Core Needle Biopsy of Breast Cancer Tumors Increases Distant Metastases in a Mouse Model[1,2]

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Abstract

INTRODUCTION: Incisional biopsies, including the diagnostic core needle biopsy (CNB), routinely performed before surgical excision of breast cancer tumors are hypothesized to increase the risk of metastatic disease. In this study, we experimentally determined whether CNB of breast cancer tumors results in increased distant metastases and examine important resultant changes in the primary tumor and tumor microenvironment associated with this outcome. *METHOD:* To evaluate the effect of CNB on metastasis development, we implanted murine mammary 4T1 tumor cells in BALB/c mice and performed CNB on palpable tumors in half the mice. Subsequently, emulating the human scenario, all mice underwent complete tumor excision and were allowed to recover, with attendant metastasis development. Tumor growth, lung metastasis, circulating tumor cell (CTC) levels, variation in gene expression, composition of the tumor microenvironment, and changes in immunologic markers were compared in biopsied and non-biopsied mice. *RESULTS:* Mice with biopsied tumors developed significantly more lung metastases compared to non-biopsied mice. Tumors from biopsied mice contained a higher frequency of myeloid-derived suppressor cells (MDSCs) accompanied by reduced CD4+ T cells, CD8+ T cells, and macrophages, suggesting biopsy-mediated development of an increasingly immunosuppressive tumor microenvironment. We also observed a CNB-dependent up-regulation in the expression of *SOX4*, *Ezh2*, and other key epithelial-mesenchymal transition (EMT) genes, as well as increased CTC levels among the biopsy group. *CONCLUSION:* CNB creates an immunosuppressive tumor microenvironment, increases EMT, and facilitates release of CTCs, all of which likely contribute to the observed increase in development of distant metastases.

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Introduction

Breast cancer is the most common cancer affecting women and accounts for the second highest incidence of cancer-related death, after lung cancer [1]. There are numerous factors known to influence the metastatic potential of any given breast cancer (tumor size, receptor status, lymph node involvement at the time of diagnosis, age of the patient, menopausal status, and family history) [2–5]. However, the most consistent predictors of metastasis continue to be tumor size and lymph node involvement at the time of diagnosis [6,7]. Because of this, breast cancer screening and early detection are critically important in improving outcomes for women with breast cancer.

Suspicious lesions detected on screening mammograms are generally biopsied to confirm or rule out a diagnosis of cancer. The most common form of biopsy administered today is a core needle biopsy (CNB) [8–14]. A CNB is a form of incisional biopsy whereby a portion of a tumor is removed for histologic evaluation leaving the remainder *in vivo* to be removed at a later date following a definitive diagnosis. Typically with breast cancer, tissue samples are collected by administering three to nine passes with a 14G biopsy needle [15,16]. Biopsy needle sizes can range from 9G to 18G depending on the particular form of image guidance and system of sample acquisition used for the core biopsy [17–19].

Other breast cancer biopsy techniques include complete excisional biopsy, open incisional biopsy, and fine needle aspiration. CNB and fine needle aspiration are favored over open incisional or excisional biopsies because they are less invasive, produce a smaller cosmetic post-operative footprint, and result in faster patient recovery. This is particularly appealing given that the majority of biopsied breast lesions are ultimately ruled benign [20,21]. Furthermore, in addition to distinguishing invasive from non-invasive cancer, tissue obtained from CNB can be used to perform nucleic acid analysis, immunohistochemistry, or analysis of prognostic biomarkers [22–24]. As a consequence, stereotactic or ultrasonographically guided CNB is currently the predominant biopsy method employed in breast cancer management [16].

Incisional surgical procedures, including incisional biopsies, on cancers have historically been associated with higher local recurrence rates and elevated incidence of lymph node metastasis [25–29]. There is also a growing body of evidence suggesting that surgical trauma in the presence of an established neoplasm can potentiate its growth and metastatic proliferation [30–33].

Current literature also increasingly notes the occurrence of post-surgical immunosuppressive changes and their relevance to metastatic spread and disease recurrence [33]. These observations suggest that surgically instigated changes in the tumor and subsequent host interaction with residual disease can influence the tumor's metastatic potential.

The clinical impact and potential risk associated with performing a CNB has long been debated. There is compelling evidence that CNB increases the risk of needle track seeding and local tumor recurrence in patients with breast cancer [28,34,35]. There is also little question that cancer cells from both invasive and non-invasive breast cancers enter lymphatic channels and migrate to lymph nodes following a biopsy procedure [27,36]. However, whether or not cancer cells displaced into lymphatic and vascular channels are capable of effectively establishing distant metastases remains unproven [34,37–39].

To test the hypothesis that surgically initiated changes in the tumor microenvironment due to CNB results in increased metastatic spread, we used the 4T1-BALB/c mouse model, a well-established, immune-competent, cancer animal model considered to closely mimic metastatic breast cancer in humans. Murine mammary 4T1 tumor cells were orthotopically implanted in BALB/c mice and tumors large enough to biopsy developed within 2 to 3 weeks. Biopsies were then performed in a manner designed to experimentally replicate the human clinical experience of using CNB for the diagnostic workup of breast cancer as well as study the impact of the CNB on metastatic outcomes. In this model, tumors spontaneously metastasize from the mammary fat pad to lymph nodes, lung, and bone in a similar pattern to that observed in human breast cancers [40]. This immunologically intact model also enabled study of the immunologic changes associated with CNB within the local tumor microenvironment, in distant organs, and peripheral circulation. These changes were assessed to detect those events that might be associated with tumor progression and metastasis [41]. Gene expression profiles were evaluated to detect changes in known key epithelial-mesenchymal transition (EMT) genes [42]. Recent studies have drawn associations between surgical procedures and increased levels of tumor cells in circulation [43]. In consideration of this finding, and the documented link between circulating tumor cell (CTC) levels and metastasis [44,45], the impact of CNB on CTC levels was also measured.

Methods

Cells

Metastatic murine breast cancer cell line 4T1 (ATCC CRL-2539) cells were cultured in RPMI-1640, supplemented with 10% FBS and antibiotic-antimycotic, at 37°C at 90% humidity and 5% CO_2. All media, sera, and supplements used for the murine metastasis model were purchased from Invitrogen, Life Technologies (Burlington, Ontario).

Animals

Female BALB/c mice were obtained from Charles River Laboratory (St-Constant, Canada) and housed in the Carleton Animal Care Facility at Dalhousie University. After a 1 week acclimatization period, the mice were used in the study. All protocols (No. 09-044 and No. 13-088) were approved by the Dalhousie University Committee on Laboratory Animals in keeping with guidelines established by the Canadian Council on Animal Care (CCAC).

Metastasis Model

Mice received a subcutaneous orthotopic injection of 7×10^3 4T1 tumor cells into the third thoracic mammary fat pad. Primary tumors were palpable in 10 to 16 days and reached the biopsy size of 6 to 8 mm by 15 to 20 days after implantation. Mice with slow-growing tumors that failed to achieve the required size for experimentation were excluded from the study (this was typically no more than 3 mice from a starting set of 23 mice). Tumor growth was determined by measuring two axes of the tumor and determining the mean tumor diameter. At the CNB time point, mice were alternately assigned to biopsy and non-biopsy groups to eliminate bias. Mice assigned to the biopsy group were fully anesthetized using inhaled isoflurane (2% body weight) and their biopsy sites were shaved and subjected to aseptic surgical preparation; they then received CNB with an 18-gauge needle attached to a 5-ml syringe. Six to eight cutting passes were administered in a palmate-radiating pattern through a single cutaneous insertion point so as to reduce injury to the skin. On occasion, when a tumor was multilobed or irregularly shaped, an

additional skin puncture was required to achieve six to eight passes distributed through the tumor with the biopsy needle. The non-biopsy (control) groups received no biopsy but were subjected to an equal duration of complete anesthesia in a modified induction chamber. Mice were maintained in a recovery area for 24 hours post procedure and observed to assure return to normal grooming and feeding behavior. All mice were monitored regularly throughout the experiment for any signs of illness or distress and were killed if and when they became moribund, according to CCAC guidelines.

In the clinical setting, breast cancers are generally biopsied using image-guided CNB technology to confirm the diagnosis with definitive (excisional) surgical treatment typically performed 2 or more weeks following performance of a diagnostic CNB. To reproduce this process, tumors were excised in both biopsy and non-biopsy control mice groups 7 days post biopsy/non-biopsy assignment (Figure 1A). Mice were anesthetized and tumors were surgically (and aseptically) excised, removing all tumor tissue to

prevent regrowth. Incisions were sutured shut using Novaril 4. Mice were given analgesic Ketoprofen (5 mg/kg), monitored regularly for any signs of illness, and were killed if judged moribund according to CCAC guidelines.

Excised tumors were divided with a 2 mm × 2 mm × 6 mm sliver of tissue being snap frozen in TRIzol and RNAlater (Qiagen GmbH, Hilden, Germany) for later RNA extraction and quantitative polymerase chain reaction (qPCR) gene expression analysis, as described in the Quantification of Gene Expression by qPCR section. The remaining tumor tissue was placed in 10% formalin buffered with sodium acetate for paraffin embedding and histologic analysis.

Histologic Quantification of Pulmonary Metastasis and Tumor Necrosis

To compare the level of metastasis and associated necrosis between biopsied and non-biopsied mice, a total of 84 mice in four cohorts underwent the metastasis model described above. Cohort size was
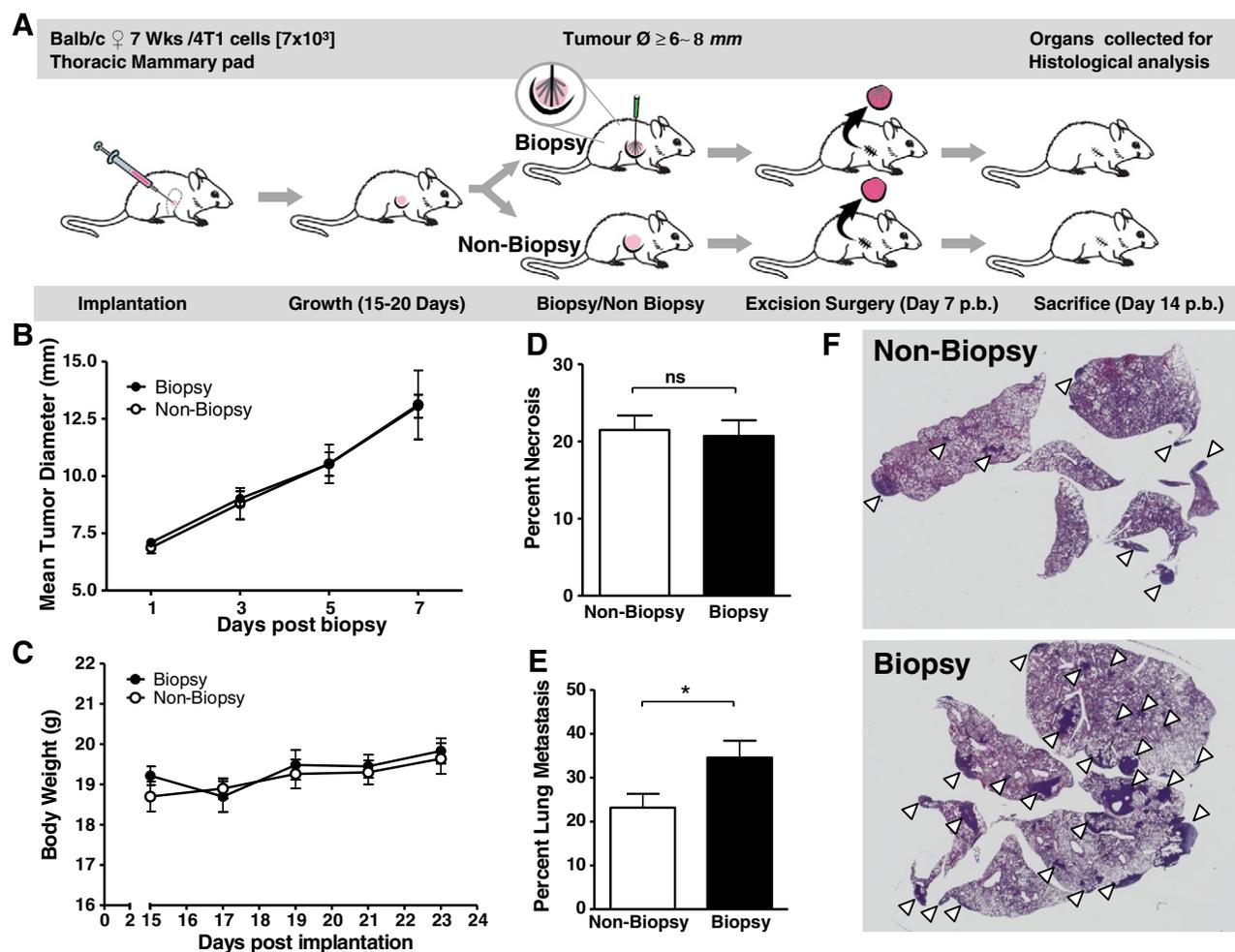

Figure 1. CNB significantly increases lung metastasis. (A) Experiment schematic mimicking the format of progression in the human scenario Detection > Biopsy > Excision > Outcome. Mice were implanted with 4T1 cells, and after 6- to 8-mm diameter tumors developed, the mice are alternately assigned to two groups, a biopsied group ($n = 40$), and a non-biopsy group ($n = 44$). This was followed by surgical excision of the tumors 7 days post biopsy and sacrifice of the mice at 14 days post biopsy (circular inset: details radiating a palamate biopsy pattern). (B) Tumor growth and (C) body weight were monitored post biopsy. (D) Percent necrosis of excised tumors and (E) percent metastasis of the lungs were determined by H&E staining of fixed tissue sections. (F) Representative section of lungs from non-biopsy and biopsy mice groups. White pointers indicate lung metastases. Significance determined by an unpaired t test, *$P \le .05$; error bars represent the SEM.

designed to allow manageable numbers of mice and enable consistent treatment and surgical handling. Seven days after the tumor excision, each cohort of mice consisting, on average, of biopsy ($n = 10$) and non-biopsy ($n = 11$) mice was sacrificed. The draining lymph nodes and lungs were harvested, formalin fixed, and paraffin embedded with each block assigned an anonymizing numerical identifier to allow unbiased evaluation. The anonymized lungs, lymph nodes, and tumors were serial sectioned into 5-μm thin tissue sections and stained with hematoxylin and eosin (H&E) for histologic evaluation. Imaging was performed using a Zeiss Axiocam HRC Color, mounted on a Zeiss Stemi. The percentage of metastatic (lung and lymph node) and necrotic (tumor) tissue areas relative to total section area was established using an overlay grid imposed on images captured from H&E-stained tissue sections.

Quantification of CTCs in Peripheral Circulation post Biopsy Using 4T1^{neo} Cells

To quantify CTCs in circulation, we generated neomycin cassette-bearing 4T1 cells using the Clontech retroviral expression system (Clontech Laboratories, Mountain View, CA; Cat. No. 634401). The cells were selected for stable expression of the neomycin resistance gene by multiple passages under RPMI-1640 with 100 μg/ml G418 sulfate (Fisher Scientific Co., Fair Lawn, NJ), and the resulting cells were designated 4T1^{neo} cells.

To assess changes in the level of CTCs, the 4T1^{neo} cells were implanted in the mammary fat pad of a single group of 12 BALB/c mice using a procedure identical to that used for unmodified 4T1 cells. The mice developed similar sized tumors in the same time frame as unmodified 4T1 implanted mice. The 12 mice implanted with 4T1^{neo} cells were biopsied when the 6- to 8-mm tumor diameter threshold was achieved. Groups of mice ($n = 4$) were processed for each post-biopsy time point (6 hours, 24 hours, and 10 days). Mice were bled through facial vein with 200 μl of blood collected and immediately mixed with 1 ml of TRIzol reagent. This was snap frozen in liquid nitrogen and stored at –80°C until RNA was extracted and qPCR was performed for neomycin gene expression. Mice were sacrificed, and their tumors harvested and processed in RNAlater for subsequent RNA extraction and gene expression analysis by qPCR.

Quantification of Gene Expression by qPCR

Immediately after blood collection for analysis, mice from biopsy ($n = 5$) and non-biopsy ($n = 5$) groups were sacrificed for the 0-, 3-, 12-, and 24-hour post biopsy time points ($n = 40$). Sections of tumor and lung were harvested and snap frozen in TRIzol and RNAlater (Qiagen Inc). Frozen tissue samples were later thawed, the tissue was crushed, and the RNA extracted using Purelink RNA Kit (Invitrogen Inc). Generation of cDNA was performed using Superscript II (Invitrogen Inc). For qPCR analysis of relative expression of genes of interest, specific primers for neomycin resistance gene (*Neo*), S100A8, Ly-6G (Gr-1), CXCL2 (MIP-2), CCL3 (MIP-1a), FOXP3, transforming growth factor–β (TGF-β), SOX-4, Ezh2, SNAI2, ZEB2, CDH2 (N cadherin), Interleukin-1 beta (IL-1β), IL-10, Tumor Necrotic Factor-alpha (TNF-α), matrix metalloproteinase 9, and urokinase plasminogen activator were designed on the basis of RefSeq data for *Mus musculus* using the NCBI Primer-BLAST online primer designing tool (Table S1).

Using the Bio-Rad CFX-96 system, qPCR assay was performed and analysis of results was done using the CFX manager software applying $\Delta\Delta C_t$ analysis with relative normalization calculations based on the murine glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *β-actin* housekeeping genes.

Flow Cytometry

To study variation in immune cellular populations in the tumor and lung microenvironments and systemic immune changes related to performance of a CNB, a single cohort of 60 mice was used. Twenty mice from the biopsy ($n = 10$) and non-biopsy ($n = 10$) groups were sacrificed on each assigned post-biopsy time point (days 3, 7, and 10). Sections of tissue from tumor, lung, spleen, and lymph node were harvested and immediately processed and labeled with CD4– fluorescein isothiocyanate, CD3– Phycoerythrin (PE), CD8– PerCP, Dx5 (Pan-Natural Killer [NK])– APC, CD45– fluorescein isothiocyanate, Ly6G (Gr-1)–PE, CD11b-PerCP, and F4/80-APC antibodies (eBioscience Inc., San Diego, CA) allowing quantification of CD4+ T cells, CD8+ T cells, NK cells, macrophages, and myeloid-derived suppressor cells (MDSCs). Using a FACScalibur flow cytometer (BD Biosciences, CA), data were collected and analyzed using CellQuest Pro (BD Biosciences), FCS express ver. 4 (DeNovo Software, CA), and Flowing Software ver. 2.5 (University of Turku, Turku, Finland).

Statistical Analysis

Statistical significance was established relative to baseline values at 0 hour post biopsy or values recorded for non-biopsied samples and determined using non-parametric two-tailed t test performed on GraphPad Prism software. For all analyses, a P value greater than .05 was considered not significant, while significant P values were demarcated using the standard increasing asterisk scale to indicate level of significance, $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

Results

CNB Is Associated with Significantly Increased Pulmonary Metastases

To investigate whether CNB has an effect on the development of pulmonary metastasis, we first implanted the mammary fat pads of BALB/c mice with 4T1 murine breast cancer cells. Tumors were allowed to grow to 6- to 8-mm diameter at which point CNB was performed in half the mice and the tumor growth rate was quantified in all mice for the following week (Figure 1*A*). Our results illustrate that tumor growth rates were not significantly affected by CNB (Figure 1*B*). There was also no significant difference between the body weights of the biopsied and non-biopsied groups (Figure 1*C*).

Next, to reproduce the human experience, we surgically removed the tumors from all the mice (biopsied and non-biopsied) and the mice were allowed to recover. Histologic comparison of excised tumors from biopsied mice *versus* those from non-biopsied mice showed similar levels of necrosis (Figure 1*D*). One week following surgical excision of their tumors, all mice were sacrificed and the lungs were analyzed for metastasis. Mice that had undergone CNB demonstrated significantly greater lung metastasis (Figure 1, *E* and *F*). Importantly, this suggests that CNB performed before surgical excision of a tumor may unintentionally promote metastatic dissemination.

CNBs Create an Immunosuppressive Tumor Microenvironment

It is well established that an immunosuppressive tumor microenvironment facilitates malignant progression by promoting tumor immune evasion, angiogenesis, and metastasis [46–48]. Furthermore, surgical procedures are known to induce suppressive immune conditions including infiltration by immune cells with suppressive capacities such as MDSCs [46,49]. Therefore, following the regimen illustrated in Figure 2*A*, we investigated whether the increased metastasis observed in mice after CNB is also associated
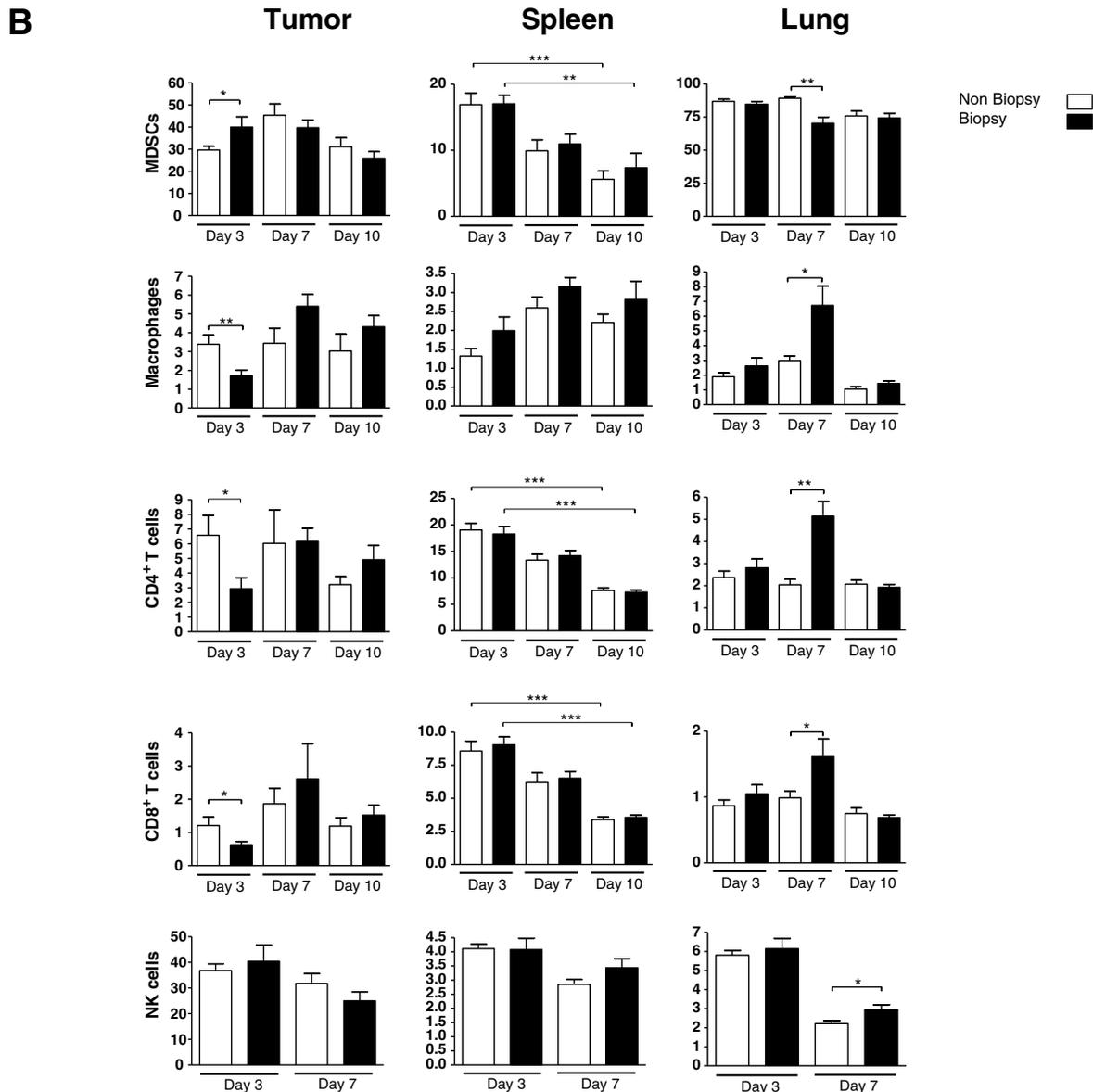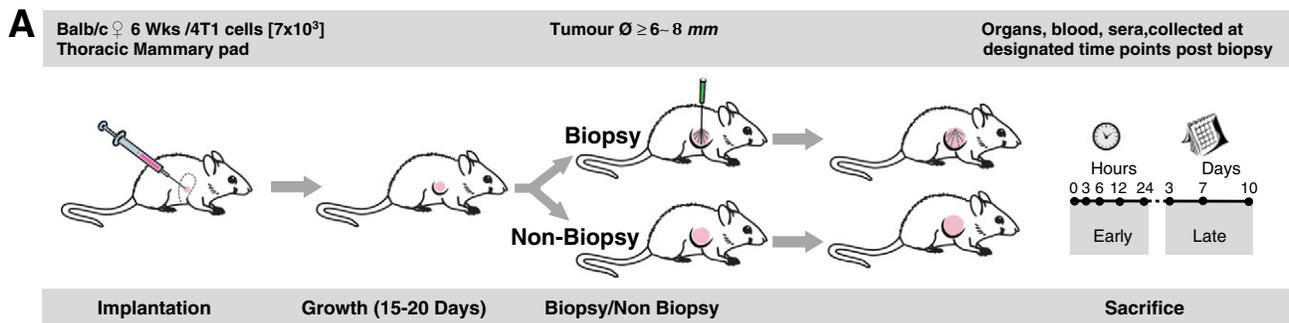
Figure 2. CNBs induce an immunosuppressive tumor microenvironment. (A) Mice were implanted with 4T1 cells, and after 6- to 8-mm diameter tumors developed, half the mice were biopsied ($n = 30$), while the other half were not biopsied ($n = 30$). The tumor, spleen, and lungs were collected from 10 mice from each group for flow cytometry analysis of the indicated immune cells at 3, 7, and 10 days after the biopsy date. NK cells were only monitored on days 3 and 7 post-biopsy date. Significance determined by unpaired t test, $*P \le .05$; error bars represent SEM.

with the development of a pro-tumor immunosuppressive microenvironment. Flow cytometry was used to quantify macrophages (CD45+, CD11b^{low}, F4/80+), MDSCs (CD45+, CD11b+, Gr-1+),

CD4+ T cells, CD8+ T cells, and NK cells (Pan-NK, CD3+) (Figure S1A). Data were collected on the frequencies of these cells in the primary tumor, spleen, lymph node, and lungs. It was found that

on day 3 post biopsy there was a significant increase in the frequencies of MDSCs with a concurrent decrease in macrophage, CD4 + T cell, and CD8 + T cell frequencies in the tumors of mice from the biopsy group compared to non-biopsied controls. On day 7, CD4 + and CD8 + T cell and macrophage levels were significantly higher in lung tissue from the biopsy group compared to non-biopsy controls (Figure 2B). Additionally, during the post-biopsy period, there was a gradual decrease in the frequencies of MDSCs, CD4 +, and CD8 + T cells and an increase in macrophages in the spleen in both groups. Furthermore, monitoring the frequencies of the NK cells during the first 7 days post biopsy revealed that there were higher frequencies of these innate immune cells in the lung tissue from the biopsy group on day 7 post biopsy compared to non-biopsied controls (Figure 2B). Although trends were observed in the spleen (Figure 2B) and lymph nodes (Figure S1B), both showed no significant difference in recorded immune cell frequencies when comparing biopsy and non-biopsy groups over 10 days post CNB.

Together, our data demonstrate that the CNB procedure impacts the level of immune cells at both local and systemic sites and promotes the development of an immunosuppressive immune milieu in the local tumor microenvironment immediately following CNB.

CNB Alters the Expression of Cytokines in the Local Tumor Microenvironment

The recruitment, pathophysiology, and functionality of immune cells are dictated by various soluble mediators (cytokines and chemokines) [50]. Hence, we next investigated whether CNB altered the expression of various pro-inflammatory factors such as S100A8, CXCL1, CXCL2, IL-1β, TNF-α, and COX2 that can influence the recruitment and survival of MDSCs, macrophages, and other immune cells at the tumor site [51–53]. To this end, tumor samples collected at various time points from biopsied or non-biopsied groups were processed and analyzed for the expression of a panel of cytokines. The qPCR analysis of these samples revealed a significant elevation in the expression of S100A8, CXCL2 (MIP-2α), CCL3(MIP-1α), and COX2 in tumor tissues as early as 3 hours post biopsy, with this elevation persisting up to 24 hours post biopsy (Figure 3, A–F).

EMT-Related Changes in Gene Expression Occur within Hours of a CNB

Activation of the processes of EMT promotes tumor-cell metastasis through development of dysfunctional cell-cell adhesive interactions, loss of cell-cell junctions, and restructuring of the cytoskeleton. These changes, along with the loss of apical polarity, signal the development of an invasion-permissive, and hence pro-metastatic, microenvironment [54]. To determine if EMT contributes to the changes that occur post biopsy, we assessed the change in gene expression levels of known EMT regulators. Our analysis showed that tumor samples from the biopsied group had significantly increased expression of TGF-β and SOX-4 compared to that of tumors from the non-biopsied control group, as early as 3 hours post biopsy (Figure 4A). This was followed by a significant increase in expression of Ezh2 (a member of the transcription repressive Polycomb group family) by 24 hours post biopsy (Figure 4A). Expression of downstream EMT genes SNAI2 (Slug), ZEB2, and CDH2 (N-cadherin) (Figure 4B) was also statistically significant at 24 hours post biopsy. The timing of TGF-β/SOX-4/Ezh2 expression changes is consistent with involvement of a TGF-β–initiated, Sox4/Ezh2, EMT pathway. These results suggest that the

CNB enhances the expression of genes involved in the development of EMT within the tumor.

CNB Increases the Number of CTCs

Recent studies have highlighted the prognostic relevance of CTCs for metastatic breast cancer [55,56]. Reports of generating increased CTCs as a consequence of incisional biopsies and surgical manipulation raise concern about increased metastasis as a consequence of biopsy-generated CTCs [43]. We therefore sought to determine whether CNB affects the level of CTCs in our experimental model. To accomplish this, we first modified the 4T1 cells to express a neomycin resistance gene (4T1^{neo} cells) that could be detected sensitively by qPCR. The 4T1^{neo} cells were implanted and processed as in previous experiments (Figure 2A). At 6 hours, 24 hours, and 10 days post biopsy, blood was collected and the relative expression of the neomycin resistance gene was quantified as a measure of the number of 4T1^{neo} cells in peripheral circulation. A significant elevation in expression of the neomycin resistance gene was seen in the biopsied group at the 6-hour ($P = .008$) and 24-hour ($P = .034$) time points (Figure 5). These data indicate that the performance of a biopsy is associated with significantly higher number of CTCs as early as 6 hours and persisting to beyond 24 hours post biopsy.

Discussion

CNB, a type of incisional biopsy procedure, is commonly performed in the diagnostic workup of breast cancer. We tested the hypothesis that surgically initiated changes in the tumor microenvironment resulting from a CNB and the ensuing host's interaction with the residual cancer influence the metastatic potential of that tumor resulting in increased metastatic spread. Using a 4T1:BALB/c murine metastatic breast cancer model, we evaluated the impact of performing a CNB on the following: 1) the development of distant metastases that occur over and above those expected in this metastatic model, 2) the status of immune mediators (cells and cytokines) within the tumor microenvironment, 3) the expression of molecules involved in EMT, and 4) the number of CTCs.

Our findings demonstrate that CNB performed on malignant breast cancers significantly increased the incidence of distant metastases above that which would be expected without biopsy. The observed increase in metastases is associated with early immunosuppressive changes within the tumor microenvironment that facilitate metastatic progression. These changes include recruitment of MDSCs and a concomitant down-regulation of CD4+ T cells, CD8+ T cells, and NK cells. Additional findings that support the hypothesis of a CNB-facilitated metastatic process include the up-regulation early post biopsy of genes responsible for EMT including involvement of a TGF-β–initiated, SOX-4/Ezh2, EMT pathway.

Significant decreases in splenic CD4+ T cells and CD8+ T cells, with associated increase in macrophages, have previously been recorded in tumor-bearing mice with no biopsy performed demonstrating that these changes would occur independent of any biopsy-associated effects [57,58].While these changes in spleen cellular population are presumed to be a consequence of dynamic interaction between the tumor microenvironment and the immune system, the specific mechanism or exact implications of the changes remain unknown.

In keeping with the cellular changes demonstrated in the post-biopsy tumor microenvironment, we observed an up-regulation of
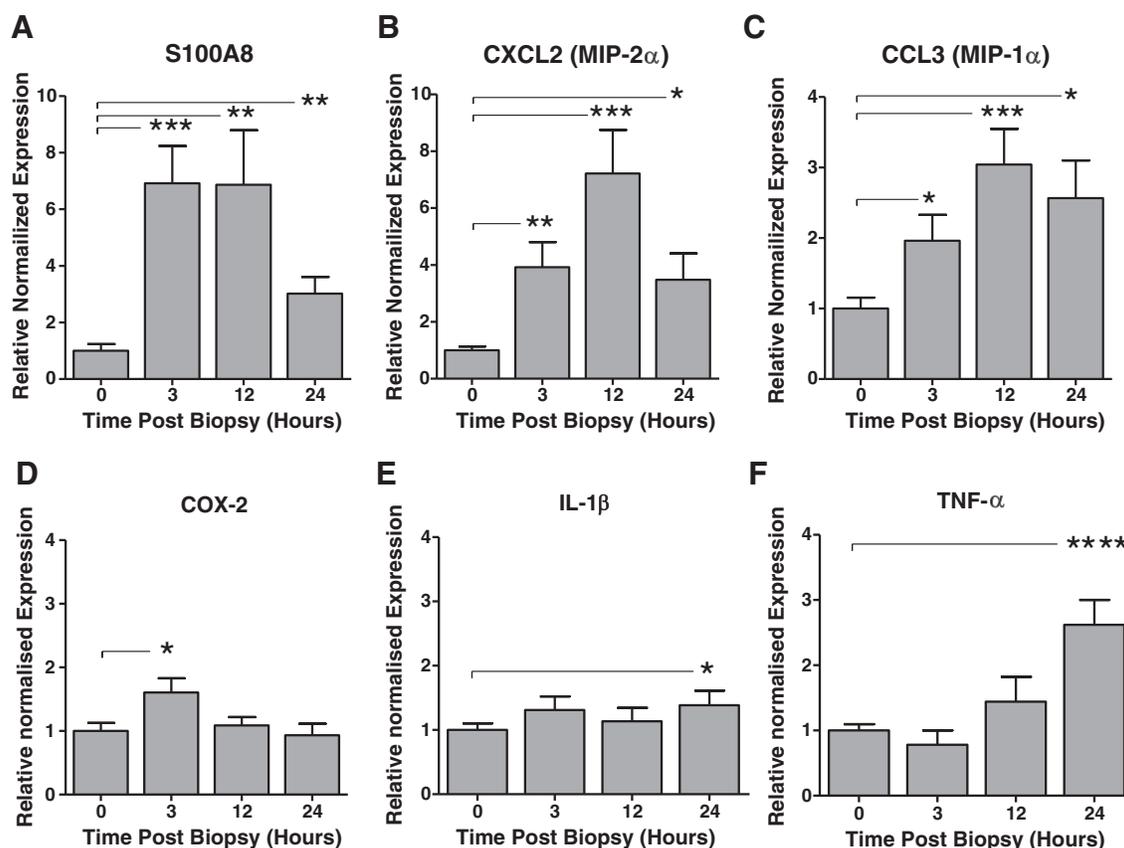
Figure 3. CNB modulates the expression of pro-inflammatory factors within the local tumor microenvironment. Tumor samples collected from biopsied and non-biopsied mice were processed, and RNA was extracted, purified, and reversed transcribed using random primers. Real-time qPCR was conducted on the following gene-specific primers: (A) S100A8, (B) CXCL2 (MIP-2α), (C) CCL3(MIP-1α), (D) COX2, (E) IL-1β, and (F) TNF-α. Gene expression at 0, 3, 12, and 24 hours post biopsy was analyzed using $\Delta\Delta C_t$ analysis with relative normalization with murine GAPDH and β-actin ($n = 5$; significance is determined relative to levels at 0 hour post biopsy by unpaired t test, *$P \leq .05$; error bars represent SEM).

many key inflammatory cytokines and mediators including S100A8, CXCL1, CXCL2, IL-1β, TNF-α, and COX2, which are responsible for the recruitment of multiple inflammatory and immune cell populations including neutrophils, macrophages, MDSCs, and Tregs. Increased S100A8 expression is reportedly associated with chemotactic recruitment of MDSCs as well as the promotion of increased vascular permeability [49,59]. Increased expression of CCL3 and CXCL2 also indicate enhanced chemoattraction of granulocytes and MDSCs. COX2 stimulates production of prostaglandin E2 (PGE2) and has been reported to increase recruitment of EP2-expressing FoxP3 + Tregs to the tumor through a PGE2/EP2 interaction–dependent pathway [53]. Collectively, this post-biopsy cytokine profile favors a pro-metastatic tumor microenvironment.

We established that TGF-β expression was significantly increased post biopsy as was the *SOX-4* gene, a master EMT regulator and a member of the C subgroup of SRY-related HMG box (SOX) transcription factor family [60]. Further investigation showed accompanying increases in expression of the epigenetic modifier Ezh2 as well as increases in the expression of other attendant downstream EMT genes (*ZEB2*, *SNAI2*, and *CDH2*). These findings suggest involvement of the TGF-β/SOX-4 EMT pathway, which would contribute to the increased metastasis we observed through increased migration and extravasation. Previous studies have shown

that SOX-4 expression is associated with poor metastasis-related outcomes among early-stage lymph node–negative patients [42,61], while a separate study correlates SOX-4 expression with triple-negative breast cancer patients and consequently poor prognosis [60]. In conjunction with these studies, our findings suggest a mechanistic role for EMT and related molecules in CNB-induced breast cancer metastasis.

The observed cytokine and EMT-associated gene expression changes also correlated with significantly elevated CTCs from biopsied tumor-bearing mice that persisted up to 24 hours post biopsy. Importantly, other studies have demonstrated that CTC persistence post-surgery extending to, and beyond, 24 hours is considered a robust predictor for metastatic recurrence [62]. The relevance of the CTC/EMT relationship and its role in determining metastatic outcomes in breast cancer is explored in a recent study by Yu et al., which highlights the dynamic relationship between EMT and CTC level and shows the association between cancer subtype, treatment response, and stage of disease. They reveal that EMT, and consequently mesenchymal CTCs, occur more prevalently in aggressive disease subtypes typically characterized as being highly pro-metastatic [44,63]. The persistent increase in the total CTC numbers we observed in biopsied mice likely has a cumulative impact that favors the possibility of successful metastatic colonization (Figure 6).
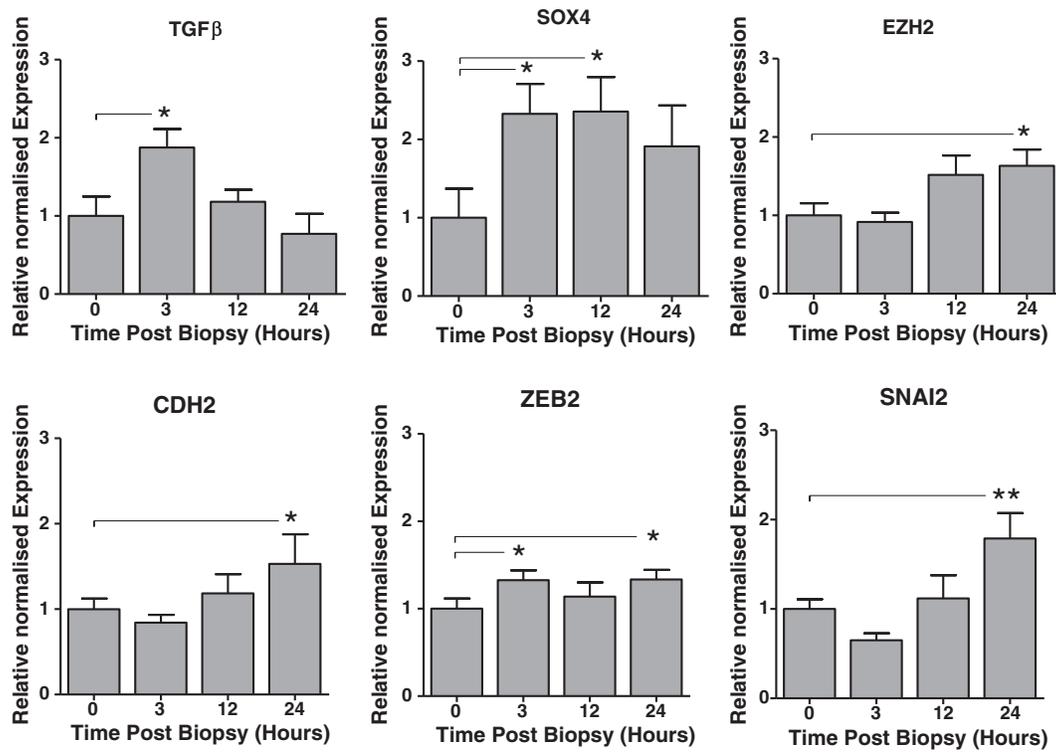
Figure 4. Increase in SOX-4/TGF-β and EMT marker expression post biopsy. qPCR was conducted on tumor-derived cDNA samples from biopsied or non-biopsied mice, and the relative gene expression of TGF-β, SOX-4, EZH2, CDH2, ZEB2, and SNAI2 was normalized to GAPDH and β-actin and analyzed at 0, 3, 12, and 24 hours post biopsy ($n = 5$; significance is determined relative to levels at 0 hour post biopsy by unpaired t test, $*P \leq .05$; error bars represent SEM).

Conclusion

Our experimental findings demonstrate for the first time that in the setting of malignant breast cancer, performance of a CNB is associated with a significantly increased incidence of pulmonary metastases. We also show that an additional impact of CNB includes creation of a distinctly immunosuppressive and pro-metastatic tumor microenvironment with elevated TGF-β/SOX-4–associated EMT and significantly higher CTC levels.

In this era of digital mammography when smaller breast cancers are being detected, presumably in a pre-metastatic state, biologic knowledge of the potential harms associated with the traditional workup of breast cancer through the application of a CNB needs to be considered. Further research to better understand the biologic pathways associated with surgically induced metastases needs to be conducted.

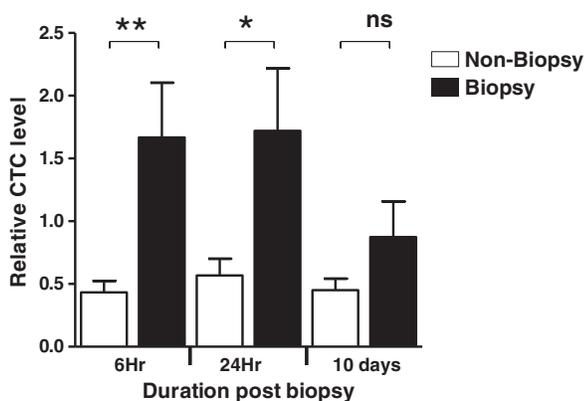Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.09.004.

Figure 5. CNB promotes increased frequency of CTCs in peripheral blood. Blood collected from biopsied and non-biopsied mice at 6 hours, 24 hours, and 10 days post biopsy was analyzed using real-time qPCR for the relative expression of neomycin (neo) resistance gene as a measure of the levels of 4T1^{neo} cells in the peripheral circulation ($n = 15$; significance determined by unpaired t test, $*P \leq .05$, $**P \leq .01$; error bars represent SEM).
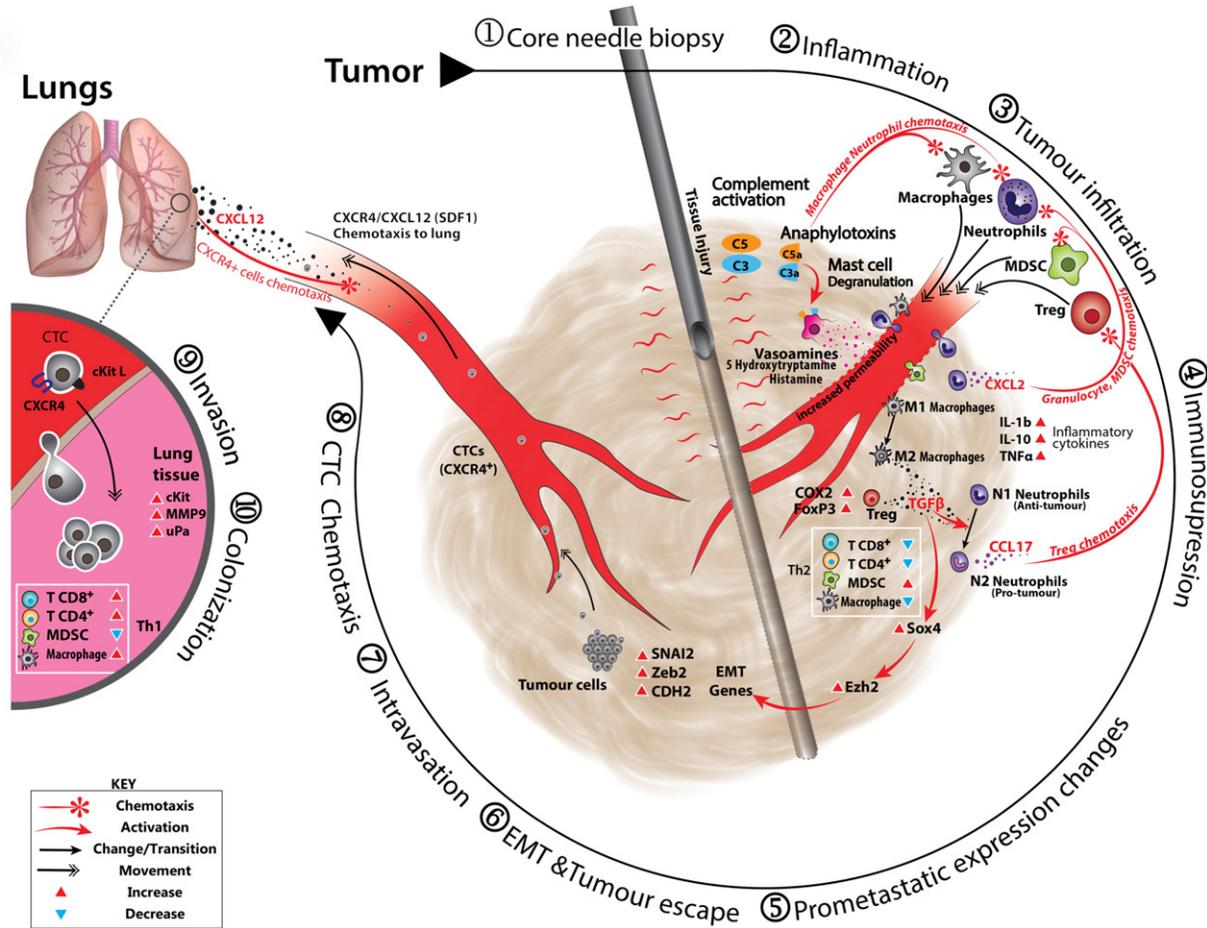
Figure 6. Hypothetical schematic for biopsy-associated metastasis. Clockwise: (1) CNB, with attendant tissue injury, is rapidly followed by (2) inflammation, acute inflammatory reaction with complement-mediated anaphylatoxin release, macrophage and neutrophil chemoattraction accompanied by associated mast cell degranulation, vasoamine release, and corresponding increase in vascular permeability [64,65]. (3) Tumor infiltration, this adds to the population of leucocytes already present in the tumor and initially involves anaphylatoxin and leukotriene chemotaxis drawing neutrophils, other granulocytes (including MDSCs), more mast cells and monocytes, and macrophages; T lymphocytes would typically arrive later, but these are already well represented in the tumor's Cell Mediated Immunity (CMI) environment [66,67]. (4) Immunosuppression, events occur that drive the tumor milieu toward an immunosuppressive phenotype. They include conversion of tumor-associated macrophages from an M1 to an M2 phenotype that increases TGF-β expression [68,69]. This COX2 expression in the tumor stimulates conversion of nascent naïve CD4 + T cells to FoxP3 + Tregs as well as their recruitment through interaction between COX2-stimulated PGE2 and Treg-expressed EP2. Elevated MDSC and Treg populations drive the immunosuppression process. The increase in TGF-β expression allows conversion of tumor-associated neutrophils from N1 to N2; N2 tumor-associated neutrophils express CCL17, a Treg chemotactic accelerating Treg infiltration [53,70]. (5) Pro-metastatic expression changes. Increase in TGF-β stimulates up-regulation of master EMT regulator, SOX-4. SOX-4 directly regulates expression of the *Ezh2* gene that encodes the Polycomb group histone methyltransferase and epigenetically modifies expression of several EMT genes [42,60]. (6) EMT and tumor escape, EMT allows the conversion of tumor cells toward an invasive mesenchymal phenotype allowing (7) intravasation into the circulatory system and (8) CTC chemotactic migration to the lung through the CXCR4/SDF1 axis [71–73], (9) invasion (extravasation), once in the lung the cells invade the lung tissue at the metastatic niche expressing Extra-cellular Matrix (ECM) degrading matrix metalloproteinase 9 and urokinase plasminogen activator completing the process of [74–76] (10) metastasis by successful colonization and formation of metastases.

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