The lymphatic system is an important route for cancer dissemination, and lymph node metastasis (LNM) serves as a critical prognostic determinant in cancer patients. We investigated the contribution of COX-2–derived prostaglandin E₂ (PGE₂) in the formation of a premetastatic niche and LNM. A murine model of Lewis lung carcinoma (LLC) cell metastasis revealed that COX-2 is expressed in DCs from the early stage in the lymph node subcapsular regions, and COX-2 inhibition markedly suppressed mediastinal LNM. Stromal cell–derived factor-1 (SDF-1) was elevated in DCs before LLC cell infiltration to the lymph nodes, and a COX-2 inhibitor, an SDF-1 antagonist, and a CXCR4 neutralizing antibody all reduced LNM. Moreover, LNM was reduced in mice lacking the PGE₂ receptor EP3, and stimulation of cultured DCs with an EP3 agonist increased SDF-1 production. Compared with WT CD11c⁺ DCs, injection of EP3-deficient CD11c⁺ DCs dramatically reduced accumulation of SDF-1⁺CD11c⁺ DCs in regional LNs and LNM in LLC-injected mice. Accumulation of Tregs and lymph node lymphangiogenesis, which may influence the fate of metastasized tumor cells, was also COX-2/EP3–dependent. These results indicate that DCs induce a premetastatic niche during LNM via COX-2/EP3–dependent induction of SDF-1 and suggest that inhibition of this signaling axis may be an effective strategy to suppress premetastatic niche formation and LNM.
Introduction

It is widely accepted that many tumors tend to metastasize to specific organs (1). The mechanisms that guide tumor cells to specific tissues are largely unknown, although recent evidence suggests that it may involve molecular differences inherent in the tumor cells themselves, modulated by the activities of immune cells, hematopoietic cells, and other tissue components. Lymph node metastasis (LNM) is a critical prognostic factor in cancer patients, and lymphatic vessels serve as an important route for the spread of cancer cells (1). Paget reported that tumor cells might prepare the lymph nodes for their future arrival, giving a new interpretation to the seed-and-soil hypothesis (2). The formation of a premetastatic niche, suitable for the arrival of the first tumor cells, facilitates metastasis via the bloodstream (3, 4). However, data regarding the factors involved in lymph node premetastatic niche formation are limited (5). Tumor-associated lymphangiogenesis may enhance metastasis to the regional lymph nodes; however, the involvement of lymph node premetastatic niche formation in the metastatic process is unclear.

The cellular components involved in tumor cell metastasis to a predetermined location are largely unknown. However, Kaplan et al. (4) demonstrated that bone marrow–derived hematopoietic progenitor cells expressing VEGFR1 (also known as FLT1) home to tumor-specific premetastatic sites and form cellular clusters before the arrival of tumor cells. Their findings demonstrated a requirement for VEGFR1–hematopoietic progenitors in the regulation of metastasis, and suggested that expression patterns of fibronectin and clusters positive for VEGFR1 and VLA-4 (also known as integrin αβ) dictate organ-specific tumor spread. However, the involvement of other cellular components in premetastatic niche formation is largely unknown. Among the many cellular components within the tumor microenvironment, dendritic cells (DCs) exert profound effects on T cells (6). The mediators that regulate DC function may also modulate niche formation. Prostaglandin E2 (PGE2) and the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) exert strong effects on the maturation and function of DCs (7).

Authorship note: Fumihiro Ogawa, Hideki Amano, and Koji Eshima contributed equally to this work.
Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: October 1, 2013; Accepted: August 21, 2014.

Figure 1. COX-2 induction in regional lymph nodes and the effect of COX-2 inhibition on regional LNM. (A) Immunohistochemical COX-2 staining of the regional lymph nodes after lung LLC cell implantation. In vehicle-treated mice, COX-2–positive cells were localized in the subcapsular regions. Celecoxib was given orally throughout the experimental period. Scale bar: 50 μm. (B) RT-PCR analysis of Cox2. Cox2 mRNA levels were significantly reduced in the celecoxib-treated group compared with the vehicle-treated group. n = 15 per group. *P < 0.0001 (ANOVA). (C) The percentage total cell population of COX-2–positive cells was significantly reduced in the celecoxib-treated group compared with the vehicle-treated group. n = 15 per group. *P < 0.0001 (ANOVA). (D) Loupe images by typical H&E staining obtained on day 10 after injection. Scale bars: 5 mm. (E) Fluorescence microscope images obtained after implantation of GFP-positive LLC cells into the lung. Scale bars: 3 mm. (F) Temporal changes in the percentage of regional LNM–positive mice. Metastasis in the vehicle-treated group was compared with that in the celecoxib-treated group. n = 15 per group. *P < 0.0001 (χ² test). (G) Percentage of regional LNM–positive mice. Metastasis was compared between Ep3 KO mice and WT mice. WT, n = 15; Ep3 KO, n = 5. *P < 0.05 (χ² test). Error bars indicate the mean ± SD. N indicates the number of mice tested. S, subcapsular regions; C, cortex; PT, primary tumor; LN, lymph node; T, thymus.
The SDF-1/CXCR4 axis governs the accumulation of LLC cells in the lymph node premetastatic niche. To determine whether the SDF-1/CXCR4 axis induces premetastatic niche formation, we injected a CXCR4 antagonist, AMD3100, following the injection of LLC cells. There was no significant difference in tumor size between vehicle- and AMD3100-treated mice during the experimental period (Figure 2E). However, LNM was completely suppressed in mice treated with AMD3100 or CXCR4 neutralizing antibody on day 10 and day 14 (Figure 2F).

Furthermore, the accumulation of SDF-1/CXCR4–double positive cells in the regional lymph nodes (Supplemental Figure 2D) was suppressed in AMD3100-treated mice (Figure 2G). This reduction was also seen in Ep3 KO mice (Figure 2H and Supplemental Figure 2D). These results suggested that LNM driven by COX-2/EP3 is highly dependent on the SDF-1/CXCR4 axis.

COX-2-positive DCs accumulate in the lymph node premetastatic niche and produce SDF-1 via EP3 signaling. To clarify the cellular components relevant to premetastatic niche formation, we examined the cell surface marker profiles of COX-2–expressing cells. We found that DC marker–positive cells accumulated in the subcapsular regions of the lymph nodes (Figure 3A). Among the DC markers, CD11c, which is a type I transmembrane protein, is highly expressed on most DCs. Furthermore, indoleamine 2,3-dioxygenase (IDO) is also considered to be a good marker for DCs, and PGE2 was reported to induce IDO in tolerogenic DCs (28). In the present study, immunohistochemical analysis revealed that COX-2–positive cells were also positive for CD11c and IDO (Figure 3, A and B). The accumulation of CD11c- and IDO-positive cells was suppressed in cebecixib- or AMD3100-treated mice and in Ep3 KO mice (Figure 3, C and D).
pared with vehicle mice (Supplemental Figure 3C). This result indicated that the induction of DCs associated with LNM was dependent on COX-2–derived PGE2-EP3 signaling. To examine whether PGE2 induces SDF-1 in DCs, we isolated immature myeloid DCs from the bone marrow and cultured them in the presence of PGs. The mRNA and protein levels of SDF-1 increased significantly following PGE2 stimulation (Supplemental Figure 3, D and E). To identify the receptor mediating this action, we added EP agonists (EP1–EP4), which are highly selective for each receptor subtype. Quantification of CD11c/IDO–positive cells revealed that the reduction in the accumulation of CD11c/IDO–positive cells by COX-2 inhibition, CXCR4 blockade, and lack of EP3 signaling began at day 5 or day 7 (Figure 3, E and F). The CD11c-positive cells were also positive for SDF-1 (Figure 3G), suggesting that DCs are sources of SDF-1 in the premetastatic niche.

Moreover, the expression of CCR7, important for the adhesion and chemotaxis of leukocytes and DCs toward lymph nodes, was also suppressed in celecoxib-treated mice and Ep3 KO mice compared with vehicle mice (Supplemental Figure 3C). This result indicated that the induction of DCs associated with LNM was dependent on COX-2–derived PGE2-EP3 signaling. To examine whether PGE2 induces SDF-1 in DCs, we isolated immature myeloid DCs from the bone marrow and cultured them in the presence of PGs. The mRNA and protein levels of SDF-1 increased significantly following PGE2 stimulation (Supplemental Figure 3, D and E). To identify the receptor mediating this action, we added EP agonists (EP1–EP4), which are highly selective for each receptor subtype.

Figure 2. COX-2/EP3 enhances SDF-1 expression in lymph nodes. (A) Immunofluorescence images of CXCR4 in cultured GFP-positive LLC cells. Scale bar: 20 μm. (B) SDF–positive cell population in the regional lymph nodes. n = 15 per group. *P < 0.05 (χ² test). (C) Immunofluorescence images showing COX-2/SDF–double staining in the regional lymph nodes. COX-2/SDF–double positive cells accumulated before arrival of GFP-negative LLC cells. Naive: Images from mice with no treatment. Scale bars: 50 μm. (D) Accumulation of SDF-1/GFP–double positive LLC cells in subcapsular regions in regional lymph nodes at day 14. Celecoxib treatment reduced them. Scale bars: 50 μm. (E) Temporal changes in tumor size from vehicle-treated mice and AMD3100-treated mice. Vehicle, n = 15; AMD3100, n = 10. (F) Temporal changes in the percentage of regional LNM–positive mice. AMD3100 and a CXCR4 neutralizing antibody were given throughout the experimental period. Vehicle, n = 15; AMD3100, n = 10; CXCR4 neutralizing antibody, n = 5. *P < 0.05 (χ² test). (G) Temporal changes in the SDF-1–positive cell population in the regional lymph nodes. Vehicle, n = 15; AMD3100, n = 10. *P < 0.0001 (ANOVA). Student’s t test was used to evaluate significant differences at days 3 and 5 (P < 0.05). (H) Temporal changes in the SDF-1–positive cell population in the regional lymph nodes. The results from Ep3 KO mice were compared with those from WT mice. WT, n = 15; Ep3 KO, n = 5. *P < 0.0001 (ANOVA), P < 0.05 (Student’s t test). Values are mean ± SD. N indicates the number of mice tested. Ex, extracellular matrix; S, subcapsular regions; C, cortical regions.
Figure 3. Effect of the SDF-1/CXCR4 axis on LNM. (A and B) COX-2/CD11c (A) and COX-2/IDO (B) double immunofluorescent staining of the subcapsular regions in regional lymph nodes at 7 days. Scale bars: 20 μm. (C and D) Localization of CD11c-positive (C) and IDO-positive (D) cells in the subcapsular regions in regional lymph nodes at 7 days. Images are typical results from each group. Scale bars: 50 μm. (E) Temporal changes in the CD11c-positive cell population in the regional lymph nodes. Vehicle, n = 15; celecoxib, n = 15; AMD3100, n = 10; Ep3 KO, n = 5. *P < 0.0001 (ANOVA). Student’s t test was used to evaluate significant differences at days 3, 5, and 7 (P < 0.05). (F) Temporal changes in the IDO-positive cell population in the regional lymph nodes. Vehicle, n = 15; celecoxib, n = 15; AMD3100, n = 10; Ep3 KO, n = 5. *P < 0.0001 (ANOVA). Student’s t test was used to evaluate significant differences at days 3, 5, and 7 (P < 0.05). (G) SDF-1/CD11c double immunofluorescence staining of the subcapsular regions in regional lymph nodes at 7 days. Scale bar: 20 μm. (H) Induction of SDF-1 in cultured DCs by stimulation with an EP3 agonist. The level of SDF-1 in the culture medium was determined with a specific ELISA. n = 6. *P < 0.05 (ANOVA). Student’s t test was used to evaluate significant differences at each time point (*P < 0.05). (I) SDF-1 levels in cultured DCs isolated from the bone marrow of Ep3 KO mice. The experimental conditions were the same as those in H. n = 6. Error bars indicate the mean ± SD. S, subcapsular regions; C, cortical regions.
SDF-1 protein levels in the culture media increased upon incubation of DCs from WT mice with an EP3 agonist, ONO-AE-248 (Figure 3H); however, DCs from Ep3 KO did not secrete SDF-1 (Figure 3I). Treatment with EP agonists for EP1, EP2, and EP4 did not increase SDF-1 secretion (Supplemental Figure 3F). These results suggested that DC accumulation in the premetastatic niche is dependent on COX-2/EP3 and SDF-1, and that induction of SDF-1 in DCs is dependent on EP3 signaling.

EP3 signaling facilitates DC recruitment to the regional lymph node premetastatic niche. To clarify the process of DC recruitment to the premetastatic niche, we injected mice i.v. with GFP-positive DCs following the injection of GFP-negative LLC cells. The GFP-positive DCs from WT mice were recruited to the subcapsular regions at day 10 (Figure 4A). This accumulation was significantly reduced following injection of GFP-positive DCs from Ep3 KO mice (Figure 4A). In the early phase (premetastatic phase), DCs were recruited to subcapsular regions in an EP3 signaling–dependent manner too (Supplemental Figure 4, A and B). Furthermore, we estimated double staining by GFP and LYVE-1 in regional lymph nodes. GFP-positive DCs from WT mice (Supplemental Figure 4C) were significantly induced to LYVE-1–positive lymph vessels compared with GFP-positive DCs from Ep3 KO mice (Supplemental Figure 4D) at subcapsular regions of regional lymph nodes.

The WT DCs recruited to the subcapsular regions expressed higher levels of SDF-1 than those from Ep3 KO mice. The percentage of LNM was higher following the injection of WT DCs than after the injection of vehicle or Ep3 KO DCs (Figure 4B). Most of the accumulated DCs were CXCR4-positive (Figure 4C), suggesting that the DCs expressing EP3 enhanced the recruitment of DCs via the generation of SDF-1. These findings imply a positive-feedback loop in the EP3/SDF-1 interplay.

Both COX-2-EP3 signaling and SDF-1 increase the recruitment of Tregs to the lymph node premetastatic niche. Previous studies show that the increased production of PGE2 by tumors is associated with the induction of Tregs and with T cell inhibition (29, 30). In the present study, we examined whether COX-2 inhibition could reduce Treg recruitment to the regional lymph nodes. As shown in Figure 5A, a substantial number of FOXP3+ Tregs accumulated in the subcapsular regions of the regional lymph nodes; this accumulation was suppressed by celecoxib, AMD3100, and a lack of EP3 signaling. These results were confirmed by quantitative analysis (Figure 5B) and immunofluorescent analysis (Supplemental Figure 5, A and B). Taken together, these findings suggest that the accumulation of Tregs in lymph nodes is dependent on both COX-2/EP3 signaling and the SDF-1/CXCR4 axis. Since many of the FOXP3+ cells expressed CXCR4 (Supplemental Figure 5C), it is possible that DC-derived SDF-1 may attract Tregs to the premetastatic niche. It was previously reported that immature DCs secrete TGF-β to induce Treg proliferation (31). Therefore, to examine whether TGF-β1 was secreted by DCs in the premetastatic niche, we performed immunofluorescence analysis of TGF-β1 expres-
tion by CD11c-positive DCs. We found that CD11c-positive cells expressed TGF-β1 and that the number of CD11c/TGF-β1-double positive cells was reduced in Ep3 KO mice compared with that in WT mice (Figure 5C). We also measured TGF-β1 secretion by DCs cultured in the presence of an EP3 agonist. WT DCs secreted large amounts of TGF-β1 in response to EP3 stimulation, but DCs from Ep3 KO mice did not (Figure 5D). TGF-β expression was significantly suppressed in celecoxib- or AMD3100-treated mice and in Ep3 KO mice (Figure 5E). These results suggested that the accumulation of Tregs in the premetastatic niche was COX-2/
Figure 6. COX-2–derived PGE₂-EP3 signaling induces lymphangiogenesis. (A–C) Temporal changes in the VEGF-C–positive cell (A), the VEGF-D–positive cell (B), and the VEGFR3–positive cell population (C) in the regional lymph nodes. N indicates the number of mice tested. Vehicle, n = 15; celecoxib, n = 15; Ep3 KO, n = 5. Error bars indicate the mean ± SD. *P < 0.0001 (ANOVA). Student’s t test was used to evaluate significant differences at days 3, 5, and 7 (P < 0.05). (D) Immunofluorescence images of regional lymph nodes stained with the LYVE-1 antibody. Lymphangiogenesis was observed at the efferent side of the regional lymph nodes in vehicle-treated mice from day 3, and was markedly reduced by celecoxib treatment. Scale bars: 100 μm. (E) A schematic presentation of COX-2/SDF-1–dependent premetastatic niche formation. COX-2 and EP3 mediate the increase of SDF-1 at the premetastatic site before the arrival of tumor cells to the regional lymph node. CXCR4-positive tumor cells were mobilized to SDF-1–generating sites. Simultaneously, CXCR4/EP3–positive DCs are recruited to generate SDF-1 in response to EP3 signaling. (F) Tregs are recruited to the premetastatic niche via SDF-1/CXCR4 signaling. The DCs recruited to the premetastatic niche secrete TGF-β1 in an EP3/SDF-1–dependent manner. Thus, COX-2/PGE₂–EP3 signaling together with SDF-1/CXCR4 signaling may decide the fate of the colonizing LLC cells. (G) Fate of metastasized tumor cells. COX-2/EP3 signaling upregulates lymph node lymphangiogenesis in the efferent side of the subcapsular region, and this facilitates tumor cell growth in the regional lymph nodes and lymph node or systemic metastasis.
PGE2-EP3 signaling as a regulator of lymph node lymphangiogenesis. Once the metastatic cells arrive at the lymph nodes, lymph node lymphangiogenesis may facilitate further metastasis to other organs, such as the distal lymph nodes and lungs. It was reported that no metastases were observed in distant organs in the absence of lymph node metastases (32). We determined the expression of prolymphangiogenic growth factors, VEGF-C and VEGF-D, in the regional lymph nodes and found that the gene expression of these growth factors was significantly increased before the arrival of the LLC cells, but was suppressed in celecoxib-treated mice and in Ep3 KO mice (Figure 6, A and B). Moreover, VEGFR3, expressed on lymphatic endothelial cells, was increased in the regional lymph nodes from day 3, but was reduced in celecoxib-treated mice and Ep3 KO mice (Figure 6C). VEGF-C-, VEGF-D-, and VEGFR3-positive cells were costained against CD11b, a macrophage marker, and CD11c in the efferent side of subcapsular regions and analyzed by immunofluorescence (Supplemental Figure 6, A and B). These results suggested that COX-2/PGE2-EP3–dependent induction of VEGF-C/D in macrophages and DCs induces lymphangiogenesis in the efferent side of subcapsular regions. Furthermore, the size of regional lymph nodes and lymph vessel area were suppressed in Ep3 KO mice and celecoxib-treated mice compared with vehicle- and celecoxib-treated mice (P < 0.05, ANOVA; Figure 6D and Supplemental Figure 7, A and B). In contrast, there were no significant differences among vehicle- and celecoxib-treated mice and Ep3 KO mice in primary lesion (Supplemental Figure 7C). These results suggested that PGE2-EP3 signaling induces lymphangiogenesis in regional metastatic lymph nodes before the arrival of LLC cells.

Discussion

Our study demonstrates that the formation of the premetastatic niche in regional lymph nodes is dependent on COX-2/PGE2-EP3 signaling, and that SDF-1–expressing DCs have a significant effect on the accumulation of CXCR4-expressing LLC cells. COX-2/EP3–dependent induction of chemokine was initiated from the early stage in which there is no metastasis in regional lymph nodes. During these steps, there was no significant difference in primary tumor size under COX-2 inhibition and EP3 receptor knockout. EP3 signaling was also active to accumulate DCs in subcapsular regions of lymph nodes for future accumulation of LLC cells. We observed an increase in the release of SDF-1 from DCs in lymph nodes following LLC cell injection. In addition, we observed significant Treg recruitment to the premetastatic niche; this process was dependent on COX-2/EP3 and SDF-1. Lymph node lymphangiogenesis, which may facilitate further metastasis, was also induced in a COX-2/EP3–dependent manner. These findings indicate that prostanooid signaling plays a significant role in LNM.

Prostanoids, including prostaglandins, are generated from arachidonic acid, a process regulated by the rate-limiting COX enzymes. COX-2 can be induced in most tissues, where it mediates the production of proinflammatory PGs during inflammation and in tumorigenic settings. Neoplastic tissues, such as human colon cancers, contain high concentrations of PGs, and the protumorigenic effects of COX-2 are largely attributed to its role in PGE2 production (8). COX-2 expression is associated with poor patient prognosis and survival (33, 34). We previously reported that, in addition to tumor COX-2 expression, COX-2 present in stromal tissues had a significant effect on tumor-associated angiogenesis (35). Further studies showed that COX-2 induced lung metastasis via EP3 signaling (14). Iwata et al. previously reported that COX-2 inhibitor suppressed tumor lymphangiogenesis and LNM by using a gastric carcinoma cell line (36); however, little is known about the involvement of COX-2 and PG receptors in forming the premetastatic niche during LNM. The current study confirmed the report and revealed that COX-2 and EP3 signaling play a role in LNM, and showed that COX-2 and EP3 signaling mediate the induction of SDF-1 and CXCR4 to establish the premetastatic niche.

The cellular components involved in the formation of premetastatic niches are largely unknown. In the present study, we showed that DCs play an important role in the formation of the lymph node premetastatic niche. DCs are bone marrow–derived cells that are present in all issues (37, 38). Lymph output of DCs in the steady state, continual traffic of DCs from peripheral tissue, is constant and is further augmented during inflammatory conditions (39, 40). Mouse models of cancer reveal that DCs capture tumor antigens released from tumor cells and cross-present these antigens to T cells in the tumor-draining lymph nodes, thereby generating tumor-specific cytotoxic T lymphocytes, which play a role in tumor rejection (41, 42). DCs sample the environment and transmit the gathered information to cells of the adaptive immune system (T cells and B cells). DC differentiation from hematopoietic stem cells and DC function are sensitive to lipid mediators, such as PGs, and DCs express several receptors involved in PG signaling pathways (43). PGE2 is a key modulator of DC differentiation, maturation, migration, and cytokine production (44–48). In contrast to its inhibitory effects on monocytes, PGE2 activates immature DCs. As a first step toward the transition from an adhesive to a highly migratory state, DCs lose their specific integrin- and actin-rich adhesive structures, called podosomes, within minutes following PGE2 stimulation (47). This rapid response to PGE2, is mediated by increased intracellular levels of cAMP, activation of the small GTPase RhoA, and subsequent induction of actomyosin contraction, ultimately leading to rapid podosome dissolution (48). In the present study, we identified a novel function of DCs in premetastatic niche formation in the regional lymph nodes in tumor-bearing mice. As shown in Figure 3, A and B, COX-2 induction in DCs was observed at the early stages of the experiment, suggesting that COX-2 regulates the formation of the premetastatic niche in the regional lymph nodes. SDF-1 induction in DCs was dependent on COX-2 and EP3 signaling, and blockade of the SDF-1/CXCR4 axis suppressed the formation of the premetastatic niche.

The chemokine receptor CCR7 is known as important for the adhesion and chemotaxis of leukocytes and DCs toward lymph nodes. Forster et al. reported that DC migration to lymph nodes was strictly dependent on CCR7 and its ligand in both the steady state and inflammation, and accordingly Ccr7 KO DCs failed to migrate to lymph nodes. Thus, by bringing together T cells, B cells, and DCs to form functional microenvironments in secondary lymphoid organs, CCR7 has been identified as a major homing receptor and important regulator for initiating an antigen-specific
immune response (49, 50). The association of COX-2 and CCR7 with LNM of breast cancer has been already reported (51). In the current study the expression of CCR7 in regional lymph nodes was suppressed in celecoxib-treated mice compared with vehicle-treated mice. This result showed the possibility that COX-2–derived PGE₂ may induce DC migration that depends on CCR7.

FACS analysis was useful for characterizing and quantitating cell subsets in lymph nodes. Though we performed FACS for regional lymph nodes with the IntraStain FACS kit, we could not detect and obtain the expected results. As a substitution for this FACS analysis, we picked up mediastinal lymph nodes from the vehicle group and the Ep3 KO group at 5 days and sorted CD11c-positive cells by FACS after homogenization; when we assayed the sorted cells for Cox2 and Sdf1 by RT-PCR, the message of COX-2 and SDF-1 was significantly suppressed in the Ep3 KO group compared with the vehicle-treated group (Supplemental Figure 3, A and B). We thought that the quantitative analysis by RT-PCR was more sensitive, and we could substitute it for FACS analysis.

In vitro assays using isolated DCs showed that EP3 signaling upregulated SDF-1 expression (Figure 3, H and I). These findings revealed the crucial role of COX-2 and EP3 signaling in the formation of a premetastatic niche via the SDF-1/CXCR4 chemokine axis. The significance of EP3 signaling for DCs can be tested in mice with DC-specific conditional knockout. Use of Tpfl/fl mice in combination with Cd11c-Cre mice will provide a good opportunity to test the function of EP3 in DCs. At present, there is no good marker for DCs besides CD11c. But CD11c is expressed on other types of cells than DCs. We expect more suitable approaches to test DC-specific inhibition of EP3 signaling that regulates premetastatic niche formation.

We further demonstrated that Tregs were recruited to the regional lymph nodes via COX-2/EP3 and SDF-1. Tregs, formerly known as suppressor T cells, are a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens, and abrogate the development of autoimmune disease (52). Studies in mouse models revealed the potential of Tregs for the treatment of autoimmune diseases and cancer, and for organ transplantation. Tregs were selectively recruited within lymphoid infiltrates and activated by mature DCs, likely through the recognition of tumor-associated antigen presentation, resulting in the prevention of effector T cell activation, immune escape, and, ultimately, tumor progression (53). Mansfield et al. reported that FOXP3⁺ cells were associated with more advanced disease in breast cancer, a finding that is proven to be true in many other cancers (54). DCs have been found to promote Treg differentiation and may become a suitable target to abrogate the development of T cell tolerance and to promote an effective immune response to breast cancer (54). As shown in Figure 5B, the recruitment of Tregs was observed in the regional lymph node premetastatic niche, and it was suppressed by COX-2 inhibition, EP3 blockade, and SDF-1 blockade. To our knowledge, this is a novel finding, showing the interplay between prostanoids and Tregs. Tregs expressed CXCR4 (Supplemental Figure 4D); thus, SDF-1 secreted by DCs may act as a major attractant of Tregs to the premetastatic niche. This may increase the immunosuppressive potential of Tregs and facilitate further metastasis.

Furthermore, TGF-β, which is a kind of cytokine produced by Tregs, has been implicated in tumor progression. The TGF-β pathway has been implicated in many of these metastatic processes and has been shown to dramatically impact the ability of tumor cells to spread throughout the body (55–58).

We previously reported that lymphangiogenesis in a Matrigel subcutaneous implantation model, which mimics tumor stromal reactions, was enhanced by COX-2 and EP3/4 signaling (12). In addition, topical implantation of both VEGF-C– and VEGF-A–overexpressing tumor cells induced lymphangiogenesis in sentinel lymph nodes (32). However, a more precise evaluation of lymph node lymphangiogenesis showed that lymphangiogenesis took place in the effferent lymphatic area (Figure 6D), whereas the premetastatic niche was formed in the subcapsular regions of the sentinel lymph nodes. The positive cells of prolymphangiogenic factors are tumor cells, macrophages, and DCs. Supplemental Figure 6 shows that VEGF-C, VEGF-D, and VEGFR3-positive cells, accumulated in the effferent side of lymph nodes, were positive for CD11b and CD11c. Those results suggested that lymphangiogenesis induced by CD11c-positive and CD11b-positive cells was dependent on COX-2/PGE₂–EP3 signaling. We previously reported that PGE₂ stimulated the induction of VEGF-A/ C, and that VEGF-A induced COX-2 (12, 59–61). COX-2/EP3–dependent induction of SDF-1 in DCs localized in subcapsular regions may induce formation of the premetastatic niche for future colonization by LLC cells. It will be important to determine the exact mechanisms by which lymph node lymphangiogenesis induces further tumor cell metastasis to the lymph nodes and other tissues. Although the induction of lymph node lymphangiogenesis and subsequent DC motility following adjuvant-induced skin inflammation has been described (62), our study demonstrated that COX-2–positive SDF-1–producing DCs are the major cell component driving niche formation, whereas PGs regulate lymph node lymphangiogenesis. Lymph node lymphangiogenesis may decide the fate of the colonizing tumor cells, and blockade of PG biosynthesis and receptor signaling may prevent further tumor cell metastasis to the lymph nodes and other tissues.

In conclusion, our study shows that endogenous COX-2–derived PGE₂, stimulated the EP3 receptor on DCs, leading to the upregulation of SDF-1 expression in the subcapsular regions in regional lymph nodes following LLC cell injection into the lungs. SDF-1 upregulation facilitated the accumulation of CXCR4⁺ LLC cells and the formation of the regional lymph node premetastatic niche (Figure 6E). We also found that accumulation of Tregs and lymph node lymphangiogenesis, both of which may decide the fate of metastasized tumor cells, were COX-2/EP3–dependent (Figure 6, F and G). Taken together, our findings suggest that PGE₂ inhibitors, together with SDF-1 receptor antagonists and EP3 antagonists, may be promising agents for the suppression of premetastatic niche formation and LNM.

Methods
Supplemental Methods are available online with this article; doi:10.1172/JCI73530DS1.

Cell lines. LLC cells (cell number LL/2 [LLC1] [ATCC CRL-1642]) originally isolated from C57BL/6 mice were cultured at 37°C in DMEM (Gibco; Life Technologies) supplemented with 10% FBS (Gibco; Life Technologies) in a humidified atmosphere containing 5% CO₂.

LLC cells were purchased from Riken BioResource Center Cell Bank (RBRV-RCB2638).
**Retroviral transfection of GFP gene.** Murine GFP cDNA was cloned into a deficient retroviral vector pLEGFP (Clontech; Takara), then transfected into PT67 cells (Clontech; Takara), followed by G418 (Roche) selection. The resulting temporary infectious recombinant virus containing GFP was infected into NIH/3T3 cells, followed by G418 selection in order to evaluate the infectious titer of those viruses, and the resulting titer was approximately 1 × 10^6 CFUs/ml. LLC cells were infected with these temporary infectious retroviruses and selected with G418, then designed as LLC-GFP.

**Animals and drugs.** Male C57BL/6 mice, 6–8 weeks old and weighing 20–25 g, were obtained from CLEA Japan. Ep3 KO mice on a C57BL/6 hybrid background were generated by our research group. The mice (male, 8 weeks old) were maintained at a constant humidity (60% ± 5%) and temperature (20°C ± 1°C) and were kept continuously on a 12-hour light/dark cycle. All animals were provided with food and water ad libitum.

A COX-2 inhibitor, celecoxib (100 mg/kg per day; Pyser), and a CXCR4 antagonist, AMD3100 (10 mg/kg per day; Sigma-Aldrich), were administrated orally every day. A CXCR4 neutralizing antibody (10 mg per mouse; clone 2B11; BD Biosciences) was injected i.p. daily.

KO mice were sacrificed using (10 mg per mouse; clone 2B11; BD Biosciences) was injected i.p. daily. Harvested cells were washed 3 times with PBS solution and homogenized using a QIA Shredder (QIAGEN). Total RNA was extracted from the homogenized tissues and cells using the RNeasy Mini Kit (QIAGEN), and single-stranded cDNA was generated from 1 μg of total RNA via reverse transcription using ReverTra Ace (TOYOBO Co. Ltd.), according to the manufacturer’s instructions. Quantitative PCR amplification was performed using SYBRR Premix Ex Taq (Takara Bio Inc.). The RT-PCR primers for Cox2, Tgfβ, Sdf1, Vegf, Vegfa, and Vegfr5 were designed using Primer3 software (http://primer3.sourceforge.net) on the basis of data obtained from GenBank.

The following primers were used for real-time RT-PCR: Gapdh forward 5′-ACATCAAGAGGTGGTGAAGC-3′, reverse 5′-AAGTTGGAAGTGGGAATGG-3′; GFAP sense 5′-ACTACAAAGCGCAACGTCT-3′, antisense 5′-GGTTTCTGCTGATGTC-3′; COX-2 sense 5′-TGGCTGAGGAAAGGAATAAGG-3′, antisense 5′-CATCATATTTGACCCCTTGGG-3′; SDF-1 sense 5′-CAGAGCCAACTACGCAAGC-3′, antisense 5′-AGGATCTCTTG-GATCCAC-3′; TGFβ-1 sense 5′-AAACATCTCCGCGCTTACCTT-3′, antisense 5′-TGATTTCCGTCCTTTTGTC-3′; VEGF-C sense 5′-TCGTCGTCGGCAATCGTATG-3′, antisense 5′-GCTCCCT-GTCCCTGTATTGAG-3′; VEGF-D sense 5′-CCATTGACAGCT-GTGGGAT-3′, antisense 5′-GTCGTTCTCCGGTGAAGAG-3′; VEGFR3 sense 5′-TTTATGTCCACCCCTCCTAC-3′, antisense 5′-GGCTGAGCTACAAGCAATCG-3′; CCR7 sense 5′-TATCATGGCGCAGGCAAGAC-3′, antisense 5′-ATACATGAGGAGCAG-GAACCA-3′ (Sigma-Aldrich).

**Bone marrow transplantation.** Bone marrow transplantation was performed as previously described (63). Donor bone marrow cells from Ep3 KO mice and their WT counterparts were harvested, and the bone marrow mononuclear cells from each donor (2.0 × 10^6 cells in 200 ml PBS) were injected into the tail veins of WT mice irradiated with 9.0 Gy using an MBR-1505R X-ray irradiator (Hitachi Medical Co.) with a filter (copper, 0.5 mm; aluminum, 2 mm), while the cumulative radiation dose was monitored.

**Bone marrow-derived DCs and cell culture.** Bone marrow cells were isolated from the femur and tibia of donor male GFP transgenic WT or GFP-positive Ep3 KO mice (a gift from M. Okabe, Genome Information Research Center, Osaka University, Osaka, Japan). GFP-positive
and Ep3 KO DCs were prepared from the mice by cross-breding of GFP transgenic mice and Ep3 KO mice. The cells were cultured (1 × 10^6 cells/ml, 5 ml/well) in GM-CSF–containing DMEM supplemented with 10% FBS at 37.1°C in 5% humidified CO2 as previously described (64). The culture medium was changed every day to remove nonadherent granulocytes. On day 8, cells were harvested by light pipetting. More than 90% of the cells expressed CD11c by FACS analysis. DCs were stimulated with PGE2 (0.1 nM per well) for 3, 6, and 24 hours.

In vitro SDF-1 and TGF-β1 expression. To assess DCs' expression of SDF-1 and TGF-β1 in vitro, DCs were stimulated with PGE2 and EP1-EP4 agonists for 3, 6, and 24 hours in serum-free medium. The culture medium was collected and stored at −20°C until analysis. The medium was used to assess SDF-1 and TGF-β1 levels using specific ELISA kits (R&D Systems). ELISA kits were also used to measure SDF-1 and TGF-β1 levels in the cell culture supernatants. This experiment was performed in duplicate of serum-free medium.

Statistics. Data are expressed as the means ± SD. Comparisons between multiple groups were performed by ANOVA followed by Scheffe’s test. Comparisons between 2 groups were made by 2-tailed Student’s t test. The correlation between the number of DCs and metastasis was analyzed using the χ2 test. P < 0.05 was considered statistically significant.

Study approval. Animal studies were approved by the local ethics committee, and all experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Acknowledgments

We thank Mieko Hamano, Michiko Ogino, and Kyoko Yoshikawa for their technical assistance, Masato Saegusa and Miki Hashimura for their suggestions on pathological technique, and Makoto Murakami and Yoshitaka Taketomi (Tokyo Metropolitan Institute of Medical Science) for their suggestions on FACS experiments. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, 23116102, 24649119, 26462132, and 26293055; Kitasato University Research Grant for Young Researchers; a grant from the Takeda Science Foundation; and a grant from Kitasato University Graduate School of Medical Sciences (Integrative Research Program 2013–2014).

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