

# Blocking IL1 $\beta$ Pathway Following Paclitaxel Chemotherapy Slightly Inhibits Primary Tumor Growth but Promotes Spontaneous Metastasis

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## Abstract

Acquired resistance to therapy is a major obstacle in clinical oncology, and little is known about the contributing mechanisms of the host response to therapy. Here, we show that the proinflammatory cytokine IL1 $\beta$  is overexpressed in response to paclitaxel chemotherapy in macrophages, subsequently promoting the invasive properties of malignant cells. In accordance, blocking IL1 $\beta$ , or its receptor, using either genetic or pharmacologic approach, results in slight retardation of primary tumor growth; however, it accelerates metastasis spread. Tumors from mice treated with combined therapy of paclitaxel

and the IL1 receptor antagonist anakinra exhibit increased number of M2 macrophages and vessel leakiness when compared with paclitaxel monotherapy-treated mice, indicating a prometastatic role of M2 macrophages in the IL1 $\beta$ -deprived microenvironment. Taken together, these findings demonstrate the dual effects of blocking the IL1 pathway on tumor growth. Accordingly, treatments using "add-on" drugs to conventional therapy should be investigated in appropriate tumor models consisting of primary tumors and their metastases. *Mol Cancer Ther*; 14(6); 1385–94. ©2015 AACR.

## Introduction

Chemotherapy is commonly used for the treatment of several malignancies. Although treatment efficacy is usually achieved, tumor relapse and metastasis spread are often observed (1). Thus, ongoing efforts are being made to overcome the resistance of cancer cells to chemotherapy, and to prevent metastases.

In recent years, some of these efforts are directed toward understanding the diverse and dual nature of the tumor microenvironment, as manifested in response to anticancer treatments. For instance, preclinical studies demonstrated that on one hand, chemotherapy supports the secretion of various protumorigenic factors from macrophages and myeloid-derived suppressor cells (MDSC; refs. 2, 3), and on the other, it acts against tumor cells by inducing the immune cell response (4, 5). In addition to the local tumor microenvironment, the host response to chemotherapy may also promote tumor regrowth and metastasis by different means, including rapid mobilization and tumor homing of

different bone marrow-derived cells (BMDC; ref. 6) and upregulation of proangiogenic and prometastatic factors (7). As such, preclinical and clinical testing of promising add-on therapies that target the induction of host response components provide ample support for their use as novel antitumor strategies (8).

The inflammatory cytokine IL1 $\beta$  has been studied in a considerable number of malignancies. Its correlation with poor prognosis in patients with cancer has established a ground for the identification of its proangiogenic and prometastatic role in cancer (9). The induction of IL1 $\beta$  expression in the spleen of mice treated with 5-FU and gemcitabine chemotherapies has led to studying whether targeting its pathway can improve therapy outcome (3). Indeed, the IL1R antagonist (IL1Ra, anakinra), which is approved for the treatment of rheumatoid arthritis in patients (10), was shown to abrogate the protumorigenic effect of naturally occurring and posttherapy-induced IL1 $\beta$  (3). However, although the prometastatic role of IL1 $\beta$  is bona fide and repeatedly reported in various preclinical models, the actual consequence of postchemotherapy-induced IL1 $\beta$  blockade has not yet been fully explored.

Here, we show that IL1 $\beta$  levels are elevated in response to paclitaxel chemotherapy. Blocking IL1 $\beta$  following paclitaxel slightly inhibits the growth of primary tumors; however, its potential as a therapeutic strategy for cancer treatment is doubtful, since it is shown to increase spontaneous metastatic spread.

## Materials and Methods

### Mice

Six- to 8-week-old C57Bl/6 IL1 $\beta$ <sup>-/-</sup> knockout mice and their wild-type counterparts (from Ben-Gurion University of the Negev, Israel), C57Bl/6, and BALB/c (Harlan) were used in experimental protocols approved by the Animal Care and Use Committee of the Technion-Israel Institute of Technology (Haifa, Israel).

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**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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### Tumor cell lines

Lewis lung carcinoma (LLC) and MDA-MB-231 human breast carcinoma (obtained from the ATCC) were cultured in DMEM supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 4 mmol/L HEPES at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. 4T1 murine mammary adenocarcinoma cancer cells (ATCC) were cultured in RPMI-1640 supplemented with 10% (v/v) FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cells were obtained between 2008 and 2010, and they were passed in culture for no more than 4 months after being thawed from authentic stocks. Cell lines were routinely tested for mycoplasma contamination.

### Drug treatment protocol

For *in vivo* studies, paclitaxel chemotherapy (25 mg/kg for BALB/c or 50 mg/kg for C57Bl/6 mice) was administered intraperitoneally as a single bolus injection, in doses previously described (6). Anakinra (Kineret, Biovitrum; 10 mg/kg) was injected intraperitoneally daily, starting one day before chemotherapy administration, and as indicated in the text. Control mice were administered with the relevant vehicles. For *in vitro* studies, cells were exposed to 200 nmol/L paclitaxel chemotherapy or 0 to 100 ng/mL anakinra. In some experiments, depletion of macrophages was achieved by using clodronate-containing liposomes (clodrolip), as previously published (11) and as detailed in Supporting Information Online. The liposomes were injected intraperitoneally to mice with 0.15 mL of either control Sham or clodrolip, at a concentration of 3 to 3.5 mg/mL, 48 hours before injection of paclitaxel chemotherapy.

### Tumor implantation

4T1 cells ( $5 \times 10^5$  cells) were orthotopically injected in 50 µL volumes into the inguinal mammary fat pad of BALB/c mice. LLC cells ( $5 \times 10^5$  cells) were subcutaneously injected in the hind flank of 8-week-old C57Bl/6 mice. The tumor size was assessed regularly with Vernier calipers using the formula  $\text{width}^2 \times \text{length} \times 0.5$ . Treatment was initiated when tumors reached either 200 mm<sup>3</sup> or 500 mm<sup>3</sup> as indicated in the text. Mice were randomly grouped ( $n = 5-7/\text{group}$ , as specified in the text) before treatment with paclitaxel, anakinra, paclitaxel, and anakinra, or vehicle control. For an experimental metastasis model, LLC cells ( $2.5 \times 10^4$ ) stably transfected with plasmid for cytoplasmic GFP expression, were intravenously injected via the tail vein ( $n = 4-6$  mice/group). Mouse survival was monitored.

### Bone marrow transplantation

Bone marrow transplantation was performed as previously described (6, 7). Briefly, bone marrow cells were obtained by flushing femurs and tibias of 8-week-old donor C57Bl/6 WT and C57Bl/6 IL1β<sup>-/-</sup> mice. The bone marrow cells ( $5 \times 10^6$  per recipient) were transplanted by tail vein injection into lethally irradiated C57Bl/6 WT recipients (1,000 cGy total body irradiation (250 cGy/minute) using Elekta Precise (Elekta Oncology Systems) linear accelerator 6MeV photon beam radiation (Department of Radiation Therapy, Rambam Medical center, Haifa, Israel). Experiments were performed 4 to 6 weeks after bone marrow cell reconstitution.

### Macrophage isolation and culture

Eight-week-old BALB/c mice were injected intraperitoneally with 3 mL 4% thioglycollate solution (BD Biosciences). Three days later, animals were euthanized and peritoneal lavage was performed. Peritoneal exudate cells ( $5 \times 10^6$  cells) were plated in 60-mm dish (Corning) for 24 hours and cultured in RPMI complemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 4 mmol/L HEPES. Nonadherent cells were removed after 24 hours by washing. For macrophage stimulation, cells were incubated with medium supplemented with 1 mmol/L arginine and LPS (0.5 µg/mL) for 4 hours following one hour exposure to ATP (5 mmol/L) or paclitaxel (200 nmol/L). In some experiments, macrophages were cultured in the presence of recombinant murine IL1β (1 pg, 10 pg, or 100 pg, Peproteck). All experiments were performed in triplicates.

### Modified Boyden chamber assay

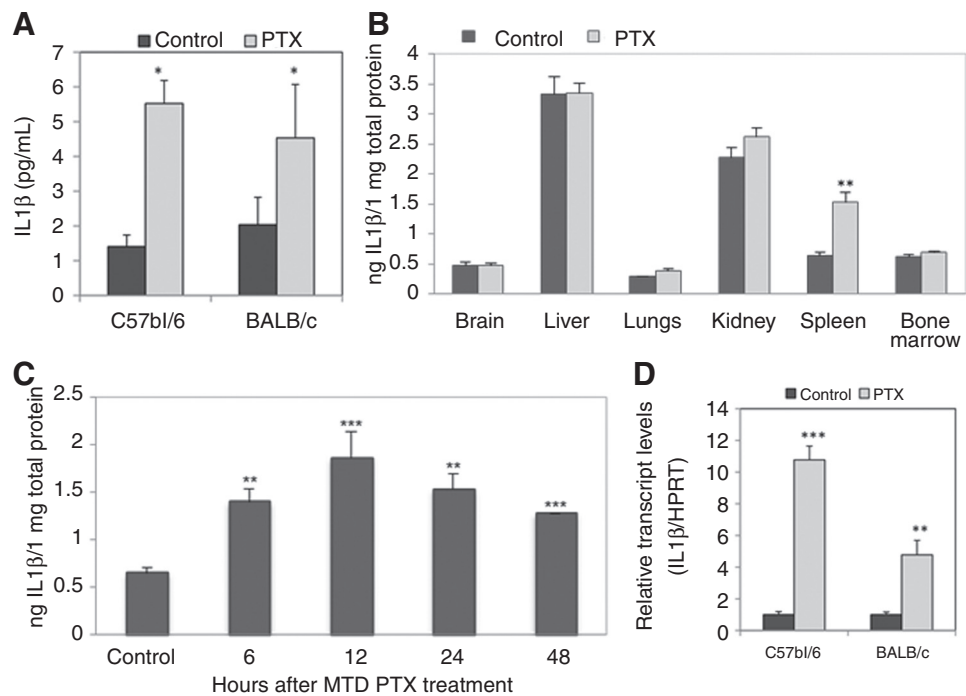
Invasion assay was performed using modified Boyden chambers as previously described (7). Briefly, filters (6.5 mm in diameter, 8 µm pore size) were coated with Matrigel (BD Biosciences) and dried for 2 hours at 37°C. Serum-deprived LLC and MDA-MB-231 cells ( $2 \times 10^5$ ) were seeded on the upper compartment of the chamber. The lower compartment was filled with serum-free DMEM that contained 10% plasma from non-tumor-bearing mice that were treated for 24 hours with paclitaxel, anakinra, combined treatment of paclitaxel and anakinra, or vehicle control; with plasma obtained from WT IL1β or IL1β<sup>-/-</sup> bone marrow transplanted mice; with peritoneal macrophages, recombinant IL1β (in escalating concentrations), or the combination of the two; or with plasma depleted from IL1β using a specific neutralizing antibody (15 µg; R&D systems) followed by immunoprecipitation and depletion using Protein A/G mixed Sepharose beads. After incubation for 6 and 18 hours at 37°C in a 5%CO<sub>2</sub> incubator, the cells that invaded to the bottom filter, were stained with 0.5% crystal violet (Sigma) and counted under an inverted microscope (Lieca DMIL LED) per  $\times 100$  objective field. All *in vitro* Boyden chamber experiments were performed in triplicates.

### ELISA

Non-tumor-bearing paclitaxel-treated and control C57Bl/6 and BALB/c mice were euthanized by isoflurane inhalation and blood was collected via cardiac puncture. For BALB/c mice, organs such as brain, liver, lungs, kidney, spleen, bone marrow, and blood were obtained from the mice at several time points after they were treated with paclitaxel or vehicle control ( $n = 5$  mice/group). Organs were homogenized in PBS containing 20 mmol/L HEPES, 100 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton, and a protease inhibitor mixture (Roche Diagnostics). After lysate centrifugation, supernatant was collected. In separate experiments, a total of 200 to 300 mm<sup>3</sup> of LLC tumor tissue ( $n = 5-6$  samples/group) or cultured peritoneal macrophages were collected and homogenized as described above. Equal amounts of protein were applied on a mouse IL1β ELISA (R&D Systems, Inc.) in accordance with the manufacturer's instructions. For detection of IL1β levels in plasma, pooled plasma samples were concentrated through Amicon ultra-0.5 centrifugal filter device (at 7-10-fold) before use in order to reach the detection threshold. All experiments detecting IL1β expression levels were performed in triplicates.

**Figure 1.**

Paclitaxel induces host-dependent IL1 $\beta$  production. A and B, eight- to 10-week-old non-tumor-bearing C57Bl/6 and BALB/c mice were treated with vehicle (control) or paclitaxel (PTX) chemotherapy ( $n = 5$  mice/group). After 24 hours, mice were sacrificed and the level of IL1 $\beta$  from pooled plasma (A) and from various organs (for BALB/c; B) was evaluated by ELISA. C, BALB/c mice were treated with vehicle or paclitaxel ( $n = 5$  mice/group) and spleens were removed at 0, 6, 12, 24, and 48 hours posttreatment for the assessment of IL1 $\beta$  expression by ELISA. D, relative IL1 $\beta$  transcript levels in spleens of C57Bl/6 and BALB/c mice treated with paclitaxel for 24 hours were assessed by quantitative RT-PCR. HPRT served as the internal control. ( $n = 3$  repeats/group). Error bars,  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



### Flow-cytometric analysis

M1 and M2 macrophages were quantitated using flow cytometry as described (6, 12). Briefly, tumors and lungs from treated and nontreated mice were collected and prepared as single-cell suspensions as previously described (6, 13). M1 macrophages were identified as F4/80<sup>+</sup>, CD11c<sup>+</sup>, CD206<sup>-</sup> CD45<sup>+</sup>; and M2 macrophages were identified as F4/80<sup>+</sup>, CD11c<sup>-</sup>, CD206<sup>+</sup>, CD45<sup>+</sup>, using BioLegend and BD Biosciences monoclonal antibodies conjugated with fluorescent dyes as follows: F4/80- Phycoerythrin (PE); CD11c-Peridinin Chlorophyll Protein (PerCP); CD206-Allophycocyanin (APC); CD45-APC-Cyanin-7 (APC-Cy7). For clodronate liposomes-treated mice, the verification of macrophage depletion was performed using the F4/80 surface marker. Flow-cytometric experiments were performed on Cyan-ADP flow cytometer (Beckman Coulter) and analyzed with Summit Version 4.3 (Beckman Coulter) and FlowJo Version 8 (Ashland) flow-cytometric analysis software.

### Quantitation and visualization of tissues

Tissue processing was performed as previously described (6). Briefly, tissue cryosections (10  $\mu$ m) were used to analyze blood vessel perfusion by the DNA-binding dye Hoechst 33342 (40 mg/kg; Sigma-Aldrich) or lung metastasis using hematoxylin and eosin (H&E) or DAPI. Vessels were immunostained with endothelial cell-specific antibody (anti-CD31, 1:200 ratio, BD Biosciences), and the number of vessel structures per field were counted and plotted (5 fields/tumor;  $n > 20$  fields per group). Metastatic lesions were counted per field, and GFP<sup>+</sup> lung metastases were analyzed by flow cytometry as described above. Sections were visualized under a Leica CTR 6000 microscope (Leica Microsystems) using Leica Application Suite Version 3 or the 3DHISTECH Panoramic MIDI system using a HITACHI HV-F22 color camera and the Panoramic Viewer visualization and analysis software.

### Gene expression analysis by RT-PCR

RNA was extracted from spleen or blood obtained from mice as indicated in the text, using the RNeasy Mini Kit (QIAGEN), and subsequently converted into cDNA by reverse transcriptase (Promega). The gene expression of mouse IL1 $\beta$  was determined by 7000 Real-Time PCR system (Applied Biosystems). For more details, see Supporting Information Online.

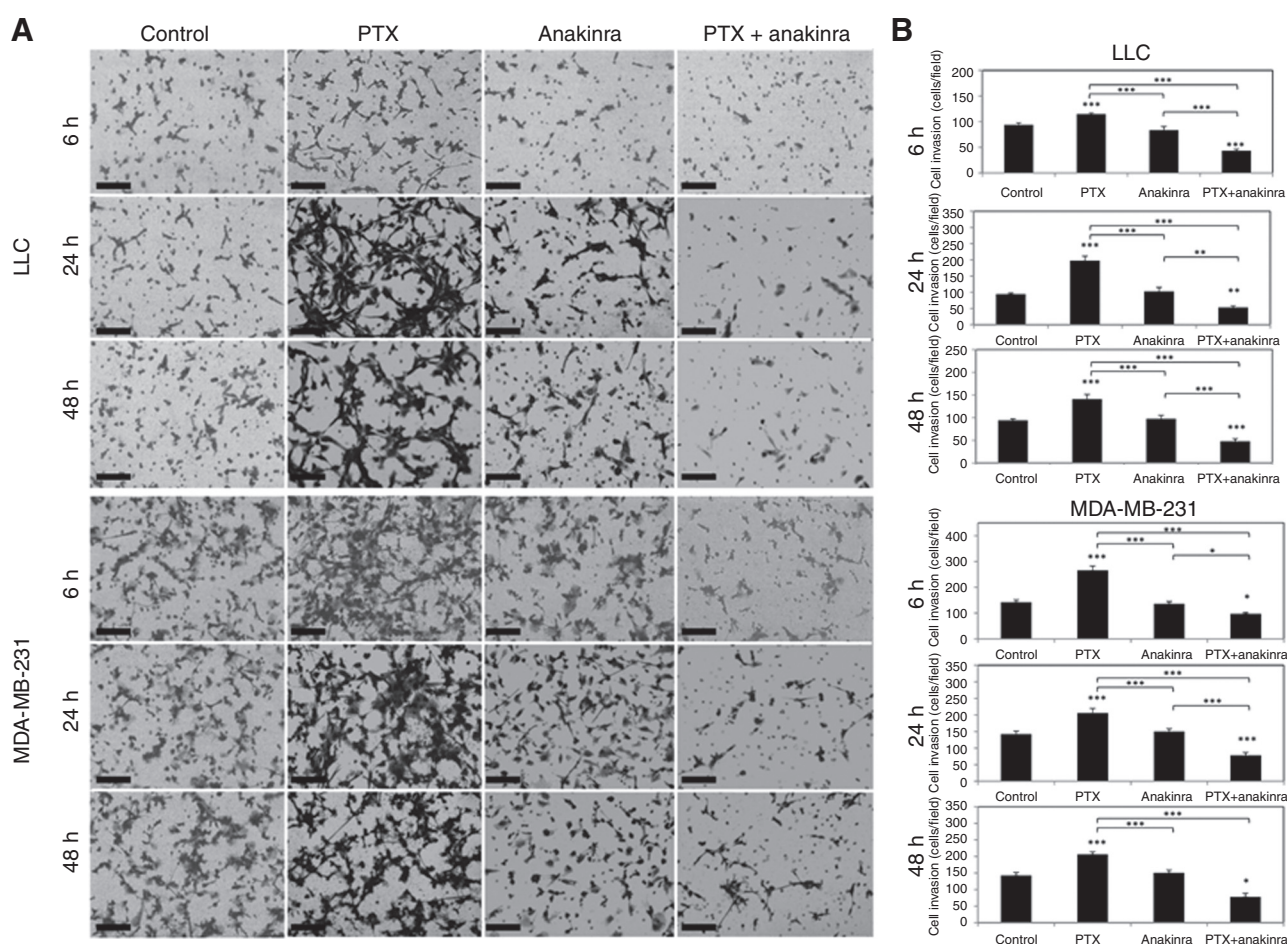
### Statistical analysis

Data are expressed as mean  $\pm$  SD, and the statistical significance of differences was assessed by one-way ANOVA, followed by Newman-Keuls ad hoc statistical test using GraphPad Prism 4 software (GraphPad Software, Inc.). Differences between all groups were compared with each other, and were considered significant at values of \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ .

## Results

### Host response to paclitaxel chemotherapy contributes to tumor cell invasiveness in an IL1-IL1R-dependent manner

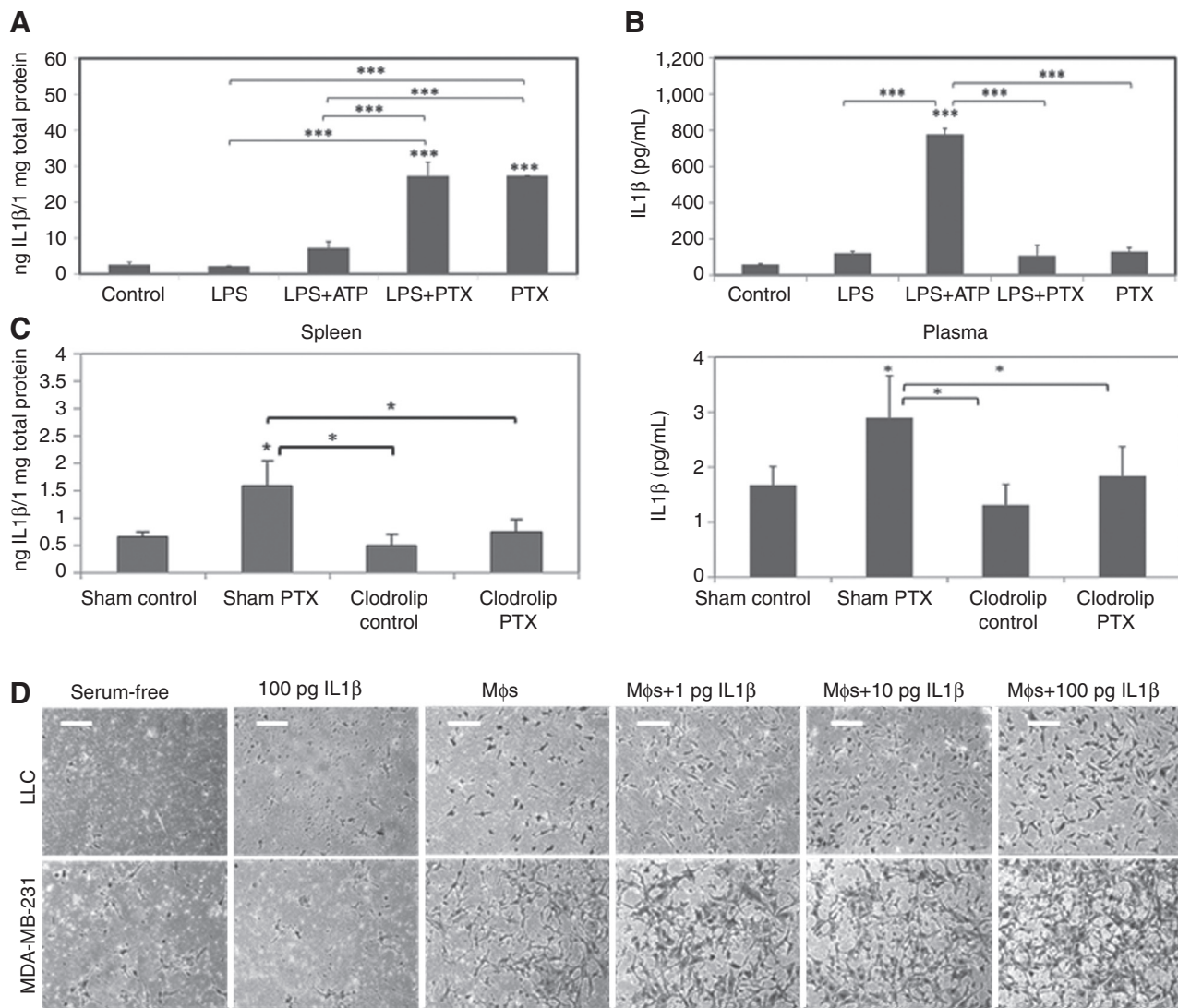
We previously demonstrated that paclitaxel chemotherapy administered to non-tumor-bearing mice induces the production of systemic factors that promote tumor cell migration, invasion, and metastasis (7). In order to identify such factors and to distinguish between host and tumor cell-derived effects following chemotherapy, we screened plasma samples from non-tumor-bearing C57Bl/6 and BALB/c mice administered with paclitaxel. IL1 $\beta$  levels were significantly elevated 24 hours following paclitaxel administration (Fig. 1A). This induction in IL1 $\beta$  levels was blocked by concomitant administration of paclitaxel and anakinra (Supplementary Fig. S1A). Interestingly, levels of IL1 $\beta$  in plasma from anakinra-treated mice were undetectable, but this was not due to the direct effect of anakinra on detection of IL1 $\beta$  by ELISA (data not shown). Of note, levels of matrix metalloproteinase-9 (MMP9) were substantially increased in the plasma from



**Figure 2.** Anakinra blocks invasive paclitaxel-induced tumor cell invasion *in vitro*. Eight- to 10-week-old non-tumor-bearing BALB/c mice were treated with vehicle (control), paclitaxel, anakinra, or the combination of paclitaxel and anakinra. Plasma was extracted at 6, 24, and 48 hours after treatment administration. A, invasion properties of murine LLC and MDA-MB-231 human breast cancer cells were evaluated by the modified Boyden chamber assay in the presence of the plasma samples (magnification,  $\times 100$ ; scale bar, 200  $\mu\text{m}$ ). B, quantification of invading cells was performed using ImageJ software (NIH). Error bars,  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

paclitaxel-treated mice when compared with their levels in control mice, similar to a previous report (7); however, MMP9 levels were decreased in plasma from mice treated with paclitaxel+anakinra (Supplementary Fig. S1B). Focusing on BALB/c mice, the analysis of IL1 $\beta$  levels in different organs revealed that the spleen was the exclusive organ exhibiting a significant increase in IL1 $\beta$  levels following paclitaxel therapy (Fig. 1B), similar to gemcitabine and 5-FU chemotherapies as previously reported (3). IL1 $\beta$  levels in the spleen but not in other organs peaked at 12 hours and remained significantly high for at least 48 hours (Fig. 1C and Supplementary Fig. S1C). These results were confirmed by analyzing IL1 $\beta$  mRNA expression in the spleen 24 hours following paclitaxel administration. However, IL1 $\beta$  mRNA expression levels were lowered when paclitaxel was administered in combination with anakinra. Comparable results were observed when C57Bl/6 mice were treated with paclitaxel as assessed by RT-PCR (Fig. 1D and Supplementary Fig. S1D). To directly assess the contribution of therapy-induced, host-derived IL1 $\beta$  to the promotion of tumor cell invasiveness as we reported in previous study (7), we performed a modified Boyden chamber assay in the presence of

plasma obtained from non-tumor-bearing mice treated with paclitaxel, anakinra, or the combination of the two drugs at 6, 24, and 48 hours posttreatment. Plasma from paclitaxel-treated mice induced LLC and MDA-MB-231 tumor cell invasion, an effect that was significantly blocked when paclitaxel treatment was combined with anakinra (Fig. 2). Of note, an increase in invasion properties of tumor cells was also demonstrated in a previous study where tumor cells were exposed to plasma from LLC tumor-bearing mice treated with paclitaxel chemotherapy (7). We verified that neither anakinra nor IL1 $\beta$ -depleted plasma directly affects tumor cell invasion (Supplementary Fig. S1E). Consistently, the motility of MDA-MB-231 cells was greater in the presence of plasma from non-tumor-bearing paclitaxel-treated mice, when compared with plasma from vehicle, anakinra, or paclitaxel+anakinra-treated mice as assessed by wound scratch assay (Supplementary Fig. S1F). We should note that the motility assay could not be performed on LLC cells due to their growth pattern in culture. Overall, these results demonstrate that paclitaxel induces host-dependent secretion of IL1 $\beta$ , which may account for cancer cell invasion properties.



**Figure 3.** Paclitaxel promotes secretion of IL1 $\beta$  from macrophages. A and B, peritoneal macrophages obtained from 8-week-old BALB/c mice ( $n = 4$  mice/group) were treated with vehicle control or paclitaxel, or stimulated for 4 hours with LPS alone or following one hour exposure to ATP or paclitaxel. The level of IL1 $\beta$  in the macrophage lysate (A) or medium from cultured macrophages (B) was assessed by ELISA. C, eight-week-old BALB/c mice underwent macrophage depletion using clodronate-containing liposomes (clodrolip) as described in Materials and Methods. Sham liposome injection was set as control ( $n = 5$  mice/group). Forty-eight hours later, the mice were treated with vehicle (control) or paclitaxel (PTX). After 24 hours, mice were sacrificed, plasma was pooled, and spleens were removed and evaluated for IL1 $\beta$  expression using ELISA. The experiment was repeated three times on pooled plasma samples. D, the invasive properties of LLC and MDA-MB-231 cells were evaluated using the modified Boyden chamber assay, with different concentrations of recombinant IL1 $\beta$  in the presence or absence of macrophages cultured in the lower chamber (magnification  $\times 100$ , scale bar, 200  $\mu\text{m}$ ). Quantification of cells/field is shown in Supplementary Fig. S2D. Error bars,  $\pm$  SD. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

Paclitaxel stimulates macrophages to secrete IL1 $\beta$  promoting the invasive properties of cancer cells

Macrophages have been described as a major source of IL1 $\beta$ , which is produced and secreted by the inflammasome-mediated inflammatory response (14, 15). Therefore, we first assessed the effects of paclitaxel on macrophage IL1 $\beta$  synthesis and secretion, in order to explain the elevation in IL1 $\beta$  levels in plasma and spleen following paclitaxel treatment. Previous work has indicated that paclitaxel increases the production of IL1 $\beta$  precursor in monocytes, but not its secretion (16). Indeed, *in vitro* stimulation of macrophages with a single treatment of paclitaxel resulted in

production of IL1 $\beta$  within macrophages, which was not further increased following activation with LPS, a proinflammatory stimulator, as evaluated in macrophage lysates (Fig. 3A). However, the increase in IL1 $\beta$  production did not result in IL1 $\beta$  secretion from the paclitaxel-treated macrophages as compared with macrophages stimulated with LPS in combination with ATP, as evaluated in the conditioned medium of macrophages (Fig. 3B). These results suggest that in the *in vitro* system used here, macrophages, on their own, do not secrete IL1 $\beta$  in response to paclitaxel due to the absence of additional stimuli that are otherwise may present *in vivo*. We therefore investigated the *in vivo* production and secretion

of IL1 $\beta$  in response to paclitaxel. To do so, macrophages were depleted from BALB/c mice using Clodronate-containing liposomes (Clodrolip) as confirmed by flow cytometry of the spleens (Supplementary Fig. S2A). The mice were then treated with vehicle control or paclitaxel and the level of IL1 $\beta$  in the spleen was assessed by ELISA. Splenic IL1 $\beta$  levels following paclitaxel treatment were significantly higher in *sham* liposome-treated mice when compared with Clodrolip-treated (macrophage-depleted) mice in which their IL1 $\beta$  levels were not significantly different from untreated control mice. Comparable results were observed in IL1 $\beta$  levels in the plasma of mice treated with Clodrolip (Fig. 3C). Moreover, the depletion of macrophages abrogated the proinvasive properties of plasma from paclitaxel-treated mice (Supplementary Fig. S2B). Collectively, these findings suggest that macrophages are a major source of paclitaxel-induced IL1 $\beta$ , at least in the spleen, and the production and secretion of IL1 $\beta$  by macrophages in response to paclitaxel promote cancer cell invasive properties.

We next asked whether IL1 $\beta$  acts directly on malignant cells to promote their invasiveness. Coincubation of LLC and MDA-MB-231 cells with a high dose (100 pg) of recombinant IL1 $\beta$  did not alter the invasive properties of these cells as assessed by the modified Boyden chamber assay (Fig. 3D), despite the expression of the IL1 receptor type I (IL1RI) in these cell lines as assessed by flow cytometry (Supplementary Fig. S2C). These results suggest that paclitaxel-induced IL1 $\beta$  possibly acts on the host to promote tumor cell invasion rather than directly on the malignant cells. Indeed, when macrophages were incubated in the lower compartment of the Boyden chamber along with escalating doses of IL1 $\beta$ , the invasive properties of both LLC and MDA-MB-231 cells were increased in an IL1 $\beta$  dose-dependent manner (Fig. 3D and Supplementary Fig. S2D). Together, these data demonstrate that paclitaxel directs macrophages toward a secretion of mediators influencing the invasive properties of tumor cells in an IL1 $\beta$ -dependent manner.

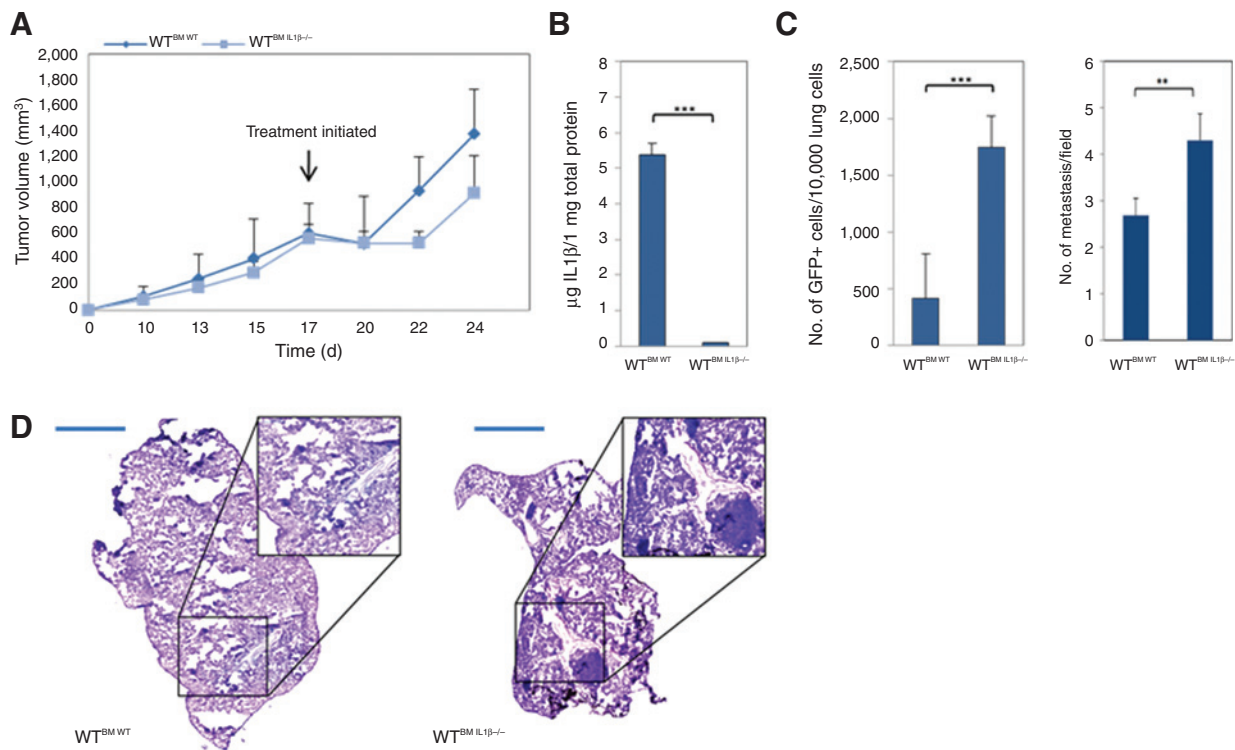
#### IL1 $\beta$ depletion in bone marrow cells or IL1 $\beta$ inhibition using IL1Ra slightly delays primary tumor regrowth following paclitaxel treatment, but increased spontaneous metastases

Having established that paclitaxel administration results in the induction of host-derived IL1 $\beta$ , which potentially accounts for tumor cell invasive phenotype, we proceeded to investigate whether depletion of host-derived IL1 $\beta$  before paclitaxel administration affects tumor progression. To this end, we generated bone-marrow (BM) transplanted WT chimeric mice, in which BMDCs are either from donor WT mice (WT<sup>BMWT</sup>) or from donor IL1 $\beta$ <sup>-/-</sup> mice (WT<sup>BM-IL1 $\beta$ <sup>-/-</sup>}), as indicated in Materials and Methods and as illustrated in Supplementary Fig. S3A. Depletion of IL1 $\beta$  from BMDCs was confirmed by RT-PCR analysis of peripheral blood cells (Supplementary Fig. S3B). The chimeric mice were implanted with LLC-GFP<sup>+</sup> cells and when tumors reached 500 mm<sup>3</sup>, the mice were treated with paclitaxel and tumor volume was assessed over time. Treatment efficacy of paclitaxel was slightly greater in the IL1 $\beta$ -depleted mice (WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) as compared with their WT counterparts (WT<sup>BMWT</sup>; Fig. 4A). Moreover, depletion of IL1 $\beta$  from bone marrow cells resulted in a dramatic reduction in the level of IL1 $\beta$  in tumors of WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) chimeric mice following paclitaxel therapy, confirming that inflammatory BMDCs are the primary source of IL1 $\beta$  in LLC tumor-bearing mice, and not tumor cells (Fig. 4B). Indeed, no significant difference in IL1 $\beta$  expression was observed between LLC tumor cell lysates from</sup></sup></sup>

control mice and mice treated with paclitaxel (Supplementary Fig. S3C). Importantly, histopathological and flow-cytometric analyses of lungs, revealed a higher number of metastatic lesions in WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) mice when compared with WT<sup>BMWT</sup> mice (Fig. 4C and D). To test whether increase in metastasis can be found in an experimental pulmonary metastasis model, indicating whether in IL1 $\beta$ -depleted mice treated with paclitaxel, tumor cell seeding is altered, LLC cells were intravenously injected to WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) or WT<sup>BMWT</sup> mice that underwent paclitaxel therapy. No significant differences in mortality rate were observed between any of the treated groups, indicating that IL1 $\beta$  may affect tumor cell dissemination from the primary tumor but not tumor cell seeding (Supplementary Fig. S3D). These results were further reinforced when plasma from WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) mice treated with paclitaxel did not induce LLC and MDA-MB-231 cell invasion when compared with plasma from paclitaxel-treated WT<sup>BMWT</sup> mice (Supplementary Fig. S3E). To rule out compensatory mechanisms that could activate IL1RI, that is, IL1 $\alpha$ , in WT<sup>BMIL1 $\beta$ <sup>-/-</sup>}) mice, anakinra, which blocks both IL1 $\alpha$  and IL1 $\beta$  signaling, was administered in combination with paclitaxel treatment in various tumor-bearing mouse cohorts. When administered in a long-term setting starting one day before chemotherapy administration followed by daily administrations to the experiment's endpoint, anakinra slightly but not significantly enhanced the antitumor efficacy of paclitaxel in primary orthotopically implanted 4T1 and subcutaneously implanted LLC tumor models, comparable with previous reported results (ref. 3; Fig. 5A and B). However, in a short-term setting, where anakinra was injected only for 3 sequential days starting one day before chemotherapy administration, it did not affect paclitaxel treatment efficacy at all (Supplementary Fig. S3F). Moreover, analysis of histopathological sections from lungs harvested at the end of long- and short-term trials, revealed a significantly higher metastatic burden in mice treated with the combination of paclitaxel and anakinra or anakinra monotherapy, similarly to the experiment performed in Figs. 4A and D and 5C and D; Supplementary Fig. S3G. Collectively, these results indicate that the long-term blockade of IL1 $\beta$  or its receptor in mice either treated with paclitaxel or vehicle control slightly but not significantly reduces primary tumor growth, but at the same time, it also significantly enhances spontaneous metastases.</sup></sup></sup></sup>

#### Administration of anakinra decreases tumor microvessel density yet promotes vessel leakiness

IL1 $\beta$  plays a major role in tumor angiogenesis, which represents one of the main mechanisms of facilitating tumor invasiveness, and its blockade by IL1Ra was shown to reduce the tumor angiogenic response (17, 18). We therefore further characterized the effect of anakinra as a single agent or in combination with paclitaxel chemotherapy on the tumor's vasculature. CD31 staining of LLC and 4T1 tumors revealed that tumor vessel density was significantly lower in tumors treated with anakinra or the combination of paclitaxel and anakinra, when anakinra was administered in a long-term setting (Fig. 6A and B). Furthermore, this reduction in tumor vessel density was accompanied by a higher percentage of perfused tumor areas as revealed by Hoechst staining (Fig. 6C). Consistently, FITC-conjugated dextran injection revealed elevated extravasation of dextran in tumors treated with anakinra or the combination of paclitaxel and anakinra (Supplementary Fig. S4A and S4B).



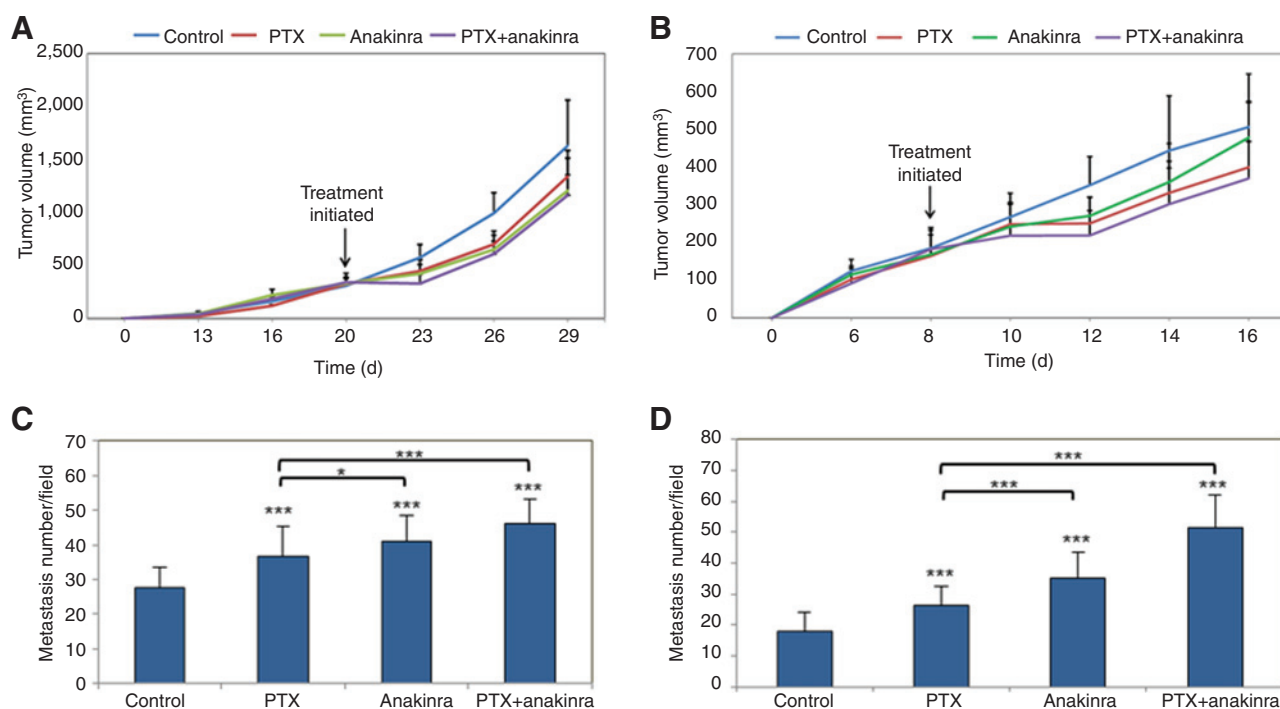
**Figure 4.** IL1 $\beta$  depletion from bone marrow cells promotes spontaneous metastasis. Half a million LLC-GFP<sup>+</sup> cells were implanted to the flanks of chimeric C57Bl/6 mice transplanted with either WT IL1 $\beta$  (WT<sup>BM WT</sup>) or IL1 $\beta$ <sup>-/-</sup> (WT<sup>BM IL1 $\beta$ -/-</sup>) bone marrow cells ( $n = 6$  mice/group). When tumors reached 500 mm<sup>3</sup>, treatment with paclitaxel (PTX) was initiated. A, tumors were measured regularly using Vernier calipers, and tumor volume was assessed over time. B, at endpoint, tumors were removed and the level of IL1 $\beta$  expression was assessed by ELISA. C, the lungs were removed and evaluated for the percentage of GFP<sup>+</sup> cells and the number of metastatic lesions. D, representative lung sections (mosaic) and high magnification (zoom factor 3) are provided (scale bar, 2,000  $\mu$ m). Error bars,  $\pm$  SD. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

Pro-tumoral (M2-like) tumor-associated macrophages (TAM) are associated with chemoresistance and a higher incidence of metastasis formation (12). M2-like TAMs may also affect vascular leakage and could thus potentially influence tumor perfusion and metastasis extravasation and dissemination (19). Because pro-inflammatory cytokines such as IL1 are abundantly expressed by antitumoral, classically activated (M1-like) macrophages (20), we sought to determine whether treatment with anakinra could alter macrophage polarization toward the M2 phenotype. Flow-cytometric analysis of freshly isolated TAMs from 4T1 tumors treated with vehicle, paclitaxel, anakinra, or a combination of the two drugs revealed M1 to M2 phenotype skewing in all treatment groups, with significance in the anakinra-treated groups when compared with control mice (Fig. 6D and Supplementary Fig. S4C). Collectively, these findings may suggest that targeting IL1 $\beta$  restrains primary tumor growth, but also skews TAM polarization toward the M2 phenotype, which in turn may account for vascular leakage and tumor cell dissemination, therefore, leading to metastasis spread.

## Discussion

This study describes a dual role for chemotherapy-induced IL1 $\beta$  in tumor progression. Our previous study has shown that paclitaxel, in addition to its anticancer therapeutic effects, may also promote invasive properties in cancer cells via the induction of

host-derived expression of MMP9 in bone marrow cells, thus facilitating tumor dissemination to distant organs (7). Our current study demonstrates that in addition to MMP9, paclitaxel also induces the production of IL1 $\beta$  by macrophages, and possibly other BMDCs (3), thereby promoting tumor cell invasiveness and angiogenesis. We found that the levels of IL1 $\beta$  in the plasma paralleled MMP9 levels, which could explain the reduced tumor cell invasiveness found in the plasma from mice treated with paclitaxel+anakinra when compared with plasma from paclitaxel-treated mice. Indeed, Huang and colleagues have recently reported that IL1 $\beta$  induces MMP9 expression, leading to metastatic phenotype of gastric adenocarcinoma cells (21). In addition, we found that other tissues, for example, kidney and liver expressed high levels of IL1 $\beta$ , yet the spleen was the exclusive organ tested that exhibited elevated levels of IL1 $\beta$  following paclitaxel therapy. Similarly, chemotherapy such as 5-FU and gemcitabine induces IL1 $\beta$  secretion from cells in the spleen, an effect that is related to the activation of the NOD-like receptor family, pyrin domain containing-3 (NLRP3) in the inflammasome (3). *In vitro*, paclitaxel induced IL1 $\beta$  production but not secretion from macrophages at early time points. It is plausible that IL1 $\beta$  is secreted at later time points when macrophages undergo apoptosis following exposure to paclitaxel or when tumor cells exposed to paclitaxel secrete additional stimuli for macrophages to secrete IL1 $\beta$ . It should be noted that IL1 $\beta$ , on its own, did not result in increased cancer cell invasion phenotype,

**Figure 5.**

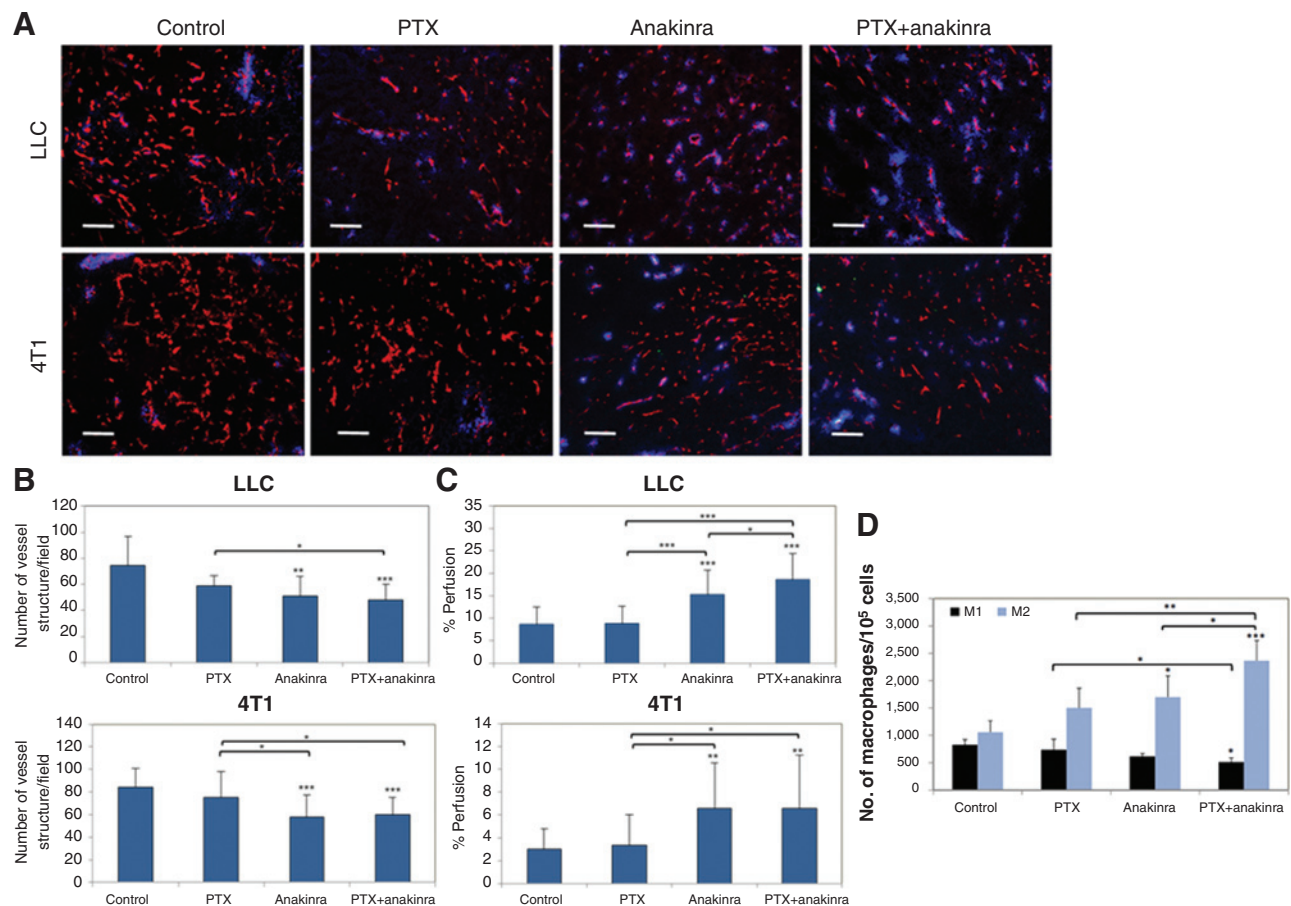
Accelerated metastasis spread in tumor-bearing mice treated with paclitaxel and anakinra. A, LLC and (B) 4T1 tumor-bearing mice ( $n = 7$  mice/group) were treated with paclitaxel, anakinra, or paclitaxel+anakinra as described in Materials and Methods. Tumors were measured regularly using Vernier calipers, and tumor volume was calculated. Tumor growth curves were not statistically different (C and D). At endpoint, lungs were removed for the evaluation and quantification of metastatic lesions in lung sections using H&E staining. Graphs are presented (A and B, relatively to C and D). Error bars,  $\pm$  SD. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ .

but only through additional possible mediators secreted by IL1 $\beta$ -activated macrophages. Overall, these *in vitro* results further suggest that additional variables play a role following the induction of macrophage-derived IL1 $\beta$  expression. To further elaborate on this point, we provide several lines of *in vivo* evidence demonstrating the role of IL1 $\beta$  secreted by macrophages in response to paclitaxel. Specifically, blocking paclitaxel-induced IL1 $\beta$  in mice, either by depleting macrophages or by treatment with a pharmacologic inhibitor, resulted in a severe reduction in IL1 $\beta$  levels in the spleen and plasma. Moreover, exposure of different cancer cell lines to the plasma of macrophage-depleted, paclitaxel-treated mice resulted in a drastic decrease in their invasive phenotype when compared with plasma from paclitaxel-treated mice that did not undergo macrophage depletion. These results are in line with studies that identified IL1 $\beta$  as an invasion mediating factor, and that its blockade may therefore prevent prometastatic effects (22). It should be noted that despite the expression of IL1R1 in the cell lines tested, the IL1 $\beta$ -dependent increase in the invasion phenotype of such cells was indirect and could probably result from the pleiotropic effect of IL1 $\beta$  on prometastatic downstream genes, for example, MMP9 or other proteolytic enzymes, expressed by different host cells, including macrophages (2, 23). We further demonstrated *in vivo*, that mice that underwent paclitaxel therapy, the depletion of IL1 $\beta$  from BMDCs or the blockade of its pathway using anakinra, paradoxically resulted in increased spontaneous metastasis and poor survival in both orthotopically and subcutaneously implanted tumor models. Consistent with our findings, Carmi and colleagues,

have already reported that in IL1 $\beta$ -depleted mice, increased metastasis lesions were observed in the lungs of mice bearing an experimental lung metastasis model (24). Taken together, our results suggest that the depletion of IL1 $\beta$  expressed specifically by BMDCs induces tumor cell invasion phenotype and therefore may account for increased metastasis spread.

In the tumor milieu, chemotherapy such as paclitaxel induces the secretion of chemotactic factors, such as colony stimulating factor-1, which then enhance the infiltration of macrophages and T cells to the treated tumor site, and further promote resistance to therapy (25). TAMs have also been shown to promote tumor growth and metastasis by the secretion of paracrine factors, including IL1 $\beta$  as we describe here, that promote angiogenesis and tumor cell invasion and migration (12). Furthermore, the increased infiltration of macrophages and MDSCs to paclitaxel-treated tumors has been recently shown to induce resistance of tumor cells to therapy, in part due to the secretion of Cathepsin-B from chemotherapy-activated TAMs (2, 3). Therefore, blocking monocyte and TAM infiltration to treated tumors, or inhibiting their activity improves tumor cell chemosensitivity and reduces vascular density (25). However, as shown here, the blockade of IL1 $\beta$  in the tumor microenvironment polarized TAMs away from the M1-like phenotype toward the prometastatic M2-like phenotype. TAM polarization toward the M2-like phenotype was previously linked to vessel abnormalization (19). Indeed, although addition of anakinra to paclitaxel decreased microvessel density in treated tumors, vessel leakiness was profound, and could explain, albeit indirectly, the increased spontaneous metastasis seen. It is





**Figure 6.** Tumors treated with the combination of paclitaxel and anakinra exhibit increased vessel perfusion and M2 macrophage colonization. A, LLC and 4T1 tumor-bearing mice ( $n = 7$  mice/group) were treated with paclitaxel (PTX), anakinra, or paclitaxel+anakinra, in the dose and schedule indicated in Materials and Methods. When tumors reached 500 mm<sup>3</sup>, they were removed and immunostained with anti-CD31 antibodies (red) to detect endothelial cells, and Hoechst (blue) to detect vessel perfusion (Scale bar, 200  $\mu$ m). Microvessel density (B) and percentage of perfusion (C) were calculated. D, in parallel, 4T1 tumors were prepared as single-cell suspensions for the evaluation of M1 and M2 macrophage colonization of tumors using flow cytometry. The number of macrophages was calculated as a percentage per total 10<sup>6</sup> cells. Error bars,  $\pm$  SD. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ .

important to note that long-term disruption of the IL1-IL1R pathway regardless of paclitaxel therapy, promoted M1 to M2 TAM phenotype skewing, leading to increased vessel leakiness and subsequent metastasis spread. Taken together, our data suggest that IL1 $\beta$  may have dual effect, as an indirect enhancer of the invasive potential of malignant cells, and as an antitumor, inflammatory-modulator of TAMs, in the tumor milieu.

Another noteworthy preclinical value that can be drawn from this study is related to the therapeutic evaluation in preclinical models of add-on drugs to chemotherapy. Bruchard and colleagues have clearly shown that IL1 $\beta$  blockade increased chemotherapy efficacy, when focusing on its therapeutic activity in primary tumors (3, 26). In our models, we found quite comparable results when treating breast or lung carcinomas with paclitaxel in combination with anakinra, although the treatment efficacy of the combination therapy did not reach statistical significance. However, the combined therapy increased metastatic burden in the mice' lungs when compared with mice treated with paclitaxel monotherapy. When the same therapy was performed on an experimental lung metastasis model, whereby tumor cells were

injected intravenously via the tail vein and seeded in the lungs, the depletion of IL1 $\beta$  actually did not significantly change the therapeutic activity of paclitaxel. The failure of reproducing therapeutic findings of preclinical studies in the clinic is a major concern, and it is related, in part, to the preclinical models used for drug testing. For example, studies have shown that the combination of several anticancer therapies improve therapy outcome at the primary tumor level, whereas this treatment is ineffective in metastatic disease (27, 28). Although anakinra, which presumably blocks the IL1 pathway, was theoretically and preclinically offered as an add-on drug to be evaluated in the clinic along with angiogenesis targeting agents (29), recent studies indicated that anakinra activity might be different between tissues, therefore allowing diverse and even opposite effects on host and tumor cells (30, 31). Indeed, our study provides preclinical evidence for the possible therapeutic disadvantage of such treatment combinations. We demonstrate that the use of long-term IL1 $\beta$  blockade alone or along with chemotherapy, although may slightly enhance the antitumor activity of primary tumors, it also paradoxically increases spontaneous metastasis. Therefore, such

treatment combinations need to be further evaluated in appropriate preclinical models. As such, investigating the correlation between IL1 $\beta$  and clinical outcome in breast cancer patients treated with chemotherapy is worthy.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** T. Voloshin, R.N. Apte, Y. Shaked

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** T. Voloshin, D. Alishkevitz, V. Miller, E. Isakov, I. Kaplanov, E. Voronov, E. Fremder

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** T. Voloshin, D. Alishkevitz, M. Benhar, Y. Shaked

**Writing, review, and/or revision of the manuscript:** T. Voloshin, R.N. Apte, Y. Shaked

**Study supervision:** Y. Shaked

**Other (responsible for the liposome production and analyses):** M. Machluf

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