Paclitaxel Therapy Promotes Breast Cancer Metastasis in a TLR4-Dependent Manner

Lisa Volk-Draper1, Kelly Hall1, Caitlin Griggs1, Sandeep Rajput1, Pascaline Kohio1, David DeNardo2, and Sophia Ran1

Abstract

Emerging evidence suggests that cytotoxic therapy may actually promote drug resistance and metastasis while inhibiting the growth of primary tumors. Work in preclinical models of breast cancer has shown that acquired chemoresistance to the widely used drug paclitaxel can be mediated by activation of the Toll-like receptor TLR4 in cancer cells. In this study, we determined the prometastatic effects of tumor-expressed TLR4 and paclitaxel therapy and investigated the mechanisms mediating these effects. While paclitaxel treatment was largely efficacious in inhibiting TLR4-negative tumors, it significantly increased the incidence and burden of pulmonary and lymphatic metastasis by TLR4-positive tumors. TLR4 activation by paclitaxel strongly increased the expression of inflammatory mediators, not only locally in the primary tumor microenvironment but also systemically in the blood, lymph nodes, spleen, bone marrow, and lungs. These proinflammatory changes promoted the outgrowth of Ly6C+ and Ly6G+ myeloid progenitor cells and their mobilization to tumors, where they increased blood vessel formation but not invasion of these vessels. In contrast, paclitaxel-mediated activation of TLR4-positive tumors induced de novo generation of deep intratumoral lymphatic vessels that were highly permissive to invasion by malignant cells. These results suggest that paclitaxel therapy of patients with TLR4-expressing tumors may activate systemic inflammatory circuits that promote angiogenesis, lymphangiogenesis, and metastasis, both at local sites and premetastatic niches where invasion occurs in distal organs. Taken together, our findings suggest that efforts to target TLR4 on tumor cells may simultaneously quell local and systemic inflammatory pathways that promote malignant progression, with implications for how to prevent tumor recurrence and the establishment of metastatic lesions, either during chemotherapy or after it is completed. Cancer Res; 74(19); 5421–34. ©2014 AACR.

Introduction

Paclitaxel is a widely used drug for breast cancer treatment with an overall response rate of 25% (1). The advanced paclitaxel formulation as albumin-embedded nanoparticles (nab-paclitaxel) increased the initial response rate to 42% (2); however, resistance occurs frequently and the evasion mechanisms remain unclear. Tumor recurrence occurs in 30% of node-negative and up to 70% in node-positive breast cancer patients (3). Only 23% of relapsed patients survive 5 years after diagnosis mainly due to metastasis to lymph nodes (LN) and distant organs (4). Several recent studies suggest that paclitaxel (5, 6) both kills and activates tumor cells thereby increasing chemoresistance and metastasis. The goal of this study was to delineate the mechanisms underlying relapse-associated metastasis possibly driven by paclitaxel therapy.

Tumor resistance to paclitaxel is currently thought to be due to either overexpression of transporters that decrease intracellular drug concentrations or preexisting mutations preventing drug binding to tubulin (7). We recently identified a novel mechanism of chemoresistance to paclitaxel relating to its activation of Toll-like receptor-4 (TLR4; ref. 8). TLR4 is expressed mainly in the antigen-presenting cells that respond to lipopolysaccharide (LPS), a component of bacterial membrane (9). Macrophages activated by the LPS–TLR4 pathway acquire the capacities to quickly migrate to an infected site, protect themselves from pathogen toxicity, destroy invaders, and restore homeostasis to the injured tissue (9). While these TLR4-dependent macrophage functions are essential for body defense, overexpression of TLR4 in tumor cells has deleterious consequences due to acquisition of a migratory, toxicity-resistant, and “wound healing”-like phenotype. Albeit not universally recognized, TLR4 is often expressed and activated in human cancers by the products of injured and dead cells typically present in the tumor environment and particularly...
increased after chemotherapy (10). In addition to endogenous TLR4 ligands, this receptor is also activated by paclitaxel (11), suggesting that this type of therapy can enhance recurrence and metastasis in TLR4-positive tumors (5, 8).

This notion is strongly supported by multiple clinical studies showing that TLR4 expression correlates with tumor dedifferentiation (12), higher clinical stage (13), faster recurrence rates (14, 15), infiltration by inflammatory myeloid cells (14), higher incidence of venous invasion (16), as well as lymphatic and distant (14, 16, 17) metastasis. Tumor expression of TLR4 also correlates with shorter relapse-free and overall survival in breast (14), colorectal (17), and pancreatic (16) human cancers. Moreover, mechanistic studies in multiple cancer models showed that LPS activation of tumor-expressed TLR4 increases expression of angiogenic and inflammatory factors (5, 18), immune evasion (18), cell survival (19), invasiveness (20), and metastatic spread (15). Studies in human ovarian cancer models were first to show that both LPS and paclitaxel cause significant inflammation and protection from cell death mediated by an intracellular adaptor of TLR4, MyD88 (5, 21). The latter is highly upregulated in clinical ovarian cancer and strongly correlates with decreased patient survival (22, 23). Knockdown of TLR4 restored sensitivity to paclitaxel (5, 21) and conferred a less metastatic phenotype (24) compared with isogenic TLR4-rich tumors.

In line with this evidence, we recently reported that TLR4 overexpression in a negative breast cancer line HCC1806 substantially increased inflammation and chemoresistance to paclitaxel therapy (8). Consistently, suppression of TLR4 expression in a positive MDA-MB-231 line significantly improved response to paclitaxel, resulting in a 6-fold increase in the number of tumor-free mice (8). Here, we aimed to delineate the mechanisms by which paclitaxel in conjunction with TLR4 impairs tumor response to paclitaxel therapy. Among various functional consequences of TLR4 activation, we considered that LPS-activated macrophages rebuild vascular networks through recruitment of bone marrow progenitors (25). Extrapolating from that, we postulated that paclitaxel-driven TLR4 activation in tumor cells may recapitulate this phenotype, leading to tumor recruitment of provascular progenitors, increased vessel formation, and tumor spread.

To test this hypothesis, we analyzed the effects of paclitaxel therapy on inflammation, tumor vascularization, growth, and metastasis in both syngeneic mouse and human breast cancer models with differential expression of TLR4. We found that paclitaxel increases local and systemic inflammation concomitant with expansion of tumor vascular networks, increases lymphatic metastasis, and significantly worsens outcome in TLR4-positive tumors as compared with TLR4-negative or untreated tumors. These findings suggest that blocking the TLR4 pathway may restore sensitivity to paclitaxel therapy and significantly improve cancer patient survival.

Materials and Methods

Materials

TRI reagent and protease inhibitors were purchased from Sigma-Aldrich. DMEM and supplements were from Lonza. Paclitaxel albumin-bound nanoparticles (nab-paclitaxel) were obtained from a local hospital pharmacy.

Antibodies

Primary antibodies were rat anti-mouse CD11b, Ly6C, Ly6G and MECA-32 (BioXCell, NH); rabbit anti-mouse Lyve-1 and Prox1 (Angiobio); mouse anti-human HLA (ATCC); and PE-rabbit anti-mouse Ly6C (BD Bioscience). Anti-species-specific secondary antibodies conjugated to 488, 549, and 650 dyes were from Jackson Immunoresearch Laboratories.

Cell lines

Luciferase-tagged human MDA-MB-231 and HCC1806 cell lines were stably modified to either suppress or upregulate TLR4 expression as described previously (8). Modified lines and the corresponding controls were designated as 231TLR4+ and 231TLR4− (derived from MDA-MB-231) or 1806TLR4+ and 1806TLR4− (HCC1806). MMTV-PyMT mouse breast cancer line was generated in Dr. David DeNardo’s laboratory. Other mouse breast cancer lines were obtained from ATCC. All lines were cultured in 5% DMEM with standard additives at 37°C in 10% CO2. All human lines were authenticated by ATCC in 2013 and tested for mycoplasma using a kit from R&D Systems.

Generation of dominant-negative TLR4 and MyD88 cells

MDA-MB-231 cells were stably transfected with dominant-negative (DN)-TLR4 and DN-MyD88 using pZero-hTLR4a and pdn-MyD88, respectively. Control lines were similarly generated using corresponding empty plasmids (pZero-MCS and pDeNy-MCS) from Invivogen.

Animal studies

Tumor growth was monitored as described previously (8, 26). Briefly, 4 × 106 (human) or 0.5 × 106 (mouse) cells suspended in 50% Matrigel were implanted orthotopically into 4- to 6-week-old female SCID, C57BL/6, or BALB/c mice (Taconic) followed by measurements 3 times a week using digital calipers. When tumors reached 150 or 500 mm3, mice were treated with 10 to 30 mg/kg nab-paclitaxel for 2 cycles of 5 consecutive days with 1 week rest between the cycles. Tumors, serum, and organs were collected when tumors reached 1,800 mm3. Animal care was in accordance with institutional guidelines.

Determination of metastatic burden

Tissues were homogenized in ice-cold lysis buffer (Promega) containing protease inhibitors. Luciferase substrate (50 μL) was mixed with lysates (10 μL) followed by luminescence detection using a luminometer (Berthold). Extracts with luciferase activity of 1,000 relative luciferase units (RLU)/s above background were considered positive for metastasis. Data are expressed as the mean RLU/s ± SEM from duplicate readings normalized per μg of total protein determined by Bradford assay.

qRT-PCR

RNA was extracted from tumors, lymph nodes, and lungs by TRI reagent and reverse transcribed using RevertAid cDNA

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qRT-PCR

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Synthesis Kit (Fermentas). Primers were designed on the basis of complementary DNA sequence of targets found in the NCBI database. All primer sequences are listed in Supplementary Table S1, and were validated to be human or mouse specific. Transcripts were analyzed using GoTaq Green Master Mix (Promega) and MasterCycle realplex PCR machine (Eppendorf). Data were normalized to β-actin, and relative changes in mRNA expression were determined using the ΔΔCt method.

**Measurement of cytokine concentration by ELISA**

Serum and tumor lysates were collected from tumor bearing mice treated with sterile endotoxin-free saline or 10 to 30 mg/kg of nab-paclitaxel and sacrificed on day 6 or 14 posttreatment. IL6, IL8, CCL2, and IL1β concentrations were measured by ELISA according to the manufacturer’s instructions (Peprotech). All experiments were performed in duplicate and reproduced twice. Results are presented as mean pg ± SD normalized per mg of protein.

**Measurement of spleen weight**

Spleens were harvested from untreated or nab-paclitaxel treated mice bearing 1806TLR4+ or 1806TLR4− tumors. Results are presented as the mean spleen weight ± SD for each group (n = 3–5 mice).

**Flow cytometry**

Flow cytometry was performed on whole bone marrow, spleen-derived mononuclear cells, and CD11b+ cells from tumors. Bone marrow was collected by flushing the bones with 2% FBS and 1 mmol/L EDTA in PBS. Spleen mononuclear cells were obtained from a single-cell suspension following by a Ficoll–Paque gradient. Tumor-associated CD11b+ cells were isolated using rat anti-CD11b magnetic beads (Miltenyi Biotec) from digested tumors by 225 U/mL collagenase type III and 100 U/mL hyaluronidase (Sigma-Aldrich). After blocking Fc, cells were incubated with anti-CD11b, Ly6C, and Ly6G for 30 minutes on ice followed by incubation with secondary 488- and 650-conjugated anti-rat antibodies. Expression was analyzed using AccuriC6 flow cytometer (BD Accuri Cytometers) and FlowJo software. Results are expressed as mean% of marker expression per group (n = 5) ± SEM.

**Immunohistochemical analysis of blood vessels, lymphatic vessels, and macrophages**

Acetone-fixed 8 µm thick frozen tumor sections were rehydrated in PBS with 0.1% Tween20 (PBST) before incubation with anti-mouse CD11b, Ly6C, MECA-32, Lyve-1 diluted 1:100, or w6/32 hybridoma supernatant used undiluted. All antibodies were incubated with tissues for 1 hour at 37°C with a 10-minute wash in PBST between incubations. After the last wash, slides were mounted in VectaShield medium containing 4,6-diamidino-2-phenylindole nuclear stain (Vector Labs). Images were acquired on an Olympus BX41 microscope equipped with a DP70 digital camera and DP Controller software (Olympus). Images were analyzed by ImageJ software to determine fluorescent intensity for individual pixels. The amount of pixels acquired from positive staining was divided by the total pixels per field yielding the area-normalized% of positive staining for each specific marker. The results are presented as mean percent of marker expression per field ± SD (3 mice/group).

**Quantification of blood and lymphatic vessel densities**

Blood vascular density (BVD) and lymphatic vessel density (LVD) were determined from four images per slide captured at ×200 magnification in tumor sections stained with either anti-MECA-32 or anti-Lyve-1 antibodies (3 mice/group). Vascular structures were enumerated using ImageJ. BVD is presented as the mean number of vessels per field ± SEM, and LVD is the mean number of Lyve-1+ structures normalized per mm² of the section area ± SEM.

**Quantification of vascular invasion**

Tumor sections were double-stained with anti-human HLA and anti-mouse MECA-32 or Lyve-1 antibodies as described above. Blood and lymphatic vessels with open lumens were identified and enumerated in each section. The percentage of open-lumen vessels invaded by tumor cells was normalized per section area. Results are presented as the mean percentage of invaded vessels from total open-lumen blood or lymphatic structures normalized per section area ± SD.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software. Results are expressed as mean ± SEM or ± SD. Statistical significance for continuous variables and categorical covariants was determined by the Student paired t test and χ² test, respectively. Metastatic burden and incidence was assessed by the Mann–Whitney and Fisher exact tests, respectively. P values ≤ 0.05 were considered statistically significant.

**Results**

**TLR4 expression in breast cancer cells dictates the response to paclitaxel therapy and metastatic spread**

We previously showed that paclitaxel elicits strong inflammatory responses and promotes tumor growth in TLR4-positive MDA-MB-231 and HCC1806 models, but not in isogenic controls with low or no TLR4 expression (8). Here, we confirmed that paclitaxel effect is largely mediated via TLR4 because MDA-MB-231 cells overexpressing dominant-negative (DN) TLR4 or its intracellular adaptor MyD88 had substantially lower responses to paclitaxel than vector-transfected controls (Supplementary Fig. S1). We also confirmed that the proinflammatory response is not restricted to nab-paclitaxel as both Taxol (8) and docetaxel (Supplementary Fig. S2) similarly activate TLR4-positive tumor cells. While our first study (8) focused on the effects of TLR4 and paclitaxel therapy on growth and recurrence of primary tumors, here we used the same breast cancer models to analyze the effects on metastasis. Lymph node and lung metastasis was analyzed in mice with orthotopically implanted 231TLR4+. 

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231TLR4−, 1806TLR4−, or 1806TLR4+ tumors that were either treated with nab-paclitaxel (10 mg/kg for 10 days) or left untreated. Consistent with prior results (8), TLR4 expression in both breast cancer models was associated with 2- to 3-fold accelerated tumor growth and a higher resistance to nab-paclitaxel compared with controls (Figs. 1A and B). In addition, both TLR4 and paclitaxel strongly affected the incidence and metastatic burden. For instance, nab-paclitaxel treatment...
decreased the burden but failed to reduce 100% metastatic incidence in mice with TLR4-expressing 231TLR4+ tumors. In contrast, the same treatment of an isogenic model with 90% depleted TLR4 drastically decreased both burden and incidence of lymph node and lung metastases (Figs. 1C, E, and G; Supplementary Table S2).

The prometastatic role of TLR4 amplified by chemotherapy was even more pronounced in the HCC1806 model in which nearly all treated mice (90%) with TLR4-positive tumors were positive for lymph node and pulmonary metastases compared with less than half of the mice bearing isogenic-negative tumors (Fig. 1D). Importantly, only in 1806TLR4+ tumors, nab-paclitaxel therapy increased the burden of lymph node metastasis by 245-fold compared with untreated controls (P = 0.008; Supplementary Table S2). Chemotherapy also increased pulmonary metastasis by 12-fold in TLR4-positive and 5-fold in negative tumors (Figs. 1F and H), suggesting that some oncogenic effects of paclitaxel are TLR4 independent. Cumulatively, these results show that paclitaxel activation of TLR4 in tumor cells promotes tumor growth and metastasis, and that paclitaxel therapy may result in a strikingly different outcome depending on expression of TLR4.

Paclitaxel in concert with TLR4 promotes local inflammation in primary human xenografts

We next delineated the mechanisms by which TLR4 and paclitaxel advance metastatic spread. TLR4 is the major inducer of inflammation in injured or pathogen-attacked tissues (27). We therefore hypothesized that nab-paclitaxel activation of TLR4 promotes metastasis by increasing inflammation, to test this hypothesis, we compared the cytokine expression in untreated and nab-paclitaxel–treated TLR4-negative and -positive tumors isolated 6 days posttreatment. Tissues were analyzed by quantitative real-time PCR (qRT)-PCR for expression of 15 inflammatory cytokines typically upregulated by the TLR4 pathway (8). Chemotherapy in mice with TLR4-negative tumors increased only a third of the examined cytokines by 2- to 3-fold (Fig. 2A). In contrast, treatment of mice with TLR4-positive tumors elevated 73.3% of cytokines up to approximately 12-fold (Fig. 2B). This result demonstrates that nab-paclitaxel induces a significantly stronger inflammatory response in TLR4-positive tumors than those lacking this receptor.

Remarkably, the mere expression of TLR4 in malignant cells caused significant proinflammatory changes in the tumor environment as evident by highly increased levels of IL6 (92.6-fold), IL10 (39.3-fold), and IL1β (16.4-fold) as compared with TLR4-negative controls (Fig. 2C). This proinflammatory pattern was intensified by nab-paclitaxel that further increased IL6 and IL1β by 276-fold and 57-fold, respectively (Fig. 2D). Protein analysis by ELISA confirmed TLR4-dependent alterations in the inflammatory profiles induced by paclitaxel (Figs. 2E–H). IL6 protein was undetectable in TLR4-negative tumors, but its expression increased to 56 pg/mg in TLR4-positive ones and amplified by 470% by nab-paclitaxel treatment (Fig. 2E). The changes in IL8 expression mirrored this pattern with barely detectable protein in TLR4-negative tumors and >10-fold upregulation in the TLR4-positive ones following nab-paclitaxel treatment (Fig. 2G). The differences between TLR4-negative and -positive tumors were maintained throughout the experiment as evident by highly expressed cytokines in the TLR4-positive groups 2 weeks after treatment (Figs. 2F and H). Taken together, these data indicate that paclitaxel activation of TLR4 substantially upregulates a wide range of cytokines, causing a sustained inflammatory state in the local tumor environment that likely contributes to development of metastasis.

Paclitaxel–TLR4 pathway increases inflammation and metastasis in syngeneic mouse models

We then determined whether similar effects occur in syngeneic mouse models having intact adaptive immunity. We first screened several breast cancer mouse lines in vitro to identify lines positive for TLR4 and responsive to paclitaxel. We found that all examined lines (MMTV-PyMT, 4T1, 66.3, and EM6) highly express TLR4 as well as all major regulatory components of the TLR4 pathway (Fig. 3A and B). MMTV-PyMT and EM6 models were selected for in vivo analysis. Both models showed similar increases in intratumoral inflammatory cytokines after paclitaxel treatment (Figs. 3C and D) to those observed in human xenografts (Fig. 2). EM6 tumors were also analyzed for the effects on tumor growth and metastasis. Similarly to results in human breast cancer models, nab-paclitaxel therapy suppressed the growth of primary tumors (Fig. 3E), but significantly elevated metastasis (Fig. 3F). These data suggest that paclitaxel and TLR4 cause similar pro-oncogenic and inflammatory changes in both human xenograft and syngeneic mouse models.

Paclitaxel activation of tumor TLR4 also enhances systemic inflammation

LPS-mediated activation of TLR4 increases systemic inflammation to alert distant organs of pathogen invasion and recruit immune cells first for body defense and, subsequently, for tissue healing (28). We postulated that paclitaxel activation of TLR4 in malignant cells mimics this pattern by increasing inflammatory cytokines in the blood and distant organs responsive to TLR4 systemic signals. To test this hypothesis, we determined the proinflammatory effects of TLR4 and nab-paclitaxel on blood-circulating IL6 and IL8 and multiple cytokines in the main metastatic organs, i.e., lymph nodes and lungs.

The results showed that paclitaxel activation of TLR4 significantly changed the inflammatory milieu in both blood and distant organs. Following treatment, levels of blood-circulating IL6 and IL8 proteins were highly increased in TLR4-positive (but not negative) group from undetectable to, correspondingly, 80 and 330 pg/mL (Fig. 4A–D). These cytokines were derived from human tumor cells because ELISA was performed using human-specific antibodies.

We also analyzed the expression of inflammatory cytokines in lymph nodes and lungs from untreated and nab-paclitaxel treated mice with TLR4-positive or -negative
tumors. This analysis was performed with species-specific primers validated on mouse and human universal cDNA (Supplementary Table S1). Transcripts of human cytokines were undetectable in distant organs; however, all groups showed changes in mouse host cytokines that closely followed transcriptional alterations in primary tumors (Fig. 2). In mice with TLR4-negative tumors, therapy caused a modest 2- to 3-fold increase in isolated lymph node cytokines. In contrast, in mice with TLR4-positive tumors, 40% of the examined lymph node cytokines increased by 4- to 8-fold.

Similarly, nab-paclitaxel did not affect lung cytokines in mice with TLR4-negative tumors, but increased many cytokines 3- to 4-fold in mice with TLR4-positive tumors (Figs. 4G and H). CCL20, IL4, IL6, and IL10 were among the highest upregulated in both lymph nodes and lungs. Collectively, these data indicate that paclitaxel activation of TLR4 at the primary tumor causes profound systemic changes in expression of inflammatory mediators in the blood, lymph nodes and lungs, the two main organs that permit growth of metastatic breast cancer cells.
TLR4-mediated systemic inflammation increases generation of myeloid progenitor cells in bone marrow and spleen

TLR4-induced cytokines (e.g., IL1β, IL10, and IL6) have been reported to induce generation of myeloid progenitors that promote metastasis (29). As these cytokines were highly upregulated both locally and systemically by the TLR4–paclitaxel axis, we hypothesized that their production enhances generation of myeloid progenitor cells in the distant organs such as bone marrow and spleen. Indeed, the average spleen weight in mice with TLR4-positive tumors was 300% larger than that in mice bearing tumors lacking TLR4 (Fig. 5A and B). Moreover, nab-paclitaxel treatment doubled spleen weight in mice with 1806TLR4+ tumors, making them 6-fold larger than in untreated controls. Notably, spleens of mice with TLR4-negative tumors were unchanged by chemotherapy (Fig. 5A and B).

We next determined whether the increase in spleen size is due to generation of myeloid progenitors rather than...
edema or elevated hematopoiesis. Spleen and bone marrow-derived cells were double-stained for CD11b and the markers of immature myeloid cells, Ly6C and Ly6G, followed by FACS analysis and calculation of marker-positive fractions. FACS analysis revealed two major subsets labeled as Set 1 and Set 2 (Supplementary Fig. S3). The most conspicuous difference was detected in Set 2 that consisted of approximately 5% of double-stained CD11b\(^+\)/Ly6C\(^+\) cells in bone marrow of mice with TLR4-negative tumors, but nearly 99% in both bone marrow and spleen in mice with TLR4-positive tumors (Figs. 5C and D). Nab-paclitaxel also increased a Ly6G\(^+\) subpopulation from approximately 8% to 38% of CD11b\(^+\) cells (4.75-fold) in both bone marrow and spleen (Figs. 5E and F).

Collectively, these data suggest that paclitaxel therapy, particularly in the context of TLR4-positive tumors, significantly increases
generation of immature myeloid cells that may promote metastasis (29).

**Tumor-recruited myeloid progenitors increase BVD but not invasion of blood vessels**

We next examined the effects of the activated TLR4–paclitaxel axis on tumor recruitment of myeloid progenitors and their contribution to vascular formation. Mice with TLR4-positive and -negative tumors were treated for 6 days with nab-paclitaxel or left untreated. The excised tumors were stained with anti-CD11b, Ly6C, and Ly6G antibodies followed by quantification of pixels per field (Fig. 6). As compared with controls, a higher number of CD11b<sup>+</sup> cells were detected in TLR4-positive tumors after nab-paclitaxel treatment than in those lacking TLR4 (3.3-fold vs. 1.9-fold, respectively; Figs. 6A and B). Ly6C<sup>+</sup> and Ly6G<sup>+</sup> cell recruitment showed a similar pattern. After chemotherapy, 1806<sup>TLR4+</sup> tumors recruited approximately 7-fold more Ly6C<sup>+</sup> cells than TLR4-negative tumors (Figs. 6A and C). Following this trend, paclitaxel treatment of 1806<sup>TLR4+</sup> tumors mobilized approximately 3-fold more Ly6G<sup>+</sup> cells than negative tumors following identical therapy (Fig. 6B–D; statistically significant differences are identified by asterisks).

Because Ly6C<sup>+</sup> and Ly6G<sup>+</sup> cells have been reported to promote tumor blood vasculature (30), we determined the effects of TLR4 and paclitaxel therapy on formation of blood vessels. We found that BVD was significantly increased by 2.5-fold (P < 0.001) in treated mice with 1806<sup>TLR4+</sup>, but not in other experimental groups (Fig. 6E and F). The role of
immature myeloid cells in chemotherapy-driven vascular formation was also supported by treatment with anti-CD11b antibody, which significantly reduced intratumoral densities of both CD11b+ monocytes and blood vessels (Supplementary Fig. S4). We next determined whether increased BVD corresponded to enhanced tumor cell invasion of these vessels. Tumors were double-stained with w6/32 (anti-human HLA) and MECA-32 (anti-mouse BV marker) antibodies followed by quantifying the percent of tumor-invaded vessels of total open-lumen MECA-32+ structures. Following nab-paclitaxel treatment, TLR4-positive tumors displayed slightly higher density of invaded blood vessels (~3%, as compared with 0.2% in TLR4-negative group); however, this difference was not statistically significant (Fig. 6G). This suggested that although TLR4–paclitaxel-recruited Ly6C+ and Ly6G+ myeloid cells enhanced formation of new blood vessels they had no effect on tumor invasion of these vessels.

**Activation of the TLR4–paclitaxel axis creates de novo intratumoral lymphatics and promotes their invasion**

As increased metastasis could not be explained by enhanced invasion of blood vessels, we hypothesized that
the prometastatic effect of the TLR4–paclitaxel axis might be mediated through increased tumor cell access to lymphatics. To test this hypothesis, we restained tumors shown in Fig. 6 for lymphatic-specific markers. As previously reported (31), native HCC1806 tumors lack intratumoral lymphatics although they are rich in peritumoral Lyve-1+ structures as shown in Fig. 7A. This pattern did not change in the absence of TLR4 or omission of paclitaxel therapy. In sharp contrast, all treated mice with 1806TLR4+ tumors display extensive network of intratumoral lymphatics as shown in the last panel of Fig. 7A. The mean LVD in this group was 5.0 ± 0.7 Lyve-1+ vessels/mm², which was approximately 25-fold higher than in any other group (Fig. 7D). These de novo created vessels, residing as deep as 792 μm from the tumor margin, displayed clear lymphatic phenotype as shown by mutually exclusive staining pattern with a blood vascular-specific marker, MEGA-32 (Fig. 7B), and confirmed by another lymphatic-specific marker, Prox1 (Fig. 7C). Importantly, the new intratumoral lymphatics were highly permissive for tumor cell invasion as demonstrated by the drastically increased fraction of occluded Lyve-1+ vessels that increased from zero in TLR4-negative tumors to 42% in TLR4-positive tumors treated by nab-paclitaxel (Fig. 7D). In comparison, only 8% of lymphatic vessels were invaded in 1806TLR4+ tumors without treatment. Noteworthy that albeit TLR4-negative tumors were surrounded by Lyve-1−peritumoral vessels (Fig. 7A), most of them showed no signs of invasion (Fig. 7F). In sharp contrast, the majority of both peritumoral and intratumoral Lyve-1+ vessels in TLR4-positive tumors after chemotherapy were invaded by malignant cells (Fig. 7G). These findings demonstrate a paramount role of tumor-expressed TLR4 in paclitaxel chemotherapy-driven lymphatic metastasis.

Discussion

Emerging evidence derived from recent clinical and experimental studies highlights the dark side of chemotherapy, namely, its alarming association with metastatic relapse after treatment. In particular, clinical studies showed a link between the widely used drug paclitaxel and substantial increase in lymphatic metastasis (32), tumor recruitment of prometastatic myeloid cells (32), circulating inflammatory cytokines (33), intratumoral COX-2 (34), and resistance to cytotoxic therapy (35). Paclitaxel was also shown to significantly promote metastasis in a transgenic breast cancer model MMTV-PyMT (32), orthotopic human breast (26), and ovarian (36) xenografts as well as syngeneic models of melanoma and colon cancer (6). Mechanistically, the prometastatic effects of paclitaxel can be attributed to its ability to induce NF-κB–dependent inflammatory, angiogenic, and prosurvival factors that either directly enhance tumor aggressiveness (37) or mobilize bone marrow myeloid cells that promote tumor spread via paracrine loops (32). We recently proposed that prometastatic effects of paclitaxel are mediated by tumor-expressed TLR4 (8). Macrophage-expressed TLR4 is a naturally designed pathway that propagates inflammatory local and systemic circuits that expand pools of myeloid progenitors and recruits them to sites of inflammation to repair injured vasculature. We previously showed that TLR4-positive tumors vigorously react to paclitaxel therapy by upregulating inflammatory mediators that create a favorable environment for expansion of primary tumors (8). Here, we used mouse and human breast cancer models with differential expression of TLR4 to analyze its role in promoting vasculature that subsequently increases locoregional and distant metastasis following the paclitaxel therapy.

This analysis showed that both TLR4 and paclitaxel play paramount roles in determining the magnitude of metastatic spread. Even in the absence of treatment, TLR4 expression in tumor cells caused multifold increases in both inflammatory cytokines and metastasis compared with tumors lacking this receptor (Figs. 1 and 2). This drug-independent activation of TLR4 might be mediated by endogenous ligands overexpressed in breast cancer such as hyaluronan (38) and S100A8/A9 (39). We also observed TLR4-independent effects of paclitaxel, which was evident by moderately enhanced inflammation and metastasis in tumor models lacking this receptor (Fig. 2). This effect might be mediated by TLR2 receptor (40) or by sensory receptors such as RAGE (25) and TREM-1 (41), all of which are known to be activated by the products of dead cells generated by chemotherapy. This scenario may explain prometastatic effects of chemotherapeutics other than paclitaxel, including cyclophosphamide (42), gemcitabine (43), and cisplatin (6, 43). However, combination of paclitaxel and TLR4-positive tumors significantly increased the magnitude of the effect, resulting in a 500% increase in incidence of lymph node and pulmonary metastasis and up to 245-fold elevated nodal burden (Supplementary Table S2). This was concomitant with an approximately 300-fold increase in intratumoral inflammatory mediators (Fig. 2D), implicating the inflammatory consequence of the activated paclitaxel–TLR4 pathway as the primary cause of increased metastasis.

Notably, paclitaxel-dependent activation of TLR4 increased inflammation not only in the local tumor environment, but also in the blood, lymph nodes, bone marrow, spleen, and lungs (Figs. 4 and 5). This observation is not surprising given the natural function of macrophage-expressed TLR4 to systemically alert responsive organs for the presence of pathogens urging them to generate hematopoietic and endothelial progenitors. Under normal circumstances, these progenitors are recruited to the pathogen-invaded site to replenish the pool of immune cells, clear debris, and restore tissue homeostasis, including the repair of vasculature. Analogously, in mice with TLR4-positive tumors, paclitaxel induced strong responses in bone marrow and spleen, resulting in an approximately 20-fold increase in CD11b+/Ly6C− and CD11b+/Ly6G+ progenitors that subsequently massively mobilized to tumors (Figs. 5 and 6). These findings are consistent with widely reported paclitaxel-dependent (6, 32, 44) increases in bone marrow produced progenitors along with the evidence for their prometastatic effects upon arrival to primary (32) and distant tumor sites (44). However, this is the first evidence that both expansion of CD11b+/Ly6C−/Ly6G+ cells at TLR4-responsive organs and their recruitment to tumors is enhanced by taxane therapy and correlate with tumor-expressed TLR4. This notion
is also consistent with reported mechanisms of creating pulmonary premetastatic niche through tumor-produced G-CSF and granulocyte macrophage-colony stimulating factor that mobilize Ly6C⁺/Ly6G⁺ myeloid precursors from the bone marrow to the lung (44). Noteworthy, paclitaxel was reported to upregulate these factors by >20-fold in TLR4-positive breast
cancer lines (45). Collectively, these findings suggest that prometastatic effects of the paclitaxel–TLR4 axis are mediated, in part, by chemotherapy-induced factors in primary tumors that are released to systemic blood circulation and promote successful engraftment of malignant cells at secondary sites.

**Paclitaxel therapy of TLR4-positive tumors creates intratumoral lymphatic vessels and promotes their invasion**

One of the most remarkable and important findings in this study is the discovery that paclitaxel therapy induced de novo formation of intratumoral lymphatics in TLR4-positive (but not negative) tumors (Fig. 7). It is currently believed that peritumoral lymphatic vessels are mainly responsible for malignant spread (31), because intratumoral lymphatics are rare in most untreated tumors, including breast cancers, that preferentially metastasize through these vessels (46). However, tumors that do generate intratumoral lymphatics are much more efficient in reaching lymph nodes (47), resulting in a significantly higher risk for relapse and poorer prognosis (48). We show here that although blood and lymphatic vessels are both significantly increased in TLR4-positive tumors following chemotherapy, only the latter were invaded by tumor cells (Fig. 7). This suggests that the primary mechanism by which the paclitaxel–TLR4 axis promotes breast cancer metastasis is through creation of penetration-permissive intratumoral channels that exponentially increase the number of malignant cells transported to the local nodes. This new mechanism in tumor biology might mirror a previously established ability of the LPS-activated TLR4 pathway to promote lymphangiogenesis mediated by bone marrow-derived macrophages recruited to the inflamed site (49). Analogously, the paclitaxel–TLR4 pathway may induce intratumoral lymphangiogenesis mediated by CD11b+ myeloid progenitors mobilized to the tumor site (Fig. 6). Thus, this discovery not only provides a likely explanation for paclitaxel-mediated metastasis, but also suggests a novel way to study the formation of tumor lymphatics by comparing parallel steps of this monocyte-driven process induced by both chronic inflammation and cancer (50).

In summary, we show here that paclitaxel chemotherapy substantially increases the magnitude of lymphatic and distant metastasis through activation of the TLR4 pathway in tumor cells, which strongly elevates inflammation in the tumor environment, regional nodes, and distant organs thus favoring malignant outgrowth. The prometastatic phenotype is locally driven by de novo formation of intratumoral lymphatics and amplified systemically by expansion of myeloid provascular progenitors and their recruitment to both primary and distant sites. Collectively, these findings suggest that blocking paclitaxel-induced inflammation in patients with TLR4-positive cancers may significantly improve therapeutic outcome.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: L. Volk-Draper, S. Rajput, S. Ran

Development of methodology: K. Hall, S. Rajput, S. Ran

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Volk-Draper, K. Hall, P. Kohio

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Volk-Draper, K. Hall, C. Griggs, S. Rajput, P. Kohio, S. Ran

Writing, review, and/or revision of the manuscript: L. Volk-Draper, K. Hall, S. Rajput, S. Ran

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Hall, C. Griggs, S. Ran

Study supervision: S. Ran

Other (provided reagents for the experiments and protocols for certain aspects): D. DeNardo

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Lisa Volk-Draper, Kelly Hall, Caitlin Griggs, et al.


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