Pharmacological activation of estrogen receptor beta augments innate immunity to suppress cancer metastasis

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Metastases constitute the greatest causes of deaths from cancer. However, no effective therapeutic options currently exist for cancer patients with metastasis. Estrogen receptor β (ER β), as a member of the nuclear receptor superfamily, shows potent tumor-suppressive activities in many cancers. To investigate whether modulation of ER β could serve as a therapeutic strategy for cancer metastasis, we examined whether the selective ER β agonist LY500307 could suppress lung metastasis of triple-negative breast cancer (TNBC) and melanoma. Mechanistically, while we observed that LY500307 potently induced cell death of cancer cells metastasized to lung in vivo, it does not mediate apoptosis of cancer cells in vitro, indicating that the cell death-inducing effects of LY500307 might be mediated by the tumor microenvironment. Pathological examination combined with flow cytometry assays indicated that LY500307 treatment induced significant infiltration of neutrophils in the metastatic niche. Functional experiments demonstrated that LY500307-treated cancer cells show chemotactic effects for neutrophils and that in vivo neutrophil depletion by Ly6G antibody administration could reverse the effects of LY500307-mediated metastasis suppression. RNA sequencing analysis showed that LY500307 could induce up-regulation of IL-1 β in TNBC and melanoma cells, which further triggered antitumor neutrophil chemotaxis. However, the therapeutic effects of LY500307 treatment for suppression of lung metastasis was attenuated in IL1 β ^{-/-} murine models, due to failure to induce antitumor neutrophil infiltration in the metastatic niche. Collectively, our study demonstrated that pharmacological activation of ER β could augment innate immunity to suppress cancer metastatic colonization to lung, thus providing alternative therapeutic options for cancer patients with metastasis.

ER β | LY500307 | cancer metastasis | neutrophil | IL-1 β

Cancer metastasis is one of the most important causes of cancer-related mortalities worldwide. Clinical metastasis is described as a multistep process, and the basic steps for metastasis formation occur in the context of different organs, emerge at different rates, and are clinically managed in different ways depending on the type of cancer. A number of the metastasis-directed therapies under development are cytostatic, not cytotoxic, and in preclinical models, making their clinical validation problematic (1). Therefore, skepticism exists in the pharmaceutical industry on the druggability of the metastasis disease.

Estrogen receptors (ERs) are intracellular transcription factors whose activity is fine-tuned by the naturally occurring estrogens in the body or by synthetic, nonsteroidal, nonhormonal agonist and antagonist ligands (2, 3). Currently, there are two known ER subtypes: ER α , encoded by the ESR1 gene on chromosome 6; and ER β , encoded by the ESR2 gene on chro-

sosome 14. Both ER α and ER β are expressed in a wide range of normal tissues and cell types throughout the body. They are also widely expressed in different pathological tissues, including cancer. Previous reports showed that ~75% of all breast cancers are positive for ER α , which is positively correlated with response to endocrine therapy (4). However, 10 to 20% of all breast cancers are triple-negative breast cancer (TNBC), which lacks expression of ER α , progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification. As patients with TNBC do not benefit from targeted therapies with tamoxifen or trastuzumab, they have a poorer prognosis and a higher rate of distant recurrence than women with other breast cancer subtypes (5). In contrast to ER α , ER β has been shown to be expressed in all molecular subtypes of breast cancer, including

Significance

Cancer metastases have caused the major mortality rate for cancer patients, with limited options of treatment and unsatisfactory therapeutic efficacy. Unlike the tumor-promoting role of estrogen receptor (ER) α , ER β has shown potent antitumor effects in many cancers. In this study, we showed that the selective ER β agonist LY500307 could potently suppress lung metastasis of cancer by recruitment of antitumor neutrophils to the metastatic niche. These chemotactic effects of LY500307 for neutrophils were primarily mediated by ER β activation-induced IL-1 β release by the tumor cells. Our study provides the rationale that pharmacological activation of ER β could augment innate immunity to suppress cancer metastatic colonization to lung, implicating the potential use of selective ER β agonists for the treatment of cancer patients with metastasis.

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60% of basal-like tumors (6, 7). Thus, ER β could be an interesting therapy target for patients with TNBC. Similar to TNBC, melanoma is an aggressive cancer type, with poor clinical prognosis. ER β is the predominant ER in melanoma, and its expression decreases in melanoma progression, supporting its role as a tumor suppressor (8, 9). Thus, ER β is now considered an effective molecular target for melanoma treatment.

In this study, we used TNBC and melanoma as cancer models to study the therapeutic effects of selective ER β activation in cancer metastasis. We used LY500307 as a selective ER β agonist and evaluated its therapeutic efficacy in the lung metastasis of these two models in vitro and in vivo, and uncovered an underlying immunological antitumor program that mediated these therapeutic effects.

Fig. 1. Selective ER β agonist LY500307 suppresses lung metastasis of cancer and prolongs survival in murine models. (A) Representative photos showing the lung metastatic nodules of the 4T1 murine model. (B) The number of lung metastatic nodules in the two groups ($n = 10$ in each group) of the 4T1 murine model. (C) The overall survival of mice in the two groups ($n = 10$ in each group) of the 4T1 model. (D) The lung weight in each group of the 4T1 murine model at indicated time points ($n = 10$ in each group). (E) The body weight of mice in each group of the 4T1 model at indicated time points ($n = 10$ in each group). (F) Representative photos showing the lung metastatic nodules of the B16 murine model. (G) The number of lung metastatic nodules in the two groups ($n = 12$ in each group) of the B16 murine model. (H) The overall survival of mice in the two groups ($n = 12$ in each group) of the B16 model. (I) The lung weight in each group of the B16 murine model at indicated time points ($n = 12$ in each group). (J) The body weight of mice in each group of the B16 model at indicated time points ($n = 12$ in each group). Data are shown as mean $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$.

Results

The Selective ER β Agonist LY500307 Effectively Suppresses Cancer Metastasis to Lung. To investigate the effects of modulation of ER β in cancer metastasis, we established lung metastasis murine models using two aggressive cancer cell lines, the TNBC 4T1 cell line and the melanoma B16 cell line. In the 4T1 in vivo model, we observed that administration of the selective ER β agonist LY500307, given as continuous-release pellets for 3 d, could potentially suppress lung metastasis of 4T1 cells compared with the control-treated group (Fig. 1A). In detail, the number of lung metastatic nodules in mice of the 4T1 model treated with

LY500307 was significantly lower than that in mice of the 4T1 model treated with control (Fig. 1B; $P < 0.05$). Moreover, the overall survival in the LY500307 treatment group of the 4T1 murine model was significantly longer than that of the control group (Fig. 1C; $P < 0.05$). However, we did not observe significant differences in lung weight (Fig. 1D) or body weight (Fig. 1E) between the two groups. Similarly, in the B16 in vivo model, treatment with LY500307 significantly reduced lung metastasis of melanoma compared with the control group (Fig. 1F). Specifically, the number of metastatic nodules in the lung of the LY500307 treatment group of the B16 model was significantly

Fig. 2. Selective ERp agonist LY500307 induces apoptosis of lung metastatic cancer cells *in vivo*, but not *in vitro*. (A) Western blotting analysis of caspase 3 in 4T1 cells treated with control or LY500307 *in vitro*. (B) Immunostaining of cleaved caspase 3 in the lung metastatic niche in the LY500307-treated 4T1 murine model. (C) Flow cytometry analysis of propidium iodide (PI)/Annexin V staining in 4T1 cells treated with different concentrations of LY500307 *in vitro*. (D) Western blotting analysis of caspase 3 in B16 cells treated with control or LY500307 *in vitro*. (E) Immunostaining of cleaved caspase 3 in the lung metastatic niche in the LY500307-treated B16 murine model. (F) Flow cytometry analysis of PI/Annexin V staining in B16 cells treated with different concentrations of LY500307 *in vitro*.

fewer than that in the control group (Fig. 1G; $P < 0.001$). The overall survival of the LY500307 treatment group was also significantly longer than that of the control group (Fig. 1H). No differences in lung weight (Fig. 1I) or body weight (Fig. 1J) were observed in the two groups of B16 in vivo model. These data suggested that pharmacological activation of ER β could effectively suppress cancer metastatic colonization to lung and prolong survival in the mouse.

LY500307 Induces Non-Cell-Autonomous Apoptosis of Lung Metastatic Foci. We next investigated the underlying mechanisms of LY500307-mediated metastasis suppression. Initially, we wondered whether ER β activation induced apoptotic cell death of cancer cells. Western blotting analysis of procaspase 3 and cleaved caspase 3 in the both the 4T1 and B16 cell lines treated with LY500307 and control did not reveal any change in the protein expression level of procaspase 3 and cleaved caspase 3, indicating that treatment of 4T1 and B16 cells with LY500307 did not induce significant apoptotic cell death in vitro (Fig. 2A and D). This in vitro phenomenon was further corroborated by flow cytometry of Annexin V/propidium iodide staining analysis (Fig. 2C and F). However, we noticed that treatment with LY500307 induced cell death of tumor cells in both the 4T1 and B16 murine models, as revealed by immunohistochemical analysis of cleaved caspase 3 (Fig. 2B and E). Thus, we postulate that LY500307 might induce non-cell-autonomous cell death of lung metastatic foci to exert its metastasis suppression effects.

LY500307 Treatment Results in Infiltration of Neutrophils in the Lung Metastatic Niche of Cancer. We next asked whether the cell death-inducing effects of LY500307 for cancer metastatic foci are

mediated by the tumor microenvironment. We were particularly interested in the immune cell profile alterations in the lung of the metastasis murine model after LY500307 treatment. We screened the amount of CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, Ly6G $^{+}$ CD11b $^{+}$ neutrophils, Ly6C $^{+}$ CD11b $^{+}$ monocytes, GR1 $^{+}$ CD11b $^{+}$ myeloid-derived suppressor cells, and CD11c $^{+}$ CD11b $^{+}$ dendritic cells in the LY500307 treatment group and control group using flow cytometry analysis. Interestingly, it was found that among these immune cells, only Ly6G $^{+}$ CD11b $^{+}$ neutrophils significantly increased in the lung of the metastasis murine model following LY500307 treatment compared with control (Fig. 3A and C). We further performed detailed histopathologic evaluation of the lung metastatic foci from LY500307-treated and control-treated mice. Consistently, we observed accumulation of multi-lobed neutrophils surrounding the metastatic foci of lung in both the 4T1 and B16 murine models treated with LY500307 compared with control, as analyzed by H&E analysis (Fig. 3B and D). Moreover, immunohistochemical analysis further proved that Ly6G $^{+}$ and myeloperoxidase (MPO) $^{+}$ neutrophils infiltrated into the tumor in a time-dependent manner (Fig. 3B and D).

Subsequently, we investigated whether the supernatant from LY500307-treated tumor cells could exert chemotactic effects for neutrophils in vitro. We noticed that the supernatant from control 4T1 cells or the media containing LY500307 could not attract neutrophils to migrate to the lower layer of the Transwell chamber; however, compared with the control media, a significant increase in the number of neutrophils that migrated to the lower layer of the chamber was noticed in the group filled with the supernatant from LY500307-treated 4T1 cells (Fig. S1A and B). Similarly, it was demonstrated that the supernatant from LY500307-treated

Fig. 3. Selective ER β agonist LY500307 induces infiltration of neutrophils in the lung metastatic niche. (A) Flow cytometry analysis of dissociated 4T1 tumors treated with control or LY500307 at indicated time points (staining with CD11b and Ly6G antibodies). (B) H&E, Ly6G, and MPO staining of the lung metastatic niche in the LY500307-treated 4T1 murine model. (C) Flow cytometry analysis of dissociated B16 tumors treated with control or LY500307 at indicated time points (staining with CD11b and Ly6G antibodies). (D) H&E, Ly6G, and MPO staining of the lung metastatic niche in the LY500307-treated B16 murine model.

B16 cells could attract more neutrophils to migrate to the lower layer of the Transwell chamber compared with the control media, the supernatant from control B16 cells, and the media containing LY500307 (Fig. S1 C and D). Therefore, we concluded that LY500307 treatment could lead to neutrophil infiltration into the lung metastatic niche both in vitro and in vivo.

Neutrophil Depletion Attenuates the Therapeutic Efficacy of LY500307 Treatment for Cancer Lung Metastasis. We further examined the importance of neutrophils in the therapeutic efficacy of LY500307 treatment-mediated metastasis inhibition. We used Ly6G neutralizing antibody (1A8) to block the function of neutrophils in the lung metastasis murine model. Interestingly, while administra-

tion of Ly6G neutralizing antibody did not cause much change in lung weight in the control group, in the LY500307 treatment group, or in the Ly6G neutralizing antibody plus LY500307 treatment group in both the 4T1 and B16 murine models (Fig. 4 B and F), we noticed a significant increase of lung metastatic nodules in the group treated with both Ly6G neutralizing antibody and LY500307 compared with the group treated with LY500307 alone in the two models (Fig. 4 A, C, E, and G). Moreover, immunohistochemical analysis further demonstrated that administration of Ly6G neutralizing antibody could reduce the number of infiltrated Ly6G⁺ and MPO⁺ neutrophils and decrease the immunostaining intensity of cleaved caspase 3 in the lung metastasis niche when in combined use with LY500307 in the two models (Fig. 4 D and H).

Fig. 4. Neutrophil depletion hampers the therapeutic efficacy of LY500307 in the suppression of cancer metastasis to the lung. (A) Representative photos showing the lung metastatic nodules of the 4T1 murine model groups treated with control, LY500307, and a combination of LY500307 and 1A8 (Ly6G antibody) ($n = 10$ in each group). (B–D) Lung weight (B), number of lung metastatic nodules (C), and Ly6G, MPO, and cleaved caspase 3 staining of the lung metastatic niche (D) in each treatment group ($n = 10$ in each group) of the 4T1 murine model. (E) Representative photos showing the lung metastatic nodules of the B16 murine model groups treated with control, LY500307, and a combination of LY500307 and 1A8 (Ly6G antibody) ($n = 10$ in each group). (F) The lung weight in each treatment group ($n = 10$ in each group) of the B16 murine model. (G) The number of lung metastatic nodules in each treatment group ($n = 10$ in each group) of the B16 murine model. (H) Ly6G, MPO, and cleaved caspase 3 staining of the lung metastatic niche in each treatment group ($n = 10$ in each group) of the B16 murine model. Data are shown as mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

This phenomenon indicated that neutrophil depletion could significantly impair the therapeutic efficacy of LY500307 treatment for cancer lung metastasis, indirectly proving that the recruited neutrophils by LY500307-treated tumor cells exert antitumor functions and suppress tumor metastasis.

LY500307-Treated Cancer Cells Release IL-1 β into the Metastatic Niche. We further examined which soluble tumor-secreted factors are responsible for the neutrophil chemotaxis. RNA sequencing analysis demonstrated that LY500307 treatment in TNBC cells and melanoma cells could trigger alterations of a panel of genes on the mRNA level (Fig. 5A). Further, Venn diagram analysis showed there were eight mRNAs that significantly changed between the LY500307-treatment group and the control group in TNBC cells and melanoma cells (Fig. 5B). Among the eight overlapped genes, we were particularly interested in *IL1B* because only *IL1B* gene-encoded protein is a secreting protein (Fig. 5C and D). We further validated the correlation between *IL1B* expression and *ESR2* expression in the TNBC dataset and the melanoma dataset in The Cancer Genome Atlas (TCGA). Interestingly, we found that the expression of *ESR2* was positively correlated with the expression of *IL1B* in both the TNBC dataset (Fig. S24) and the melanoma dataset (Fig. S2B). Moreover, we validated the mRNA changes of *IL1B* in the LY500307-treated 4T1 and B16 cell lines. Consistent with our RNA sequencing results, it was demonstrated that LY500307 could potentially induce the up-regulation of *IL1B* mRNA levels in both the 4T1 cell line (Fig. 5E) and the B16 cell line (Fig. 5F). Moreover, the concentrations of IL-1 β were significantly increased in the conditioned media of LY500307-treated TNBC cells (Fig. 5G) and melanoma cells (Fig. 5H). These observations demonstrated that LY500307-treated cancer cells could release IL-1 β into the tumor microenvironment.

Neutrophils Were Recruited to the Lung Metastatic Niche of Cancer to Suppress Metastasis by LY500307-Treated Cancer-Released IL-1 β . To further characterize the functional role of IL-1 β in cancer metastasis to the lung, we next investigated whether it is essential for the chemotactic effects for neutrophils in vitro. While a significant increase in

the number of neutrophils that migrated to the lower layer of the chamber was noticed in the group filled with the supernatant from LY500307-treated 4T1 cells compared with that in the group filled with the supernatant from control 4T1 cells or the media containing LY500307, treatment with IL-1 β monoantibody effectively blocked this chemotactic effect for neutrophils (Fig. S3A and B). We observed similar results in the B16 model in vitro (Fig. S3C and D).

We next assessed the essentiality of IL-1 β in the chemotactic effects for neutrophils and the metastasis-suppressing effects of LY500307 in vivo. We used the *IL1B*-knockout mouse (*IL1B*^{-/-}) as the in vivo model for investigation. The results showed that while the number of B16 metastatic nodules in the lung was consistently reduced in the LY500307-treated wild-type (WT) mouse group compared with the control-treated WT mouse group, the number of metastatic nodules in the lung was remarkably increased in the LY500307-treated *IL1B*^{-/-} mouse group, indicating that IL-1 β might be potentially critical for the metastasis-suppressing effects of LY500307 (Fig. 6A and B). No significant changes in the lung weight or body weight among the three groups were observed (Fig. 6C and D). Functionally, we found that the number of Ly6G⁺CD11b⁺ neutrophils was significantly reduced in the lung of the metastasis model in the *IL1B*^{-/-} mouse treated with LY500307 compared with that in the LY500307-treated *IL1B* WT mouse (Fig. 6E). Moreover, we observed a decrease of multilobed neutrophils surrounding the metastatic foci of the lung in *IL1B*^{-/-} murine models treated with LY500307 compared with *IL1B* WT murine models treated with LY500307, as analyzed by H&E analysis (Fig. S4). Immunohistochemical analysis further proved that the number of infiltrated Ly6G⁺ and MPO⁺ neutrophils was reduced in the metastatic foci of the lung in *IL1B*^{-/-} murine models treated with LY500307 compared with *IL1B* WT murine models treated with LY500307 (Fig. S4). These observations illustrated that IL-1 β plays a vital role in the chemotactic effects of neutrophils to infiltrate the lung in LY500307-induced metastasis suppression (Fig. 6F).

Fig. 5. ER β activation induces IL-1 β secretion in tumor cells. (A) Heatmap showing the differentially expressed mRNAs in 4T1 and B16 cells treated with control and LY500307. (B) Venn diagram showing the genes commonly up-regulated in LY500307-treated 4T1 and B16 cells compared with control-treated 4T1 and B16 cells. (C and D) Volcano plots showing the differentially expressed mRNAs in LY500307-treated compared with control-treated 4T1 cells (C) and B16 cells (D). (E and F) qPCR assays to measure the mRNA levels of IL-1 β in control-treated and LY500307-treated 4T1 cells (E) and B16 cells (F). (G and H) The relative concentrations of IL-1 β in the conditioned media of control-treated and LY500307-treated 4T1 cells (G) and B16 cells (H). Data are shown as mean $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.

Fig. 6. *IL1B* knockout reduces infiltration of neutrophils to the lung metastatic niche and reduces the therapeutic effects of LY500307. (A) Representative photos showing the lung metastatic nodules (arrows) in *IL1B* WT, LY500307-treated *IL1B* WT, and LY500307-treated *IL1B*^{-/-} mice (*n* = 10 in each group) of the B16 model. (B–D) Number of lung metastatic nodules (B), lung weight (C), and body weight (D) of *IL1B* WT, LY500307-treated *IL1B* WT, and LY500307-treated *IL1B*^{-/-} mice in each treatment group (*n* = 10 in each group) of the B16 model. (E) Flow cytometry analysis of dissociated B16 tumors in *IL1B* WT, LY500307-treated *IL1B* WT, and LY500307-treated *IL1B*^{-/-} mice (*n* = 10 in each group) (staining with CD11b and Ly6G antibodies). (F) Schematic model for LY500307 effects on neutrophil-mediated activation of antitumor innate immunity within the tumor microenvironment, resulting in invasive cancer clearance. The blue cells with red nuclei are tumor cells. The yellow cells with green nuclei are tumor cells undergoing cell death. The white cells with lobed nuclei are neutrophils. The yellow squared particles are IL-1 β . Data are shown as mean $\pm$ SEM. **P* < 0.05.

Discussion

Cancer metastases that are resistant to conventional therapy are the major cause of death from cancer. In the majority of patients, metastasis has already occurred by the time of diagnosis. The biological heterogeneity within metastatic foci presents not only a challenge but also an opportunity for metastasis treatment in view of the complex components of both tumor-suppressive and tumor-promoting cells (10, 11). TNBCs and melanoma represent two cancer types with notoriously aggressive biological behaviors, both of which are prone to metastasize to the lung (12, 13). Currently, no efficacious therapeutic options have been provided for these cancers with metastasis. Therefore, it is imperative to identify novel treatment regimens for these deadly diseases.

TNBC is a complex and aggressive subtype of breast cancer which lacks ERs, PRs, and HER2 amplification, thereby making it difficult to target therapeutically. Moreover, TNBC has the highest rates of metastatic disease and the poorest overall survival of all breast cancer subtypes (14). Melanoma is a type of cancer that develops from the pigment-containing cells known as melanocytes. Once it becomes metastatic, melanoma is difficult to treat (15). Therefore,

there is an urgent need for the development of novel targeted therapies for metastatic diseases of TNBC and melanoma. Recent studies indicated that ER β is expressed in the majority of melanoma (9) and invasive breast cancer cases (16), irrespective of their subtype, including TNBC. Thus, ER β might be a potential target for therapy of these challenging metastatic diseases.

Since its cloning in 1996 from a rat cDNA prostate library, we have proved that ER β could be a major player in protecting against development and/or progression of cancer (3). Different from the tumor-promoting role of ER α , the presence of ER β exerts antiproliferative effects (17). Moreover, the presence of ER β seems to potentiate the antitumorigenic efficacy of tamoxifen (18). Immunohistochemical analysis of archival breast cancer samples from women treated with adjuvant tamoxifen suggested that the presence of ER β may be correlated with significantly improved patient survival (19). Large numbers of preclinical experiments have also suggested that selective targeting of ER β with an agonist may be an important therapeutic strategy for the clinical management of breast cancer (20–22). The functional role of ER β in melanoma has

also been reported. ER β is the predominant ER in melanoma, and its expression attenuates melanoma progression, supporting its role as a tumor suppressor. In vitro studies prove that ER β ligands inhibit the proliferation of melanoma cells harboring the NRAS (but not the BRAF) mutation, suggesting that ER β activation might impair melanoma development through the inhibition of the PI3K/Akt pathway. These data suggest that ER β agonists might be considered an effective treatment strategy, in combination with MAPK inhibitors, for NRAS-mutant melanomas (23). Natural compounds that specifically bind to ER β have also been identified. These phytoestrogens decrease the proliferation of melanoma cells. Importantly, these effects are unrelated to the oncogenic mutations of melanomas, suggesting that in addition to their ER β -activating function, these compounds might impair melanoma development through additional mechanisms (9). A better identification of the role of ER β in melanoma development will help increase the therapeutic options for this aggressive pathology. Here, we show that LY500307, a selective ER β agonist, generated a potent neutrophil-mediated antitumor innate immune response in the metastatic niche, resulting in metastasis suppression in both the 4T1 and B16 murine models.

Neutrophils represent the first step in the generation of an innate immune response upon an active infection and are recruited to the inflamed tissue via interaction with activated endothelial cells and chemokine gradients (24). Following recruitment/activation, neutrophils could trigger oxidative damage through reactive oxygen species (ROS) production and protease release (25, 26). In recent years, studies have demonstrated that neutrophils are detected in a variety of solid tumors and play multifaceted roles in the tumor microenvironment (27). Neutrophils are functionally capable of acquiring either proinflammatory, antitumor (N1), or protumorigenic (N2) properties, which are regulated by the chemokine context within the tumor microenvironment (28). Most published literature describing an antitumor role for neutrophils focuses around their production of ROS, causing tumor cell apoptosis (29). This antitumor cytotoxic activity has also been described as suppressing metastatic seeding of the lung, even though the inhibiting effect was only transient (30). More recent studies have demonstrated type 1 interferons (31) and the MET protooncogene (32) as requirements for driving antitumor functions and recruitment of neutrophils, respectively. Interestingly, our data demonstrated that neutrophils are recruited to the metastatic niche via IL-1 β released by selective ER β agonist-treated tumor cells. This immunological consequence of innate immunity activation by neutrophil infiltration within tumors is the suppression of tumor metastasis, further corroborating that the subtype of neutrophils recruited to the metastatic niche by ER β activation should be the N1 subtype.

Collectively, our observations revealed that pharmacological activation of ER β in tumor cells could induce release of IL-1 β . Increased concentration of IL-1 β within the tumor microenvironment augments innate immunity via recruitment of antitumor neutrophils, suppressing cancer metastatic colonization to lung. These findings lay the theoretical foundations for the use of selective ER β agonists in the treatment of cancer patients with metastasis.

Materials and Methods

Animals and Tissue Preparation. Female BALB/c and C57/BL6 (6- to 8-wk-old) mice were purchased from Vital River. IL1 β ^{-/-} mice were purchased from The Jackson Laboratory. These mice were housed in a specific-pathogen-free environment with a consistent room temperature and humidity. All animal experiments were approved by the Institutional Animal Care and Use Committee and Ethics Committee of Sichuan University. Briefly, 100 μ L of tumor cell suspension containing 5×10^5 4T1 cells or 1×10^5 B16 tumor cells were injected i.v. into the tail veins of BALB/c mice or C57/BL6, respectively. The mice were treated by inserting pellets (vehicle or LY500307) 7 d after inoculation of tumors. Hormonal treatment lasted for 3 d. Body weight was assessed every 2 d. After all animals were euthanized, the lungs were harvested, the lung weight was recorded, and the total number of lung metastases was counted.

For histological evaluation of lung micrometastases, sections of lung tissue from each mouse were stained by H&E and examined under a light microscope. For neutrophil depletion studies, Ly6G-depletion antibody (1A8; Bio X Cell), 200 μ g diluted in PBS, was administered daily via i.p. injection during the pretreatment phase for 3 d.

H&E Staining and Immunohistochemistry (IHC). Immunohistochemistry staining of lung sections was described previously (33). Some of the paraffin tumor sections were stained with H&E. The others were stained with Ly6G, MPO, and cleaved caspase 3 antibodies.

Immunoblot Analysis and ELISA. Immunoblot analysis was performed as described previously (33), with minor modifications. Briefly, 4T1 (5×10^5 cells per well) or B16 cells (3×10^5 cells per well) were seeded in a 10-mL plate for 12 h and treated with LY500307 (5 μ M). After treatment for 48 h, cells were washed twice with ice-cold PBS and lysed in RIPA buffer (Sigma-Aldrich). Antibodies to cleaved caspase 3 and β -actin (Abcam) were used. ELISA was used to measure IL-1 β concentration in the cultured medium as described elsewhere (34).

Isolation of Mouse Neutrophils and ex Vivo Neutrophil Migration Assay. Mouse neutrophils were prepared from isolated bone marrow as previously described (35). More than 85% of the pelleted cells were neutrophils as determined by flow cytometry. Transwell chamber migration assay was performed to assess the ex vivo neutrophil migratory potential as previously described (36, 37) with minor modifications. Briefly, neutrophils in 200 μ L of serum-free medium were added in the top chamber, and then 500 μ L of medium with 10% FBS, conditioned media, or LY500307 plus conditioned media was added to the bottom chamber. Different concentrations of nicosamide were added in both chambers. Neutrophils were allowed to migrate for 48 h. Nonmigrated cells in the top chamber were removed. The migrated cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Migrated cells were counted and photographed under a light microscope.

Statistical Analysis. For studies comparing differences between two groups, we used unpaired Student's *t* tests. For studies comparing more than two groups, we used ANOVA with appropriate post hoc testing. Differences were considered significant when $P < 0.05$. Data are presented as mean $\pm$ SEM. High-throughput sequencing data have been deposited in the Gene Expression Omnibus (GEO) database under accession numbers GSE110769 and GSE110770.

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