The levels of CCL20 in the mouse brain supernatants and mouse and human serum were determined using mouse and human CCL20 ELISA kits (Sigma-Aldrich). Briefly, 96-well plates were first coated with anti-CCL20 monoclonal antibodies followed by addition of 100 μ l of the supernatants and serum under investigation or the same volume of the CCL20 standards. The plate was incubated at 4°C overnight on a rocking platform. After washing the plates, the detection antibody and streptavidin solution were added to each well. After incubation, the 3,3',5,5'-tetramethylbenzidine (TMB) and stop solution were added to each well. Finally, the plates were examined at an absorbance wavelength of 450 nm. Standard curves were obtained from values generated from known concentrations of mouse and human CCL20 provided by the kits.

IGF-1 ELISA

Microglial cells (HMC3) were treated with or without nicotine (1 μ M) plus STATTIC (0.5 μ M) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for an additional 24 h. The human monocyte/macrophage cells were treated with or without nicotine for 24 h. CM was collected and analyzed by the human IGF-1 ELISA kit (Sigma-Aldrich). Briefly, 96-well plates were first coated with anti-IGF-1 monoclonal antibodies followed by addition of 100 μ l of the microglia conditional medium or the same volume of the IGF-1 standards. The plate was incubated at 4°C overnight on a rocking platform. After washing the plates, the detection antibody and streptavidin solution were added to each well. After incubation, the TMB and stop solution were added to each well. Finally, the plates were examined at an absorbance wavelength of 450 nm. Standard curves were obtained from values generated from known concentrations of mouse and human IGF-1 provided by the kit.

Phagocytosis assay

H2030BrM and LL/2 cells were labeled with PKH26 dye (Sigma-Aldrich). The labeled cells were then washed three times and cultured overnight to reduce nonspecific leaking of dye during the assay. Tumor cells were 95% viable after the incubation. Labeled tumor cells were mixed with microglia that were pretreated with or without nicotine. Co-cultured tumor and microglia cells in the culture slides were harvested after 24 h and fixed with 4% paraformaldehyde. Microglial cells were counterstained with anti-Iba1 antibody overnight. Secondary antibody conjugated with the fluorescent dye Alexa Fluor 488 (1:1,000, Invitrogen) was used to detect the microglia. The phagocytic activities were measured by immunofluorescence microscope and FACS. For quantification of phagocytosis, the phagocytic percentage was calculated as 100 $\times$ (percent Iba1⁺/

PKH26⁺ cells/[percent Iba1⁺/PKH26⁺ cells + percent Iba1⁺/PKH26⁺ cells]).

Statistical analysis

Data are represented as mean $\pm$ SD. Significance was set at $P < 0.05$. The nicotine effect on M1/M2 gene expression, effect of nicotine-derived microglial CM on tumor growth, and effect of nicotine on microglia phagocytosis ability were analyzed using the unpaired Student's *t* test. One-way ANOVA was used to analyze the effect of nicotine, PTL3397, or STATTIC treatment on M1/M2 gene polarization and tumor progression, the effect of different concentrations of IGF-1 or CCL20 on cancer progression, the effect of different natural compounds on M2 gene polarization, and the effect of nicotine or PTL treatment on M1/M2 gene polarization and cancer cell progression and nicotine effect in animal studies. Significance between groups was represented as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Online supplemental material

Fig. S1 shows the effect of smoking and nicotine on brain metastasis. **Fig. S2** demonstrates that the nicotine promotes brain metastasis in a mouse model. **Fig. S3** shows that nicotine treatment of microglia increases secretion of CCL20 and IGF-1 followed by promotion of tumor cell stemness. **Fig. S4** shows the effect of natural compounds on nicotine-mediated activity of Arg1 reporter. **Fig. S5** shows PTL suppressed nicotine-promoted brain metastasis in syngeneic mouse model.

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Author contributions: S. Wu and K. Watabe designed the study and wrote the manuscript. S. Wu, F. Xing, and K. Wu conducted experiments and acquired, analyzed, and interpreted the data. S. Sharma, A. Tyagi, and K. Wu performed animal experiments. F. Xing and Y. Liu performed intracardiac injections. T. Lycan and A. Dothard performed clinical sample analysis. S. Wu, F. Xing, S. Sharma, A. Tyagi, K. Wu, Y. Liu, D. Zhao, R.P. Deshpande, Y. Shiozawa, T. Ahmed, W. Zhang, M. Chan, J. Ruiz, and K. Watabe reviewed and edited the manuscript and interpreted the data. K. Watabe supervised the study.

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References

- Adams, S., L.J. van der Laan, E. Vernon-Wilson, C. Renardel de Lavalette, E.A. Döpp, C.D. Dijkstra, D.L. Simmons, and T.K. van den Berg. 1998. Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. *J. Immunol.* 161:1853–1859.
- Ali, A., J.R. Goffin, A. Arnold, and P.M. Ellis. 2013. Survival of patients with non-small-cell lung cancer after a diagnosis of brain metastases. *Curr. Oncol.* 20:e300–e306. <https://doi.org/10.3747/co.20.1481>
- Bacha, S., H. Cherif, D. Rabaa, S. Habibech, S. Cheikhrouhou, H. Racil, N. Chaouch, M.L. Megdiche, and A. Chabbou. 2018. Brain metastases of non-small cell lung cancer: prognostic factors and management. *Tunis. Med.* 96:165–171.
- Badie, B., and J.M. Scharfner. 2000. Flow cytometric characterization of tumor-associated macrophages in experimental gliomas. *Neurosurgery*. 46: 957–961. <https://doi.org/10.1097/00006123-200004000-00035>
- Barnholtz-Sloan, J.S., A.E. Sloan, F.G. Davis, F.D. Vigneau, P. Lai, and R.E. Sawaya. 2004. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Detroit Cancer Surveillance System. *J. Clin. Oncol.* 22:2865–2872. <https://doi.org/10.1200/JCO.2004.12.149>
- Braverman, M.T.. 1999. Research on resilience and its implications for tobacco prevention. *Nicotine Tob. Res.* 1(Suppl 1):S67–S72. <https://doi.org/10.1080/14622299050011621>
- Butowski, N., H. Colman, J.F. De Groot, A.M. Omuro, L. Nayak, P.Y. Wen, T.F. Cloughesy, A. Marimuthu, S. Haidar, A. Perry, et al. 2016. Orally administered colony stimulating factor 1 receptor inhibitor PLX3397 in recurrent glioblastoma: an Ivy Foundation Early Phase Clinical Trials Consortium phase II study. *Neuro-oncol.* 18:557–564. <https://doi.org/10.1093/neuonc/nov245>
- Cannarile, M.A., M. Weisser, W. Jacob, A.M. Jegg, C.H. Ries, and D. Rüttinger. 2017. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J. Immunother. Cancer.* 5:53. <https://doi.org/10.1186/s40425-017-0257-y>
- Carlisi, D., A. D'Anne, L. Angileri, M. Lauricella, S. Emanuele, A. Santulli, R. Vento, and G. Tesoriere. 2011. Parthenolide sensitizes hepatocellular carcinoma cells to TRAIL by inducing the expression of death receptors through inhibition of STAT3 activation. *J. Cell. Physiol.* 226:1632–1641. <https://doi.org/10.1002/jcp.22494>
- Chen, Q., A. Boire, X. Jin, M. Valiente, E.E. Er, A. Lopez-Soto, L. Jacob, R. Patwa, H. Shah, K. Xu, et al. 2016. Carcinoma-astrocyte gap junctions promote brain metastasis by cGAMP transfer. *Nature.* 533:493–498. <https://doi.org/10.1038/nature18268>
- Chen, W., Y. Qin, D. Wang, L. Zhou, Y. Liu, S. Chen, L. Yin, Y. Xiao, X.H. Yao, X. Yang, et al. 2018. CCL20 triggered by chemotherapy hinders the therapeutic efficacy of breast cancer. *PLoS Biol.* 16. e2005869. <https://doi.org/10.1371/journal.pbio.2005869>
- Cuccarese, M.F., J.M. Dubach, C. Pfirsche, C. Engblom, C. Garriss, M.A. Miller, M.J. Pittet, and R. Weissleder. 2017. Heterogeneity of macrophage infiltration and therapeutic response in lung carcinoma revealed by 3D organ imaging. *Nat. Commun.* 8:14293. <https://doi.org/10.1038/ncomms14293>
- da Fonseca, A.C., and B. Badie. 2013. Microglia and macrophages in malignant gliomas: recent discoveries and implications for promising therapies. *Clin. Dev. Immunol.* 2013. 264124. <https://doi.org/10.1155/2013/264124>
- Ellis, T.L., M.T. Neal, and M.D. Chan. 2012. The role of surgery, radiosurgery and whole brain radiation therapy in the management of patients with metastatic brain tumors. *Int. J. Surg. Oncol.* 2012. 952345. <https://doi.org/10.1155/2012/952345>
- Elmore, M.R., A.R. Najafi, M.A. Koike, N.N. Dagher, E.E. Spangenberg, R.A. Rice, M. Kitazawa, B. Matusow, H. Nguyen, B.L. West, et al. 2014. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron.* 82:380–397. <https://doi.org/10.1016/j.neuron.2014.02.040>
- Feng, X., F. Szulzewsky, A. Yerevanian, Z. Chen, D. Heinzmann, R.D. Rasmussen, V. Alvarez-Garcia, Y. Kim, B. Wang, I. Tamagno, et al. 2015. Loss of CX3CR1 increases accumulation of inflammatory monocytes and promotes gliomagenesis. *Oncotarget.* 6:15077–15094. <https://doi.org/10.18632/oncotarget.3730>

- Fidler, I.J.. 2015. The Biology of Brain Metastasis: Challenges for Therapy. *Cancer J.* 21:284–293. <https://doi.org/10.1097/PPO.0000000000000126>
- Gaspar, L., C. Scott, M. Rotman, S. Asbell, T. Phillips, T. Wasserman, W.G. McKenna, and R. Byhardt. 1997. Recursive partitioning analysis (RPA) of prognostic factors in three Radiation Therapy Oncology Group (RTOG) brain metastases trials. *Int. J. Radiat. Oncol. Biol. Phys.* 37: 745–751. [https://doi.org/10.1016/S0360-3016\(96\)00619-0](https://doi.org/10.1016/S0360-3016(96)00619-0)
- Gomez Perdiguero, E., K. Klapproth, C. Schulz, K. Busch, E. Azzoni, L. Crozet, H. Garner, C. Trouillet, M.F. de Bruijn, F. Geissmann, et al. 2015. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature.* 518:547–551. <https://doi.org/10.1038/nature13989>
- Hartmann-Boyce, J., S.C. Chepkin, W. Ye, C. Bullen, and T. Lancaster. 2018. Nicotine replacement therapy versus control for smoking cessation. *Cochrane Database Syst. Rev.* 5. CD000146. <https://doi.org/10.1002/14651858.CD000146.pub5>
- Hawkins, B.T., T.J. Abbruscato, R.D. Egleton, R.C. Brown, J.D. Huber, C.R. Campos, and T.P. Davis. 2004. Nicotine increases in vivo blood-brain barrier permeability and alters cerebral microvascular tight junction protein distribution. *Brain Res.* 1027:48–58. <https://doi.org/10.1016/j.brainres.2004.08.043>
- He, B.P., J.J. Wang, X. Zhang, Y. Wu, M. Wang, B.H. Bay, and A.Y. Chang. 2006. Differential reactions of microglia to brain metastasis of lung cancer. *Mol. Med.* 12:161–170. <https://doi.org/10.2119/2006-00033.He>
- Heishman, S.J.. 1999. Behavioral and cognitive effects of smoking: relationship to nicotine addiction. *Nicotine Tob. Res.* 1:S143–S147, NaN–S166. <https://doi.org/10.1080/14622299050011971>
- Henningfield, J.E., C. Cohen, and J.D. Slade. 1991. Is nicotine more addictive than cocaine? *Br. J. Addict.* 86:565–569. <https://doi.org/10.1111/j.1360-0443.1991.tb01809.x>
- Huang, Z., B. Sun, S. Wu, X. Meng, Y. Cong, G. Shen, and S. Song. 2018. A nomogram for predicting survival in patients with breast cancer brain metastasis. *Oncol. Lett.* 15:7090–7096. <https://doi.org/10.3892/ol.2018.8259>
- Hutter, G., J. Theruvath, C.M. Graef, M. Zhang, M.K. Schoen, E.M. Manz, M.L. Bennett, A. Olson, T.D. Azad, R. Sinha, et al. 2019. Microglia are effector cells of CD47-SIRPα antiphagocytic axis disruption against glioblastoma. *Proc. Natl. Acad. Sci. USA.* 116:997–1006. <https://doi.org/10.1073/pnas.1721434116>
- Janker, F., W. Weder, J.H. Jang, and W. Jungraithmayr. 2018. Preclinical, non-genetic models of lung adenocarcinoma: a comparative survey. *Oncotarget.* 9:30527–30538. <https://doi.org/10.18632/oncotarget.25668>
- Kakusa, B., S. Han, S. Aggarwal, B. Liu, G. Li, S. Soltys, and M. Hayden Gephart. 2018. Clinical factors associated with mortality within three months after radiosurgery of asymptomatic brain metastases from non-small cell lung cancer. *J. Neurooncol.* 140:705–715. <https://doi.org/10.1007/s11060-018-03002-0>
- Kamei, D., M. Murakami, Y. Sasaki, Y. Nakatani, M. Majima, Y. Ishikawa, T. Ishii, S. Uematsu, S. Akira, S. Hara, et al. 2009. Microsomal prostaglandin G synthase-1 in both cancer cells and hosts contributes to tumour growth, invasion and metastasis. *Biochem. J.* 425:361–371. <https://doi.org/10.1042/BJ20090045>
- Kharitonov, A., Z. Chen, I. Sures, H. Wang, J. Schilling, and A. Ullrich. 1997. A family of proteins that inhibit signalling through tyrosine kinase receptors. *Nature.* 386:181–186. <https://doi.org/10.1038/386181a0>
- Könczöl, A., J. Müller, E. Földes, Z. Béni, K. Végh, A. Kéry, and G.T. Balogh. 2013. Applicability of a blood-brain barrier specific artificial membrane permeability assay at the early stage of natural product-based CNS drug discovery. *J. Nat. Prod.* 76:655–663. <https://doi.org/10.1021/np300882f>
- Lee, P.N., B.A. Forey, and K.J. Coombs. 2012. Systematic review with meta-analysis of the epidemiological evidence in the 1900s relating smoking to lung cancer. *BMC Cancer.* 12:385. <https://doi.org/10.1186/1471-2407-12-385>
- Leskovaar, A., L.J. Moriarty, J.J. Turek, I.A. Schoenlein, and R.B. Borgens. 2000. The macrophage in acute neural injury: changes in cell numbers over time and levels of cytokine production in mammalian central and peripheral nervous systems. *J. Exp. Biol.* 203(Pt 12):1783–1795.
- Li, T., J. Zhang, J. Zhang, N. Zhang, Y. Zeng, S. Tang, Z. Tao, X. Qu, J. Jia, W. Zhu, et al. 2017. Nicotine-enhanced stemness and epithelial-mesenchymal transition of human umbilical cord mesenchymal stem cells promote tumor formation and growth in nude mice. *Oncotarget.* 9: 591–606. <https://doi.org/10.18632/oncotarget.22712>
- Lin, M., H. Bi, Y. Yan, W. Huang, G. Zhang, G. Zhang, S. Tang, Y. Liu, L. Zhang, J. Ma, et al. 2017. Parthenolide suppresses non-small cell lung cancer GLC-82 cells growth via B-Raf/MAPK/Erk pathway. *Oncotarget.* 8:23436–23447. <https://doi.org/10.18632/oncotarget.15584>
- Liu, Q., J. Zhao, R. Tan, H. Zhou, Z. Lin, M. Zheng, E. Romas, J. Xu, and N.A. Sims. 2015a. Parthenolide inhibits pro-inflammatory cytokine production and exhibits protective effects on progression of collagen-induced arthritis in a rat model. *Scand. J. Rheumatol.* 44:182–191. <https://doi.org/10.3109/03009742.2014.938113>
- Liu, X., Y. Pu, K. Cron, L. Deng, J. Kline, W.A. Frazier, H. Xu, H. Peng, Y.X. Fu, and M.M. Xu. 2015b. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat. Med.* 21:1209–1215. <https://doi.org/10.1038/nm.3931>
- Liu, B., Y. Jia, J. Ma, S. Wu, H. Jiang, Y. Cao, X. Sun, X. Yin, S. Yan, M. Shang, et al. 2016. Tumor-associated macrophage-derived CCL20 enhances the growth and metastasis of pancreatic cancer. *Acta Biochim. Biophys. Sin. (Shanghai).* 48:1067–1074. <https://doi.org/10.1093/abbs/gmw101>
- Liu, M., C. Xiao, M. Sun, M. Tan, L. Hu, and Q. Yu. 2018. Parthenolide Inhibits STAT3 Signaling by Covalently Targeting Janus Kinases. *Molecules.* 23. E1478. <https://doi.org/10.3390/molecules23061478>
- Lu, E., J. Su, Y. Zhou, C. Zhang, and Y. Wang. 2017. CCL20/CCR6 promotes cell proliferation and metastasis in laryngeal cancer by activating p38 pathway. *Biomed. Pharmacother.* 85:486–492. <https://doi.org/10.1016/j.biopha.2016.11.055>
- Malladi, S., D.G. Macalino, X. Jin, L. He, H. Basnet, Y. Zou, E. de Stanchina, and J. Massagué. 2016. Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell.* 165:45–60. <https://doi.org/10.1016/j.cell.2016.02.025>
- Marsigliante, S., C. Vetrugno, and A. Muscella. 2016. Paracrine CCL20 loop induces epithelial-mesenchymal transition in breast epithelial cells. *Mol. Carcinog.* 55:1175–1186. <https://doi.org/10.1002/mc.22360>
- Mazzaferro, S., I. Bermudez, and S.M. Sine. 2017. αβ2 Nicotinic Acetylcholine Receptors: Relationships Between Subunit Stoichiometry and Function at the Single Channel Level. *J. Biol. Chem.* 292:2729–2740. <https://doi.org/10.1074/jbc.M116.764183>
- Morioka, N., S. Harano, M. Tokuhara, Y. Idenoshita, F.F. Zhang, K. Hisaoka-Nakashima, and Y. Nakata. 2015. Stimulation of α7 nicotinic acetylcholine receptor regulates glutamate transporter GLAST via basic fibroblast growth factor production in cultured cortical microglia. *Brain Res.* 1625:111–120. <https://doi.org/10.1016/j.brainres.2015.08.029>
- Mujoomdar, A., J.H. Austin, R. Malhotra, C.A. Powell, G.D. Pearson, M.C. Shiau, and H. Raftopoulos. 2007. Clinical predictors of metastatic disease to the brain from non-small cell lung carcinoma: primary tumor size, cell type, and lymph node metastases. *Radiology.* 242:882–888. <https://doi.org/10.1148/radiol.2423051707>
- Munn, D.H., and N.K. Cheung. 1990. Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony-stimulating factor. *J. Exp. Med.* 172:231–237. <https://doi.org/10.1084/jem.172.1.231>
- Nandi, B., C. Pai, Q. Huang, R.H. Prabhala, N.C. Munshi, and J.S. Gold. 2014. CCR6, the sole receptor for the chemokine CCL20, promotes spontaneous intestinal tumorigenesis. *PLoS One.* 9. e97566. <https://doi.org/10.1371/journal.pone.0097566>
- Nguyen, D.X., A.C. Chiang, X.H. Zhang, J.Y. Kim, M.G. Kris, M. Ladanyi, W.L. Gerald, and J. Massagué. 2009. WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. *Cell.* 138:51–62. <https://doi.org/10.1016/j.cell.2009.04.030>
- Pan, Y.F., Y.X. Tan, M. Wang, J. Zhang, B. Zhang, C. Yang, Z.W. Ding, L.W. Dong, and H.Y. Wang. 2013. Signal regulatory protein α is associated with tumor-polarized macrophages phenotype switch and plays a pivotal role in tumor progression. *Hepatology.* 58:680–691. <https://doi.org/10.1002/hep.26391>
- Park, Y.S., D.J. Kim, H. Koo, S.H. Jang, Y.M. You, J.H. Cho, S.J. Yang, E.S. Yu, Y. Jung, D.C. Lee, et al. 2016. AKT-induced PKM2 phosphorylation signals for IGF-1-stimulated cancer cell growth. *Oncotarget.* 7: 48155–48167. <https://doi.org/10.18632/oncotarget.10179>
- Prinz, M., and J. Priller. 2014. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat. Rev. Neurosci.* 15:300–312. <https://doi.org/10.1038/nrn3722>
- Qin, C., W.H. Fan, Q. Liu, K. Shang, M. Murugan, L.J. Wu, W. Wang, and D.S. Tian. 2017. Fingolimod Protects Against Ischemic White Matter Damage by Modulating Microglia Toward M2 Polarization via STAT3 Pathway. *Stroke.* 48:3336–3346. <https://doi.org/10.1161/STROKEAHA.117.018505>
- Redmer, T. 2018. Deciphering mechanisms of brain metastasis in melanoma - the gist of the matter. *Mol. Cancer.* 17:106. <https://doi.org/10.1186/s12943-018-0854-5>
- Roesch, S., C. Rapp, S. Dettling, and C. Herold-Mende. 2018. When Immune Cells Turn Bad-Tumor-Associated Microglia/Macrophages in Glioma. *Int. J. Mol. Sci.* 19. E436. <https://doi.org/10.3390/ijms19020436>

- Rummel, C., R. Gerstberger, J. Roth, and T. Hübschle. 2011. Parthenolide attenuates LPS-induced fever, circulating cytokines and markers of brain inflammation in rats. *Cytokine*. 56:739–748. <https://doi.org/10.1016/j.cyto.2011.09.022>
- Sadigh-Eteghad, S., A. Majidi, J. Mahmoudi, S.E.J. Goltari, and M. Talebi. 2016. Astrocytic and microglial nicotinic acetylcholine receptors: an overlooked issue in Alzheimer's disease. *J. Neural Transm. (Vienna)*. 123: 1359–1367. <https://doi.org/10.1007/s00702-016-1580-z>
- Schaal, C., and S.P. Chellappan. 2014. Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers. *Mol. Cancer Res.* 12: 14–23. <https://doi.org/10.1158/1541-7786.MCR-13-0541>
- Schaal, C.M., N. Bora-Singhal, D.M. Kumar, and S.P. Chellappan. 2018. Regulation of Sox2 and stemness by nicotine and electronic-cigarettes in non-small cell lung cancer. *Mol. Cancer*. 17:149. <https://doi.org/10.1186/s12943-018-0901-2>
- Scholar, E.M., L.A. Violi, J. Newland, E. Bresnick, and D.F. Birt. 1989. The effect of dietary fat on metastasis of the Lewis lung carcinoma and the BALB/c mammary carcinoma. *Nutr. Cancer*. 12:109–119. <https://doi.org/10.1080/01635588909514009>
- Schouten, L.J., J. Rutten, H.A. Huveneers, and A. Twijnstra. 2002. Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma. *Cancer*. 94:2698–2705. <https://doi.org/10.1002/cncr.10541>
- Schulz, C., E. Gomez Perdiguer, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S.E. Jacobsen, J.W. Pollard, et al. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. 336:86–90. <https://doi.org/10.1126/science.1219179>
- Scott, A., S.T. Lugg, K. Aldridge, K.E. Lewis, A. Bowden, R.Y. Mahida, F.S. Grudzinska, D. Dosanjh, D. Parekh, R. Foronjy, et al. 2018. Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages. *Thorax*. 73(12):1161–1169. <https://doi.org/10.1136/thoraxjnl-2018-211663>
- Shenker, R.F., E.R. McTyre, J. Ruiz, K.E. Weaver, C. Cramer, N.K. Alphonse-Sullivan, M. Farris, W.J. Petty, M.R. Bonomi, K. Watabe, et al. 2017. The Effects of smoking status and smoking history on patients with brain metastases from lung cancer. *Cancer Med.* 6:944–952. <https://doi.org/10.1002/cam4.1058>
- Skoumal, R., M. Tóth, R. Serpi, J. Rysä, H. Leskinen, J. Ulvila, T. Saiho, J. Aro, H. Ruskoaho, I. Szokodi, et al. 2011. Parthenolide inhibits STAT3 signaling and attenuates angiotensin II-induced left ventricular hypertrophy via modulation of fibroblast activity. *J. Mol. Cell. Cardiol.* 50: 634–641. <https://doi.org/10.1016/j.yjmcc.2011.01.001>
- Sobota, R., M. Szwed, A. Kasza, M. Bugno, and T. Kordula. 2000. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. *Biochem. Biophys. Res. Commun.* 267:329–333. <https://doi.org/10.1006/bbrc.1999.1948>
- Song, J.M., X. Qian, P. Upadhyaya, K.H. Hong, and F. Kassie. 2014. Dimethylaminoparthenolide, a water soluble parthenolide, suppresses lung tumorigenesis through down-regulating the STAT3 signaling pathway. *Curr. Cancer Drug Targets*. 14:59–69. <https://doi.org/10.2174/15680096113136660108>
- Sperduto, P.W., B. Berkey, L.E. Gaspar, M. Mehta, and W. Curran. 2008. A new prognostic index and comparison to three other indices for patients with brain metastases: an analysis of 1,960 patients in the RTOG database. *Int. J. Radiat. Oncol. Biol. Phys.* 70:510–514. <https://doi.org/10.1016/j.ijrobp.2007.06.074>
- Stead, L.F., R. Perera, C. Bullen, D. Mant, J. Hartmann-Boyce, K. Cahill, and T. Lancaster. 2012. Nicotine replacement therapy for smoking cessation. *Cochrane Database Syst. Rev.* 11. CD000146. <https://doi.org/10.1002/14651858.CD000146.pub4>
- Sun, H.J., Y.F. Jia, and X.L. Ma. 2017. Alpha5 Nicotinic Acetylcholine Receptor Contributes to Nicotine-Induced Lung Cancer Development and Progression. *Front. Pharmacol.* 8:573. <https://doi.org/10.3389/fphar.2017.00573>
- Talib, W.H., and L.T. Al Kury. 2018. Parthenolide inhibits tumor-promoting effects of nicotine in lung cancer by inducing P53 - dependent apoptosis and inhibiting VEGF expression. *Biomed. Pharmacother.* 107:1488–1495. <https://doi.org/10.1016/j.biopha.2018.08.139>
- Vergier, E., M. Gil, R. Yaya, N. Viñolas, S. Villà, T. Pujol, L. Quintó, and F. Graus. 2005. Temozolomide and concomitant whole brain radiotherapy in patients with brain metastases: a phase II randomized trial. *Int. J. Radiat. Oncol. Biol. Phys.* 61:185–191. <https://doi.org/10.1016/j.ijrobp.2004.04.061>
- Wang, G.Z., X. Cheng, X.C. Li, Y.Q. Liu, X.Q. Wang, X. Shi, Z.Y. Wang, Y.Q. Guo, Z.S. Wen, Y.C. Huang, et al. 2015. Tobacco smoke induces production of chemokine CCL20 to promote lung cancer. *Cancer Lett.* 363: 60–70. <https://doi.org/10.1016/j.canlet.2015.04.005>
- Wang, B., L. Shi, X. Sun, L. Wang, X. Wang, and C. Chen. 2016. Production of CCL20 from lung cancer cells induces the cell migration and proliferation through PI3K pathway. *J. Cell. Mol. Med.* 20:920–929. <https://doi.org/10.1111/jcmm.12781>
- Wang, C., W. Gu, Y. Zhang, Y. Ji, Y. Wen, and X. Xu. 2017. Nicotine promotes cervical carcinoma cell line HeLa migration and invasion by activating PI3k/Akt/NF-κB pathway in vitro. *Exp. Toxicol. Pathol.* 69:402–407. <https://doi.org/10.1016/j.etp.2017.03.006>
- Wei, J., K. Gabrusiewicz, and A. Heimberger. 2013. The controversial role of microglia in malignant gliomas. *Clin. Dev. Immunol.* 2013. 285246. <https://doi.org/10.1155/2013/285246>
- Wu, S.Y., and K. Watabe. 2017. The roles of microglia/macrophages in tumor progression of brain cancer and metastatic disease. *Front. Biosci.* 22: 1805–1829. <https://doi.org/10.2741/4573>
- Xing, F., Y. Liu, S.Y. Wu, K. Wu, S. Sharma, Y.Y. Mo, J. Feng, S. Sanders, G. Jin, R. Singh, et al. 2018. Loss of XIST in Breast Cancer Activates MSN-c-Met and Reprograms Microglia via Exosomal miRNA to Promote Brain Metastasis. *Cancer Res.* 78:4316–4330. <https://doi.org/10.1158/0008-5472.CAN-18-1102>
- Yi, W.J., and T.S. Kim. 2017. Melatonin protects mice against stress-induced inflammation through enhancement of M2 macrophage polarization. *Int. Immunopharmacol.* 48:146–158. <https://doi.org/10.1016/j.intimp.2017.05.006>
- Yoneyama, R., K. Aoshiba, K. Furukawa, M. Saito, H. Kataba, H. Nakamura, and N. Ikeda. 2016. Nicotine enhances hepatocyte growth factor-mediated lung cancer cell migration by activating the α7 nicotine acetylcholine receptor and phosphoinositide kinase-3-dependent pathway. *Oncol. Lett.* 11:673–677. <https://doi.org/10.3892/ol.2015.3930>
- Zhang, S., and T.M. Petro. 1996. The effect of nicotine on murine CD4 T cell responses. *Int. J. Immunopharmacol.* 18:467–478. [https://doi.org/10.1016/S0192-0561\(96\)00054-9](https://doi.org/10.1016/S0192-0561(96)00054-9)
- Zhu, C., X. Qi, Y. Chen, B. Sun, Y. Dai, and Y. Gu. 2011. PI3K/Akt and MAPK/ERK1/2 signaling pathways are involved in IGF-1-induced VEGF-C up-regulation in breast cancer. *J. Cancer Res. Clin. Oncol.* 137:1587–1594. <https://doi.org/10.1007/s00432-011-1049-2>
- Zhu, Y., B.L. Knolhoff, M.A. Meyer, T.M. Nywening, B.L. West, J. Luo, A. Wang-Gillam, S.P. Goedegebuure, D.C. Linehan, and D.G. DeNardo. 2014. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* 74:5057–5069. <https://doi.org/10.1158/0008-5472.CAN-13-3723>

Supplemental material

	Adenocarcinoma	Non-adenocarcinoma
Age	62.78	65.21
Male gender (%)	94 (33%)	60 (21%)
Smoking status		
-Never/Former	149	73
-Current	43	16
Pack years		
<20	81	23
20-60	88	38
>60	23	28

Figure S1. **Effect of smoking and nicotine on brain metastasis.** Patient profile and their smoking history.

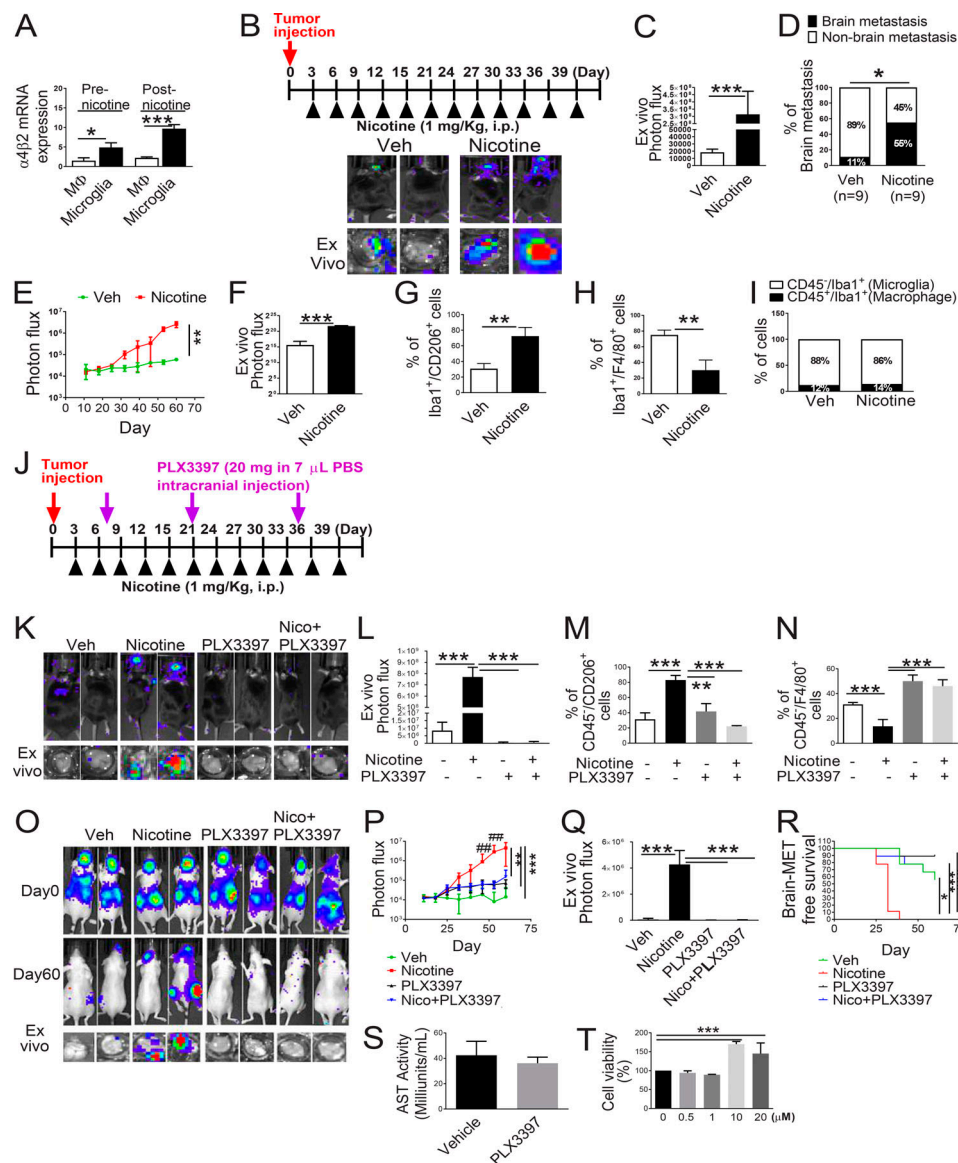


Figure S2. Nicotine promotes brain metastasis in mouse model. (A) Expression of the $\alpha 4 \beta 2$ nicotine receptor on human microglia (HMC3) and human monocyte/macrophage (THP-1) were evaluated before and after nicotine treatment by qRT-PCR ($n = 4$ /group). (B) Mouse lung cancer LL/2 cells were intracardially injected into wild-type C57BL/6 mice ($n = 9$ /group). After 3 d of intracardiac transplantation of LL/2 cells, mice received nicotine (1 mg/kg) treatment by intraperitoneal injection every 3 d. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. The lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 40). (C) Quantitative data of in vivo brain metastasis of lung cancer ($n = 9$ /group). (D) Percentage of brain metastasis of lung cancer in C57BL/6 mice with or without nicotine treatment ($n = 9$ /group). (E and F) Quantitative data of in vivo brain metastasis of lung cancer (left panel) and ex vivo signals in the brain at the end point (right panel) of the experiment conducted in Fig. 2 B ($n = 9$ /group). (G and H) Metastatic brain tumor tissues were dissociated, and the effect of nicotine was examined on Iba1⁺/CD206⁺ (G) or Iba1⁺/F4/80⁺ cells (H) by FACS ($n = 9$ /group). (I) Metastatic brain tumor tissues in Fig. 2 B were dissociated, and the Iba1⁺/CD45⁻ (microglia) and Iba1⁺/CD45⁺ (macrophage) were measured and plotted in relation to the nicotine treatment ($n = 9$ /group). (J) Scheme of the experimental design. After 3 d of intracranial transplantation of LL/2 cells, the mice received nicotine (1 mg/kg) by intraperitoneal injection every 3 d. After 1 wk of tumor transplantation, PLX3397 (20 mg/kg in 7 μ L PBS) was directly injected into the right brain of the mice every 2 wk. (K) Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. The lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 40; $n = 9$ /group). (L) Quantitative data of ex vivo brain metastasis of lung cancer at the end point. (M and N) Metastatic brain tumors in K were isolated from the brain and examined by FACS for M2 (M) and M1 (N) microglial polarization ($n = 9$ /group). (O) H2030BrM (2×10^5 cells) were intracardially injected into nude mice ($n = 9$ /group) followed by administering them with nicotine plus PLX3397 (1 mg/kg) by intraperitoneal injection every 3 d. Upper and middle panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 0 and day 60, respectively. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the endpoint (day 60; $n = 9$ /group). (P and Q) Quantitative data of in vivo brain metastasis of lung cancer and (Q) ex vivo signals in the brain at the end point (day 60; $n = 9$ /group). (R) Kaplan-Meier analysis of brain metastasis-free survival was performed. (S) Serum of mice in Fig. 2 E was collected, and an aspartate aminotransferase test (AST) was measured using the AST activity assay kit (Sigma-Aldrich) ($n = 9$ /group). (T) H2030BrM cells were incubated with the indicated concentration of nicotine for 24 h. They were then examined for cell viability ($n = 9$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus respective nicotine group. ##, $P < 0.01$ versus respective PLX3397 and Nico+PLX3397 groups.

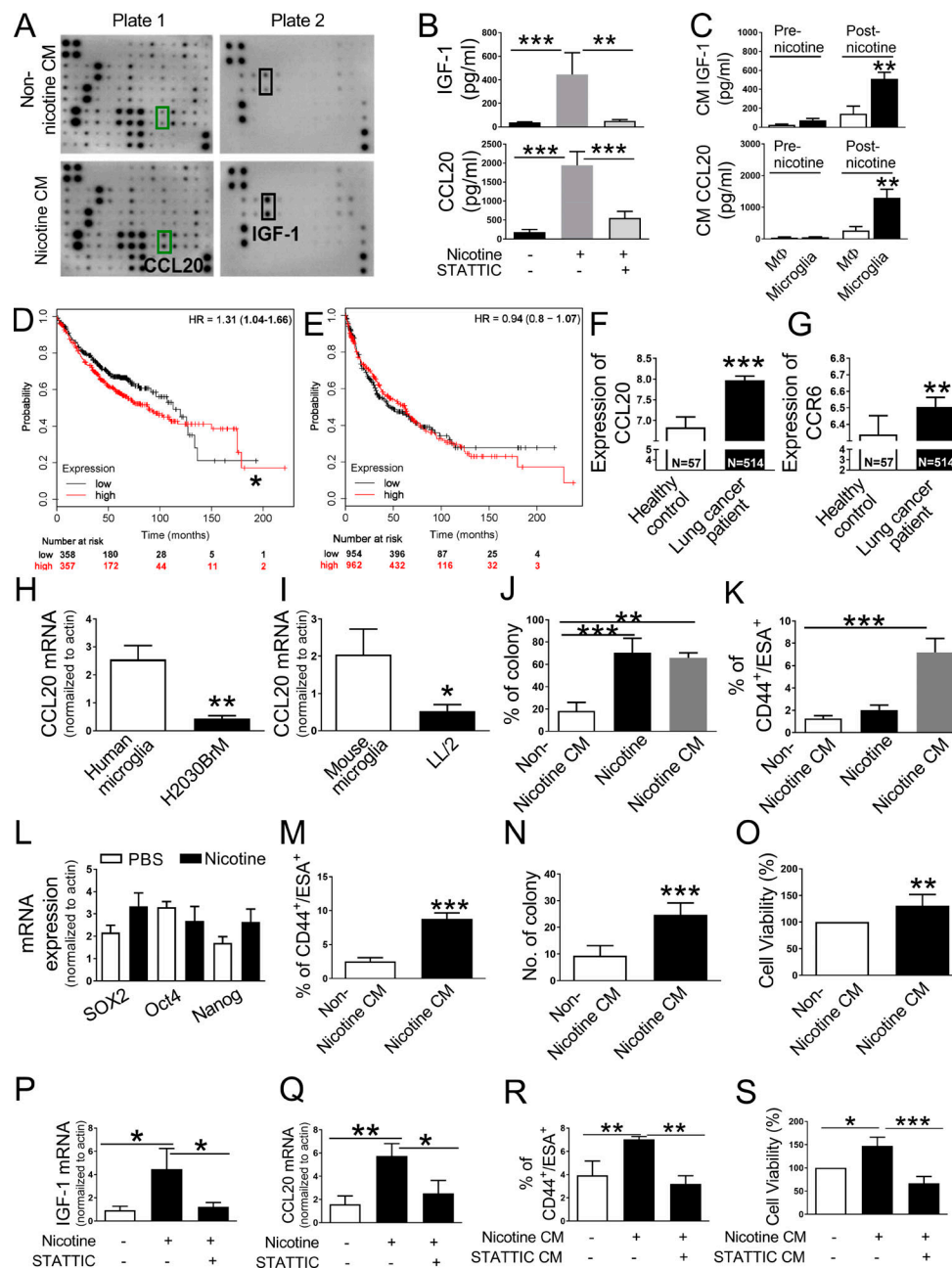


Figure S3. Nicotine treatment of microglia increases secretion of CCL20 and IGF-1 followed by promotion of tumor cell stemness. (A) Human microglial (HMC3) cells were treated with PBS or nicotine (1 μM) for 24 h. CM was collected and analyzed by using the cytokine/growth factor array (Raybio). (B) Human microglial (HMC3) cells were treated with or without nicotine (1 μM) plus STAT3C (0.5 μM) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for an additional 24 h. CM was collected and analyzed by IGF-1 and CCL20 ELISA ($n = 4$ /group). (C) Human microglial (HMC3) cells and macrophage (THP-1) were treated with or without nicotine (1 μM) for 24 h. Cells were washed with PBS twice and incubated in the fresh DMEM/F12 medium supplemented with 2% FBS for an additional 24 h. CM was collected and analyzed by IGF-1 and CCL20 ELISA ($n = 4$ /group). (D and E) Kaplan-Meier analysis was performed for examining relapse-free survival of lung cancer patients in relation to CCL20 ($P = 0.02$; D) and IGF-1 ($P = 0.36$; E) expression using the TCGA dataset. (F and G) Expressions of CCL20 (F) and CCR6 (G) were examined in lung tumor tissues from healthy individual and lung cancer patients using the GEO dataset (GSE22220). (H and I) Expression of CCL20 in human microglia and tumor cells (H) or mouse microglia and mouse tumor cells (I) were examined by qRT-PCR ($n = 4$ /group). (J-L) H2030BrM cells were treated with or without nicotine or CM extracted from human microglia that were treated with nicotine for 24 h. These cells were examined for their colony-forming ability (J), CD44⁺/ESA⁺ CSC population (K), and mRNA expression of stemness-related genes (L; $n = 4$ /group). (M) Effect of CM extracted from nicotine-treated mouse microglial cells on LL/2 mouse lung CSC population was examined by FACS. (N) Colony-forming ability of LL/2 was measured in the presence or absence of the CM derived from nicotine-treated microglia by qRT-PCR ($n = 4$ /group). (O) LL/2 cells were incubated with the CM derived from nicotine-treated microglia for 24 h. They were then examined for cell viability ($n = 4$ /group). (P and Q) Mouse microglial cells were treated with or without nicotine in the presence or absence of STAT3 inhibitor for 24 h. Cells were then examined for the expression of IGF-1 (P) or CCL20 (Q) by qRT-PCR. LL/2 cells were treated with CM derived from microglia that were treated with or without nicotine and/or STAT3C for 24 h ($n = 4$ /group). (R and S) The cells were then examined for CD44⁺/ESA⁺ CSC population (R) and for cell viability (S; $n = 4$ /group). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

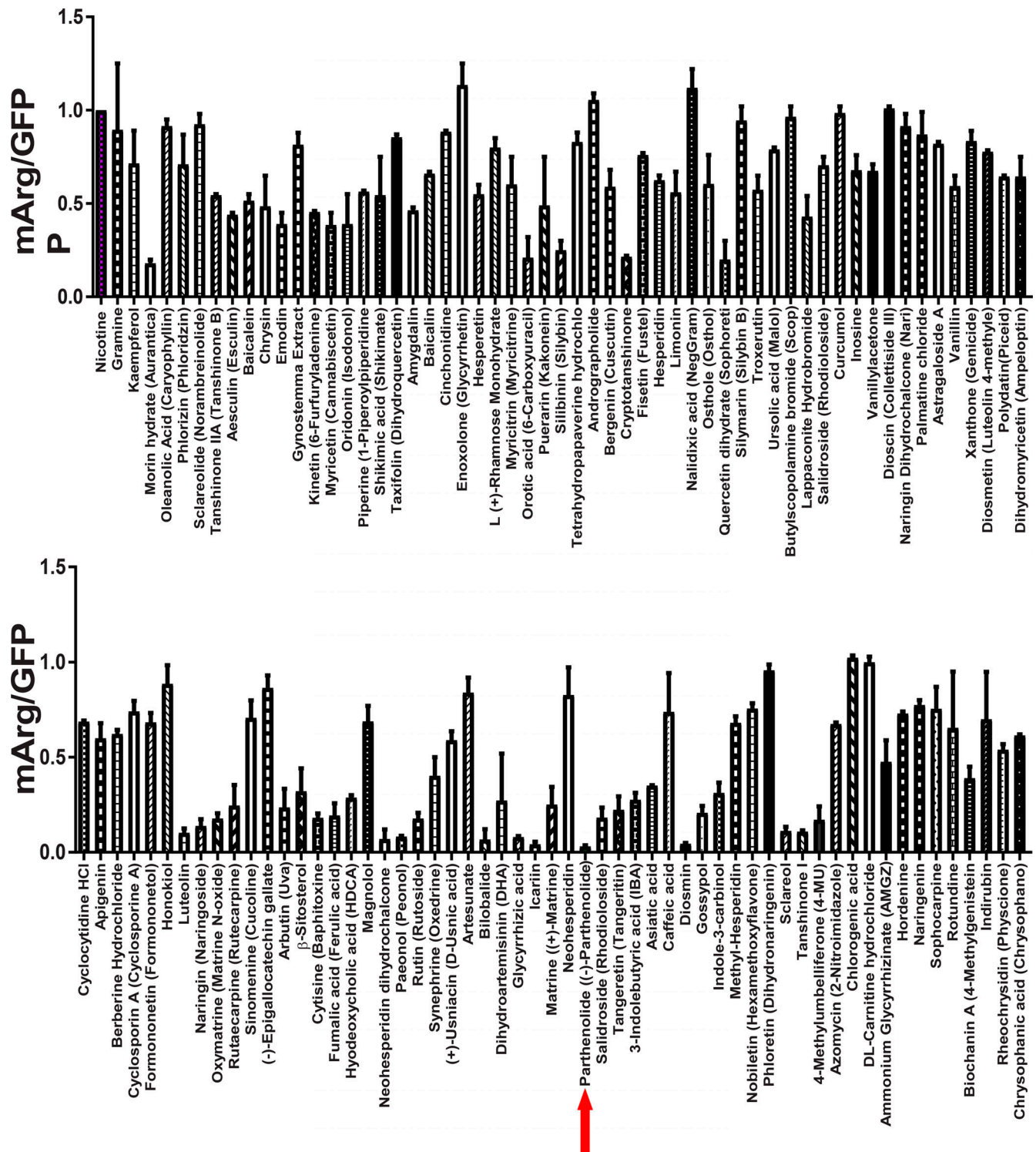


Figure S4. **Effect of natural compounds on nicotine-mediated activity of Arg1.** HMC3 cells containing Arg1 reporter plasmid were cultured in the presence or absence of nicotine (1 μ M) and/or each natural compound for 24 h. These cells were then examined for direct effect of compound on nicotine-mediated activity of Arg1 reporter ($n = 4$ /group).

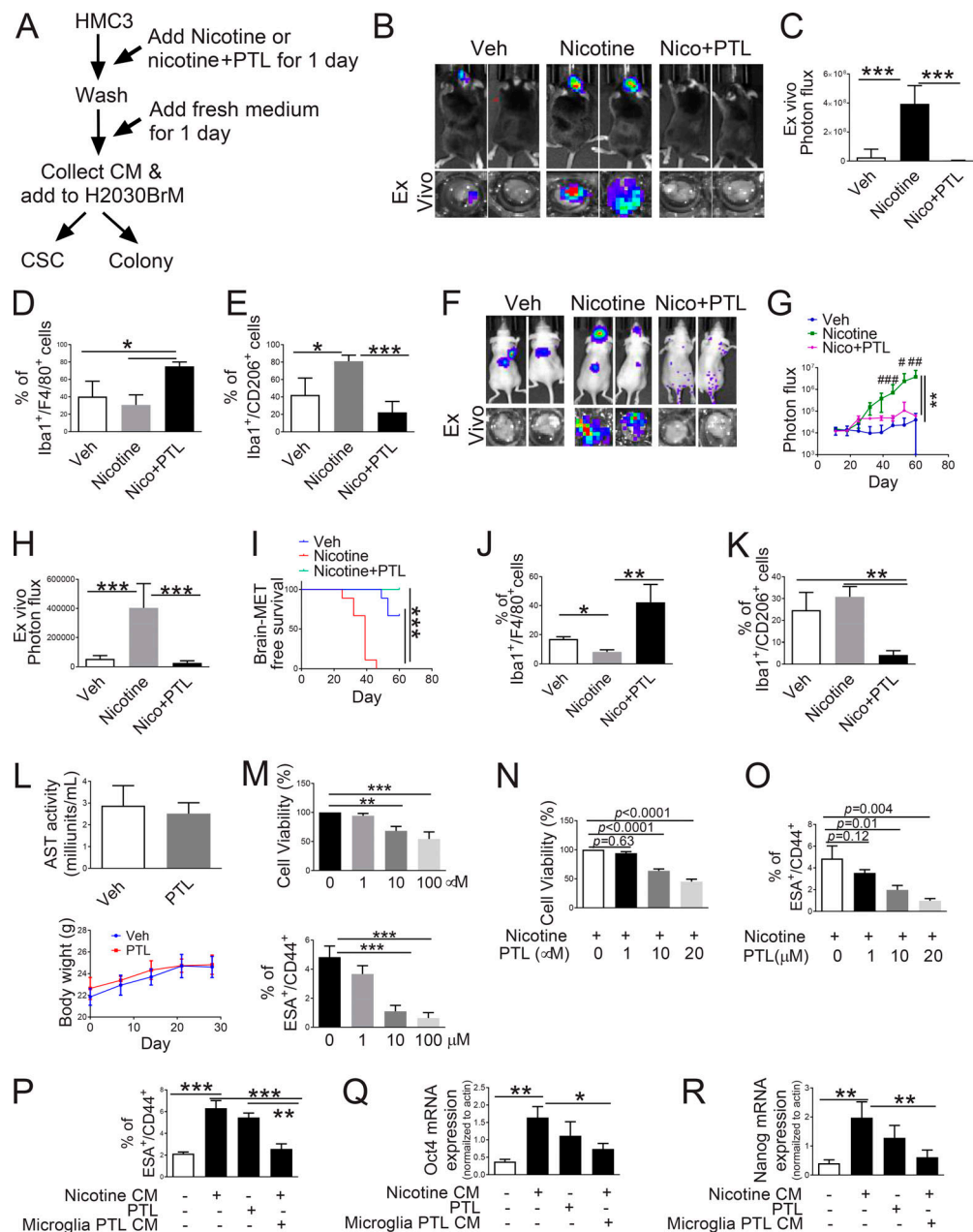


Figure S5. PTL suppressed nicotine-promoted brain metastasis in syngeneic mouse model. (A) Scheme of the experimental design. (B) Mouse lung cancer LL/2 cells were intracardially injected into wild-type C57BL/6 mice ($n = 9/\text{group}$) followed by administering nicotine (1 mg/kg) or PTL (1 mg/kg) by intraperitoneal injection every 3 d after tumor injection. Upper panels are BLI images of brain metastatic lesions of representative mice from each experimental group at day 40. Lower panels represent the total photon flux of ex vivo brain metastatic lesions as measured by BLI at the end point (day 40). (C) Quantitative data of ex vivo brain metastasis of lung cancer in the brain at the end point of the experiment conducted in B ($n = 9/\text{group}$). (D and E) Metastatic brain tumor tissues in B were dissociated and the effect of nicotine was examined for Iba1⁺/F4/80⁺ (D) and Iba1⁺/CD206⁺ (E) by FACS ($n = 9/\text{group}$). (F) H2030BrM (2×10^5 cells) were intracardially injected into nude mice followed by administering them with nicotine (1 mg/kg) plus PTL (1 mg/kg) by an intraperitoneal injection every 3 d for 60 d. Upper panel: BLI images of brain metastatic lesions of representative mice from each experimental group at day 60. Lower panel: total photon flux of ex vivo brain metastatic lesions was measured by BLI at the endpoint (day 60; $n = 9/\text{group}$). (G) Quantitative data of brain metastasis of lung cancer in vivo are shown ($n = 9/\text{group}$). (H) Ex vivo signals in the brain at the end point were examined ($n = 9/\text{group}$). (I) The Kaplan–Meier analysis of brain metastasis-free survival was performed. ($n = 9/\text{group}$). (J and K) Metastatic brain tumors were isolated from the brain and were examined by FACS for M1 (J) and M2 (K) microglial polarization using FACS ($n = 5/\text{group}$). (L) Serum of mice in Fig. 6 I was collected, and AST was measured by AST activity assay kit (Sigma-Aldrich). Body weight of the mice was also measured at the indicated time points ($n = 9/\text{group}$). (M) Direct effect of PTL on tumor cell viability by MTS assay and ESA⁺/CD44⁺ CSC population by FACS ($n = 4/\text{group}$). (N and O) H2030 cells were cultured in the presence or absence of nicotine (1 μ M) and PTL at various concentrations for 24 h. These cells were then examined for direct effect of PTL on cell viability by MTS assay (N) and on ESA⁺/CD44⁺ CSC population by FACS (O; $n = 4/\text{group}$). (P) H2030BrM cells were incubated with CM derived from microglia that were treated with or without nicotine and/or PTL (1 μ M) for 24 h, followed by examination of ESA⁺/CD44⁺ CSC population by FACS ($n = 4/\text{group}$). (Q and R) The same set of samples in P was examined for the expression of stemness-related genes, OCT4 (Q) and NANOG (R), by qRT-PCR ($n = 4/\text{group}$). The data are presented as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ versus respective Nico+PTL group.