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Enhanced survival with implantable scaffolds that capture metastatic breast cancer cells *in vivo*

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Abstract

The onset of distant organ metastasis from primary breast cancer marks the transition to a stage IV diagnosis. Standard imaging modalities often detect distant metastasis when the burden of disease is high, underscoring the need for improved methods of detection to allow for interventions that would impede disease progression. Here, micro-porous poly(ϵ -caprolactone) scaffolds were developed that capture early metastatic cells and thus serve as a sentinel for early detection. These scaffolds were used to characterize the dynamic immune response to the implant spanning the acute and chronic foreign body response. The immune cell composition had stabilized at the scaffold after approximately 1 month, and changed dramatically within days to weeks after tumor inoculation, with CD11b⁺Gr1^{hi}Ly6C⁻ cells having the greatest increase in abundance. Implanted scaffolds recruited metastatic cancer cells that were inoculated into the mammary fat pad *in vivo*,

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which also significantly reduced tumor burden in the liver and brain. Additionally, cancer cells could be detected using a label-free imaging modality termed inverse spectroscopic optical coherence tomography, and we tested the hypothesis that subsequent removal of the primary tumor after early detection would enhance survival. Surgical removal of the primary tumor following cancer cell detection in the scaffold significantly improved disease-specific survival. The enhanced disease-specific survival was associated with a systemic reduction in the CD11b⁺Gr1^{hi}Ly6C⁻ cells as a consequence of the implant, which was further supported by Gr-1 depletion studies. Implementation of the scaffold may provide diagnostic and therapeutic options for cancer patients in both the high-risk and adjuvant treatment settings.

Keywords

cancer metastasis; biomaterial implant; immune response; early detection; label free imaging

Introduction

The oncogenic progression of breast cancer from the primary tumor to distant metastatic sites is the critical event that defines stage IV disease (1-3). Currently, metastatic disease is detected through radiologic imaging modalities after the burden of distant disease has become destructive to the host organ (4-6). A limitation to the development of life-preserving timely interventions is the striking lack of robust technologies capable of early detection of metastatic events. Additionally, experimental model systems are needed that permit systematic screening and examination of factors contributing to breast cancer metastasis in a controlled setting. Detection of circulating tumor cells (CTCs) is being pursued in both the experimental and clinical settings. While promising (7, 8), the widespread use of CTC capture is not without challenges, given the high biomarker sensitivity and specificity required to capture a low number of circulating CTCs (9, 10). Furthermore, CTCs may not represent the population of cells capable of metastasis or these cells could circulate for long periods before invading distant organs. The capacity to identify metastatic cells or foci at the earliest possible time-point may permit the delivery of targeted treatment interventions prior to the compromise of distant organs, potentially translating into prolonged distant metastasis free outcomes. Thus, there is an urgent need for development of novel technologies to aid in the detection of metastatic events in the nascent setting.

An emerging approach for early detection lies with the implantation of a biomaterial scaffold that can capture metastatic cells (11). These scaffolds were modeled after the concept of the pre-metastatic niche (12, 13), echoing Paget's "seed and soil" hypothesis proposed over a century ago (12, 14-17). This paradigm proposes that, prior to colonization by metastatic cells, supportive cells (e.g., fibroblasts, immune cells, endothelial cells), soluble factors, and extracellular matrix (ECM) components establish a microenvironment conducive to tumor cell homing and colonization (12, 14-17). Importantly, these studies indicate that metastasis to specific organs is not random, but is influenced by the properties of the local environment (12, 13, 18). The initial translation of these principals led to the development and implementation of micro-porous poly(lactide-co-glycolide) (PLG) biomaterial scaffolds, which recruited metastatic breast cancer cells through the local immune response *in vivo*,

resulting in decreased tumor burden at metastatic sites (11). However, PLG scaffolds were degradable over time scales considered too short for clinical translation.

In this report, we developed micro-porous poly(ϵ -caprolactone) (PCL) scaffolds with greater stability than the PLG scaffolds, to investigate the dynamic immune response and cellular events associated with PCL-scaffold-mediated recruitment of metastatic breast cancer cells. Specifically, utilizing PCL scaffolds in metastatic breast cancer murine models we examined if (a) metastatic cells could be recruited to the scaffold (b) metastatic cells could be detected in the scaffold at a nascent stage, prior to cancer cell colonization of other major organs, using label-free imaging modalities and (c) scaffold implantation could influence survival following detection of cancer cells in the scaffold and then subsequent surgical removal of the primary tumor. The favorable translational endpoints from these studies could lead to the integration of scaffold implants, fabricated using FDA approved materials, into breast cancer disease management plans. Moreover, scaffolds could be recovered to examine the biology of metastatic tumor cells in conjunction with “niche” cells enabling the development of patient-specific treatments.

Materials and Methods

Fabrication, characterization, and implantation of micro-porous scaffolds

Scaffold Fabrication and Characterization—For preparation of microporous PCL scaffolds, PCL microspheres were first prepared by emulsifying a 6% (w/w) solution of PCL (Lactel Absorbable Polymers, Birmingham, AL; Inherent viscosity = 0.65-0.85 dL/g) in dichloromethane in a 10 % poly(vinyl alcohol) solution followed by homogenization at 10,000 rpm for ~ 1 min. The solution was then stirred for 3 h. Microspheres were collected by centrifugation and washed at least 5 times in deionized water, followed by lyophilization for 48 h. To prepare micro-porous PCL scaffolds, PCL microspheres and salt particles (size range 250 – 425 μ m) were mixed in a 1:30 (w/w) ratio and pressed at 1500 psi in a steel die for ~ 45 s. Polymer-salt discs were heated at 60 °C for ~ 5 min on each side, followed by foaming in high pressure CO₂ at 800 psi for ~ 24 h. Salt particles were removed by immersing discs in water. For experimental studies, scaffolds were sterilized using 70% ethanol, rinsed with sterile water, and dried on a sterile gauze pad. Microporous PLG scaffolds were prepared as described previously (19). Scaffolds were characterized using mechanical testing, scanning electron microscopy, and calculation of porosity. Technical details are available in Supplementary Materials and Methods.

Scaffold Implantation—Microporous scaffolds were implanted in the subcutaneous space of either female BALB/c or NOD/SCID-IL2R $\gamma^{-/-}$ (NSG) mice (8-10 week old). All animal studies were performed in accordance with institutional guidelines and protocols approved by Northwestern University and the University of Michigan Institutional Animal Care and Use Committee. NSG mice were bred in house or purchased from the Jackson laboratory. BALB/c mice were purchased from the Jackson laboratory. For the implantation procedure, mice were anesthetized with an intraperitoneal injection of Ketamine (10 mg/kg) and Xylazine (5 mg/kg). The upper back was shaved and prepped using a betadine swab followed by an ethanol swab (3 \times). An incision was made in the upper back and a

subcutaneous pocket was created on each side, into which the scaffolds were inserted (2 scaffolds per mouse). The skin was closed using wound clips (Reflex 7mm, Roboz Surgical Instrument Co.) and surgical glue (3M Vetbond Tissue Adhesive).

Tumor inoculation

Orthotopic tumor inoculation was performed one month after scaffold implantation. MDA-MB-231BR-tdTomato-luc2 cells were obtained from the Northwestern University Developmental Therapeutics Core and authenticated by short tandem repeat DNA analysis and comparison to the ATCC STR profile database in 2013 (DDC Medical). 4T1-luc2-tdTomato cells were obtained from Perkin Elmer in 2014 and were used directly without additional authentication. 2×10^6 4T1-luc2-tdTomato (Perkin Elmer) or MDA-MB-231BR-tdTomato-luc2 cells in 50 μ L sterile phosphate buffer saline (PBS) (Life Technologies) were injected into the fourth right mammary fat pad of 12-14 week old female BALB/c or NSG mice.

Flow cytometry

Mice were euthanized at indicated times and retrieved scaffolds and organs were processed according to previously established procedures, which are described in Supplementary Materials and Methods (11). Flow cytometry staining and analysis were performed according to established procedures (please see Supplementary Materials and Methods) (11).

Scaffold sectioning and fluorescence imaging

Scaffolds retrieved from mice were rinsed in PBS and then immediately flash frozen in pre-chilled isopentane. Frozen scaffolds were then embedded in optimal cutting temperature (OCT; Cardinal Health) compound with 30% sucrose and sectioned using a cryostat (Microm HM 525; Microm International) at 14 μ m. Scaffold sections were stored at -20 °C until imaging. Cryosections were air-dried at room temperature for 30 min, fixed with 10% neutral buffered formalin, washed with tap water for 5 min, DI water for 10 min (2 \times) and cover slipped with ProLong Gold antifade aqueous mounting medium containing DAPI (Molecular Probes, Grand Island, NY). DAPI fluorescence was visualized using an excitation wavelength of 358 nm, and tdTomato fluorescence in cancer cells was visualized using an excitation wavelength of 532 nm. Images were viewed using an Olympus BX43 microscope and an Olympus DP72 digital camera with CellSens Entry software (Olympus) used for image capture and co-localization.

ISOCT imaging and analysis

ISOCT imaging and analysis was performed as described elsewhere (20-22). Technical details about this procedure are provided in Supplementary Materials and Methods.

Post-surgical model of breast cancer metastasis and Gr-1 depletion

The influence of scaffold implant on survival was investigated using a post-surgical model of breast cancer metastasis (23, 24). In this model, the 4T1 primary tumor was resected 6 or 10 days post tumor inoculation. Briefly, the primary tumor area was prepped using a betadine swab followed by an ethanol swab (3 \times). An incision was made along the right side of the

lower half of the dorsal skin exposing the primary tumor. The tumor was picked up using needle nose-forceps and cut around the base using curved tip scissors. The skin was closed using MONOCRYL® (poliglecaprone 25) suture (Ethicon, Inc.) and surgical glue (3M Vetbond Tissue Adhesive). Animal health was monitored daily after the procedure for activity and responsiveness including posture, mobility, body weight, grooming behavior, and respiratory conditions. Animals were euthanized if found in a moribund condition as an experimental endpoint. Mice that evidenced primary tumor re-growth were excluded from the analysis to avoid confounding effects arising from the primary tumor. For Gr-1 depletion studies, resection was performed at day 10 as described above and mice received 300 µg anti-Gr-1 (clone RB6-8C5, Bio X Cell) via intraperitoneal injection at day 13 and 17 post tumor inoculation.

Data analysis

Data are presented as mean ± standard error (s.e.m.). Animal studies were performed with at least two independent replicates of 4–8 female 8–12-week-old mice per group with random assignment. Multiple comparisons were performed using one-way ANOVA. Comparisons post ANOVA was performed using the Tukey-HSD test. For data that did not follow a normal distribution, comparison was performed using the non-parametric Wilcoxon rank-sum test. For comparing the relative number of mice containing detectable tumor cells in organs with scaffolds, a Fisher's exact test was used to determine the p-value. Statistical analysis was performed using JMP Software (JMP Pro 11). For survival analysis, Kaplan-Meier curve was generated and statistical analysis was performed using a Log-rank test using Sigma Plot (Version 13).

Results

Micro-porous PCL scaffolds for *in vivo* recruitment of metastatic cells

We developed micro-porous PCL scaffolds (Fig. 1A, 5 mm diameter and 2 mm height) to create microenvironments *in vivo* and subsequently examine their ability to recruit metastatic tumor cells. The porous interconnected architecture of the scaffold was confirmed using SEM imaging (Fig. 1B). Micro-structural features such as porosity, pore volume, and mechanical properties (i.e., elastic modulus) were similar for PCL and previously reported PLG scaffolds (11) (Table S1). The ability of PCL scaffolds to persist and create a defined space *in vivo* was investigated by implantation into the subcutaneous dorsal space of BALB/c and NSG mice. The subcutaneous site was selected for its accessibility and amenability to non-invasive imaging. Furthermore, neither 4T1 nor MDA-MB-231BR breast cancer cells typically metastasize to the subcutaneous space, thus the presence of cancer cells in the metastatic site would likely be associated with the presence of the scaffold. PCL scaffolds retrieved after 3 months experienced minimal degradation when compared to day 0 as opposed to PLG scaffolds, which had previously been employed for *in vivo* recruitment of tumor cells (11). PLG scaffolds showed significant degradation over this time period as quantified by scaffold area (i.e., 66% in NSG and 77% in BALB/c mouse; Fig. S1).

The dynamic immune response to the biomaterial implant was investigated throughout the acute and chronic phases. Implantation of the PCL scaffold into healthy BALB/c mice

resulted in infiltration of CD45⁺ leukocytes by day 3. The number of CD45⁺ leukocytes remained relatively unchanged after day 14 post scaffold implantation (Fig. 1C). However, the relative distribution of leukocyte populations examined, including innate and adaptive immune cells, changed dynamically following scaffold implantation. The percentage of inflammatory monocytes, identified as Ly6C⁺F4/80⁻ cells, decreased after day 3 and remained relatively stable at later time points, whereas the percentage of dendritic cells, identified as CD11c⁺F4/80⁻, increased after day 3 and remained stable at later time points (Fig. 1D). These two cell populations constituted the majority of cells (i.e., 65%) observed at the PCL scaffold at later time points. The percentage of macrophages, identified as CD11b⁺F4/80⁺ cells, significantly increased through day 14 (e.g., 8.8 % at day 14 vs. 1.7% at day 3, Fig. 1D, $p < 0.05$) and then returned to levels observed at day 3 (e.g., 1.4 % at day 60, Fig. 1D, $p = 0.99$ compared to day 3). In contrast, the levels of CD11b⁺Gr-1^{hi}Ly6C⁻ cells remained low at all time points examined at 0.15 % (Fig. 1D). In the adaptive immune cell population, the percentage of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells significantly increased over time (e.g., 1 % at day 3 to 9 % at day 60 for CD4⁺ and 1.2% at day 3 to 3% at day 60 for CD8⁺ respectively, Fig. 1D, $p < 0.05$). The percentage of B cells, identified as CD19⁺, and natural killer (NK) cells, identified as CD49b⁺, increased post day 3 and returned to day 3 levels at later time points (i.e., day 30 and 60; Fig. 1D). Importantly, the relative percentages of leukocyte subpopulations were similar between day 30 and day 60 post scaffold implantation in BALB/c mice (Fig. 1D). This trend was also observed in NSG mice (Fig. S2). Based on the stabilization of cell populations after day 30, we utilized day 30 as a time point representing the chronic response to a scaffold implant in all following experiments.

We subsequently examined the recruitment of metastatic cells to a chronically implanted microporous scaffold (i.e., a scaffold that had been implanted for 30 days prior to tumor inoculation, a time corresponding to the chronic phase of the immune response). Flow cytometry and fluorescence imaging (Fig. 2) performed for scaffolds retrieved at day 15 post tumor inoculation demonstrated the presence of mouse 4T1 tumor cells in the scaffold, indicating that the local microenvironment enabled recruitment of tumor cells. Total cell infiltration was significantly greater within PCL scaffolds compared to PLG scaffolds (i.e., $\sim 6 \times 10^5$ cells in the PCL scaffold vs. $\sim 1 \times 10^5$ cells in the PLG scaffold, $p < 0.0001$, Fig. 2A) and a similar trend was observed for tumor cell recruitment (Fig. 2B, $p < 0.01$). Scaffolds were also able to recruit human MDA-MB-231BR cells in NSG mice (Fig. S4), indicating that such a system enabled recruitment of mouse and human breast cancer cells in the context of both immune competent and immune compromised mouse models, respectively.

Following tumor inoculation, the dynamics of immune cell populations at the PCL scaffold was subsequently characterized, as tumor cells are known to influence the recruitment of immune cells from the bone marrow (25). Flow cytometric analysis indicated an increase in Ly6C⁺F4/80⁻ and CD11b⁺Gr-1^{hi}Ly6C⁻ cells at the PCL scaffold site (Fig. 3C and 3D, $p < 0.0005$). For example, the numbers of CD11b⁺Gr-1^{hi}Ly6C⁻ cells increased from 0.1 % at day 0 to 17 % at day 21 post tumor inoculation ($p < 0.05$), an increase of two orders of magnitude relative to their numbers at the PCL scaffold site in tumor-free BALB/c mice (Fig. 1D, Fig. 3C). Both cell types have been implicated in the pre-metastatic niche (15,

25-28). In contrast, the percentages of CD11b⁺F4/80⁺ macrophages, CD11c⁺F4/80⁻ dendritic cells, and CD8⁺ cytotoxic T cells decreased at the PCL scaffold site (Fig. 3A, 3C, 3F, e.g., 30% at day 0 vs. 14% at day 21 for dendritic cells, $p < 0.05$). The percentage of CD19⁺ B cells, CD49b⁺ NK cells, and CD4⁺ helper T cells increased at day 3 and then decreased at later time points (Fig. 3G, 3H, 3E). Specifically, NK cells increased from 4% at day 0 to 8% at day 3, followed by a decrease to 2.5% at day 21 post tumor inoculation (Fig. 3H, $p < 0.05$). Interestingly, the immune cell dynamics at the PCL scaffold site reflected the dynamics observed in the spleen post tumor inoculation (Fig. 3 vs. Fig. S3). In summary, the changing immune microenvironment at the PCL scaffold site post tumor inoculation correlated with recruitment of 4T1 tumor cells, and is consistent with prior literature reports on the role of the immune cells in the pre-metastatic niche (15, 25-30).

Early detection of metastatic cells at the PCL scaffold

The ability to detect the presence of metastatic disease at an early stage was examined through evaluation of the percentage of tumor cells in the PCL scaffold relative to the cancer cells detected in typical metastatic sites such as the lung, liver, and brain, at day 5 post tumor inoculation. Flow cytometry analysis revealed that the PCL scaffolds had a detectable percentage of tumor cells (i.e., $0.005 \pm 0.002\%$) compared to the lung, liver, and the brain, none of which had detectable tumor cells (Fig. 4A and 4B; $N = 5$ for lung, liver, and brain, $N = 10$ for PCL scaffolds, $p < 0.05$, Fisher's exact test). The greater density of tumor cells observed at the PCL scaffold site compared to other organ sites supports the use of this tool for detecting metastatic disease at a nascent stage.

We subsequently investigated the feasibility of using a label-free imaging technique: inverse spectroscopic optical coherence tomography (ISOCT), for the early detection of metastatic disease in a chronic model of scaffold implantation. The tissue was modeled as a continuous random refractive index distribution, which enabled the refractive index correlation function shape factor D to be computed from the shape of the backscattering intensity spectrum obtained with ISOCT (20). If D has a value between 0 and 3, it has a physical meaning of a mass fractal dimension, reflecting a more clumped structure associated with higher D . Prior studies of early carcinogenesis with ISOCT and a similar spectroscopic technique, low-coherence enhanced backscattering spectroscopy (LEBS), have revealed that D measured from tissue increases with cancer progression (22, 31, 32). Thus, similar ultra-structural tissue modifications occurring in the pre-metastatic niche are likely to have an analogous effect on D . D has previously been reported to reflect mass-density distribution features at length scales of 35-350 nm (21). In addition, D values from tissue have been demonstrated as a robust biomarker of early-stage carcinogenesis (22). Consistent with these observations and data obtained via flow cytometric analysis (Fig. 4A and 4B), a significant increase was observed in average D values obtained from ISOCT measurements at the PCL scaffold site in tumor bearing mice ($N = 7$) compared to tumor free mice ($N = 8$; $p < 0.05$, Fig. 4C), confirming ultra-structural alterations to the scaffold and further indicative of the presence of tumor cells. The color map overlay of D values (Fig. 4D and 4E) demonstrated the distribution throughout the scaffold. These results suggest that ISOCT could be employed for early detection of metastatic disease at the PCL scaffold.

PCL scaffold implantation reduces tumor burden and improves disease-specific survival

We subsequently investigated the hypothesis that the recruitment of metastatic cells to the chronically implanted PCL scaffolds may reduce the tumor burden at typical metastatic sites, such as the liver, brain, and the lung at day 15 post tumor inoculation. Flow cytometry analysis indicated that the percentage of tumor cells in the liver and the brain was reduced in mice receiving a PCL scaffold versus mice undergoing a mock surgery. As stated, the tumor burden was reduced by 64% for the liver (Fig. 5A, N = 15, $p < 0.05$) and 75% for the brain (Fig. 5B, N = 8 for mock surgery, N = 6 for scaffold implant, $p < 0.05$ as determined using the Wilcoxon rank-sum test in both cases). However, in this immunocompetent mouse model, a reduction in the tumor burden in the lung was not observed (Fig. 5C, N = 11, $p = 0.7$) distinct from our previous observations in an immune compromised NSG mouse inoculated with human MDA-MB-231BR cells (11).

A post-surgical model of breast cancer metastasis was then applied to investigate the potential for PCL scaffold implants to influence survival. In this model, the primary tumor was resected at day 6 (Fig. S5) or 10 (Fig. 6A) post tumor inoculation, which corresponded to a time after which cancer cells were detectable in the scaffold by label-free imaging (i.e., day 5, Fig. 4). The resected tumor weights were comparable for both groups, with tumors from the mock surgery group weighing 0.423 ± 0.035 g versus tumors from scaffold implanted mice weighing 0.419 ± 0.029 g at day 10 post inoculation ($p = 0.93$, t-test, Fig. 6B). Kaplan-Meier survival analysis demonstrated a significant improvement in survival in mice receiving a PCL scaffold implant compared to mice receiving a mock surgery with resection at day 10 (Fig. 6C, N = 7 per group, $p < 0.05$, Log-rank test). The sacrifice end-points utilized for mice in both groups for data corresponding to Fig. 6 are described in Table S2. With resection at day 6 post tumor inoculation, ~40% of both mock and scaffold groups survived indefinitely (Fig. S5) mirroring the 40% survival in the scaffold group with day 10 resection (Fig 6C), indicating that the scaffold increased the time over which a therapeutic intervention such as surgery can be performed and provide a survival benefit.

Given the greatest increase in the abundance of $CD11b^+Gr-1^{hi}Ly6C^-$ cells (2 orders of magnitude change) at the PCL scaffold site post tumor inoculation, we hypothesized that the increased survival with scaffold implantation may reflect a differential distribution of $CD11b^+Gr-1^{hi}Ly6C^-$ cells at the primary tumor (local) and the spleen (systemic). Flow cytometric analysis indicated that the abundance of $CD11b^+Gr-1^{hi}Ly6C^-$ cells was reduced in mice receiving a scaffold implant versus mice receiving a mock surgery examined at day 10 post tumor inoculation. The burden of $CD11b^+Gr-1^{hi}Ly6C^-$ cells was reduced by 39% in the primary tumor (Fig. 7A) and 30% in the spleen (Fig. 7B, N = 7, $p < 0.05$ as determined by t-test in both cases). This result suggests that, in part, presence of the scaffold contributes to a reduction in the abundance of key niche cells locally (i.e., primary tumor site) and systemically (i.e., spleen) that support metastasis. This observation was further investigated via Gr-1 antibody depletion of $CD11b^+Gr-1^{hi}Ly6C^-$ cells *in vivo*, which was performed in the context of surgical resection (Fig. S6). Mice receiving a mock surgery with Gr-1 depletion demonstrated survival greater than 20% by day 40 (Fig. 7C), with no survival observed at this time without Gr-1 depletion (Fig. 6C). Furthermore, with Gr-1 depletion, the observed difference in survival between mock and scaffold groups was not statistically

significant (Fig. 7C), further supporting the involvement of CD11b⁺Gr-1^{hi}Ly6C⁻ cells in improving disease-specific survival in our model. Taken together, these results highlight the potential for PCL scaffold in improving disease-specific survival outcomes.

Discussion

In this study, micro-porous PCL scaffolds, implanted prior to tumor initiation, recruited metastatic cells at an early time-point in disease progression. The novel approach to this work was based on recapitulating some of the immunological aspects of the pre-metastatic niche, while prior reports have focused on materials to mimic properties of target organs (e.g., bone (33, 34), bone marrow (35)). Previous elegant studies of the pre-metastatic niche have identified some of the biological cues involved in cancer cell recruitment, such as the cellular components (e.g., hematopoietic and endothelial progenitor cells, immune cells), soluble factors (e.g., cytokines, chemokines), and ECM proteins (12, 14-17). Importantly, as indicated by Lyden (12, 18), the existence of the pre-metastatic niche implies that metastasis to a particular site is not random, but is predetermined, which supports the idea that a site could be engineered to attract metastatic cells. A synthetic scaffold provides an opportunity to create a defined environment to investigate the role of specific components involved in the colonization of metastatic cells. Scaffolds can be modified with specific niche components, such as stromal cells, ECM molecules, and cytokines to identify the key signals in the metastatic environment (36, 37), thereby providing a tool with which to advance fundamental studies of the pre-metastatic niche and tumor metastasis. Herein, the scaffold defines a site for immune cell infiltration, and we characterize the dynamic immune response associated with cancer cell recruitment.

The immune cell populations at the PCL scaffold, which had stabilized prior to tumor inoculation, were substantially altered post-tumor inoculation, suggesting that the changing foreign body response to the implant may contribute to metastatic cell recruitment. Immune cells are recognized as significant to the pre-metastatic niche (15, 25-30). As such, chemokine CCL-2 recruits inflammatory monocytes (Ly6C⁺F4/80⁻ cells) to the pre-metastatic niche enabling metastasis of breast cancer cells (28). Similarly, CD11b⁺Gr-1^{hi}Ly6C⁻ cells are recruited via inflammatory chemoattractants (e.g., S100A8 and S100A9) to pre-metastatic niches (38). In addition, CD11b⁺Gr-1^{hi}Ly6C⁻ cells are known to downregulate infiltration and suppress the function of T cells (CD4⁺ and CD8⁺ T cells) and NK cells (38-41). Consistent with these observations, we found an increase in the levels of monocytes and CD11b⁺Gr-1^{hi}Ly6C⁻ cells at the scaffold site post tumor inoculation and an associated decrease in the abundance of CD4⁺ T cells, CD8⁺ T cells, and CD49b⁺ NK cells, with the greatest change observed for CD11b⁺Gr-1^{hi}Ly6C⁻ cells (i.e., more than two orders of magnitude). Importantly, the changing immune composition as a consequence of disease progression observed in the spleen largely reflected the dynamics at the scaffold site. Taken together, these results suggest that engineering a local microenvironment may be used to identify and modulate key components of cancer-associated immunogenicity in the pre-metastatic niche.

The implantation of PCL scaffolds enhanced disease-specific survival, which we hypothesized to be related to the decreased tumor burden in major organ sites and a

reduction in systemic availability of key immune cells that support metastasis. The implantation of PCL scaffolds in the subcutaneous space reduced tumor burden in major organ sites (i.e., liver and brain) in an immunocompetent mouse model. We previously reported a reduction in burden in the lung in an immunocompromised mouse model using PLG scaffolds implanted in the intraperitoneal fat pad after tumor inoculation (11). The results of the current study extend our previous observation and importantly suggest that a scaffold-based approach can contribute to the reduction in disease burden in solid organs in both immunocompetent and compromised mouse models and when implanted at different sites. Metastatic cells could be detected within chronically implanted PCL scaffolds by day 5 following tumor inoculation using ISOCT imaging, which allowed for label free detection of metastasis through changes in the tissue ultrastructure (e.g., matrix organization) and the presence of cancer cells that have a distinct nano-scale signature relative to normal cells (22). In order to employ ISOCT analysis for diagnostic screening of early metastasis, ultrastructural parameters measured with ISOCT (D, correlation length Ln, and refractive index fluctuation Bn), which independently change in the presence of carcinogenic tissue modification, would need to be investigated to establish a multivariate diagnostic for patients with metastatic disease.

The resection of the primary tumor at day 10 post tumor inoculation resulted in increased survival in mice that received a scaffold. The increased survival may result from a decreased burden of CD11b⁺Gr-1^{hi}Ly6C⁻ cells observed locally at the primary tumor and systemically in the spleen of a scaffold-bearing mouse when compared to a mouse that received a mock surgery. Gr-1 depletion studies further supported this observation, as mice receiving a mock surgery survive longer in the context of Gr-1 depletion, and the difference between mock and scaffold groups was abrogated with Gr-1 depletion. As stated, CD11b⁺Gr-1^{hi}Ly6C⁻ cells or myeloid derived suppressor cells (MDSCs) have been implicated in the pre-metastatic niche and the reduced abundance of these cells systemically may contribute to the reduced burden in solid organs and ultimately, to enhanced survival (42-44). Finally, MDSCs have been identified in high numbers in patients with metastatic disease, correlating with clinical stage and metastatic disease burden and their levels are predictive of overall survival (45-47). Thus, a scaffold-based approach that reduces the abundance of MDSCs could, in part, explain the survival benefit observed in our studies. Taken together, the ability to detect metastatic disease at an early stage, in combination with the survival benefit provided by the scaffold highlight the potential for this technology in transforming the current detection and management of metastatic disease.

The recruitment of metastatic cells to the scaffold, combined with label-free imaging for detection of nascent stage metastatic cells, and reduced burden of disease in solid organs (i.e., liver and brain), may ultimately allow for interventions when the disease burden is low that could translate to improved disease-specific outcomes. In the clinical setting, the scaffold may be integrated into disease management plans by potentially serving as a sentinel site for detection of disease metastasis or recurrence. For example, the scaffold could be implanted in patients with a diagnosis of invasive cancer at the completion of adjuvant therapy, and monitored during scheduled follow-up visits using the optical imaging technique. If metastatic or recurrent disease was then detected, the scaffold could be explanted for cell retrieval and analysis to help guide targeted treatment decisions.

Furthermore, in the setting of metastatic disease, given the potential survival benefit of the presence of the scaffold, patients may have extended disease-specific survival with excision of the primary tumor and scaffold implantation long-term. Finally, the scaffold may be implanted prophylactically to help detect early onset metastatic disease in high-risk patients.

Our results provide the first evidence that a scaffold for capture and detection of early metastatic cells, combined with an intervention shortly after detection of early metastasis (i.e., primary tumor excision) can enhance survival. This biomaterial approach is based on the host response to an implanted scaffold, thereby avoiding the presence of potentially deleterious cellular or biological components. PCL material is currently FDA approved for applications such as drug delivery, suture material, and wound dressings, which may facilitate translation to the clinic for the capture of metastatic cells (48). Also, this material is biodegradable and would not need to be retrieved unless cancer cells are detected; and the degradation rate is relatively slow allowing the implant to be monitored for up to two years within a patient (49, 50). Clinical studies would be necessary to appropriately integrate metastatic cell-capturing scaffolds into existing breast cancer management plans. Taken together, the results of this work showing prolonged survival with scaffold implantation, hold promise for reducing breast cancer morbidity and mortality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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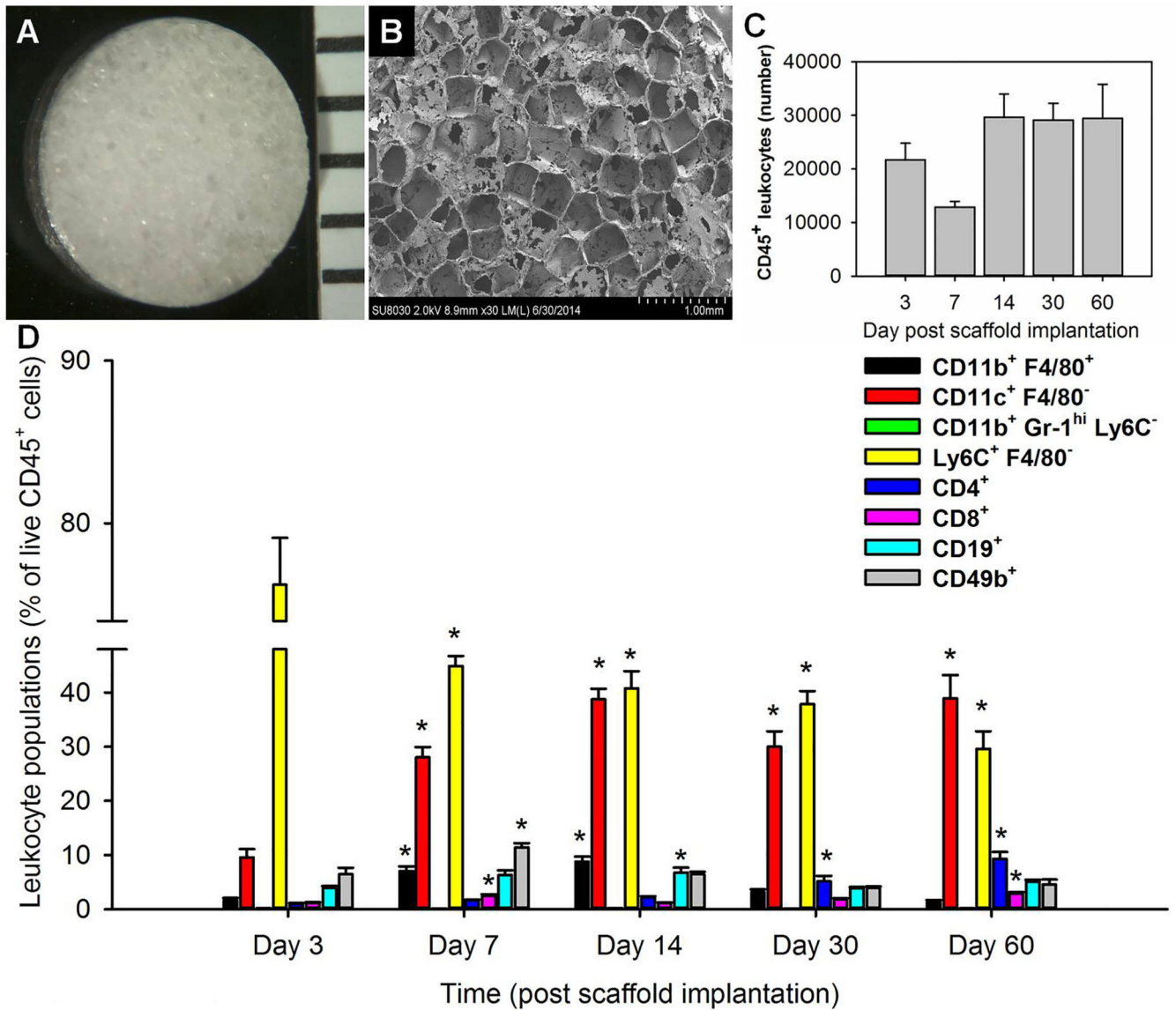


Figure 1. Physical characteristics and dynamic immune cell response following implantation of microporous PCL scaffolds into the dorsal subcutaneous space of a BALB/c mouse. Photomicrograph (A) and scanning electron micrograph (B) of a microporous PCL scaffold. SEM image shows the interconnected porous structure. (C) CD45⁺ leukocyte numbers and (D) Dynamics of CD11b⁺F4/80⁺, CD11c⁺F4/80⁻, CD11b⁺Gr-1^{hi}Ly6C⁻, Ly6C⁺F4/80⁻, CD4⁺, CD8⁺, CD19⁺, and CD49b⁺ immune cell populations expressed as a percentage of live CD45⁺ leukocytes at day 3, 7, 14, 30, and 60 post PCL scaffold implantation (N = 6 for each time point examined, *p < 0.05 compared to day 3 as determined by the Tukey-HSD test post ANOVA). Error bars denote s.e.m.

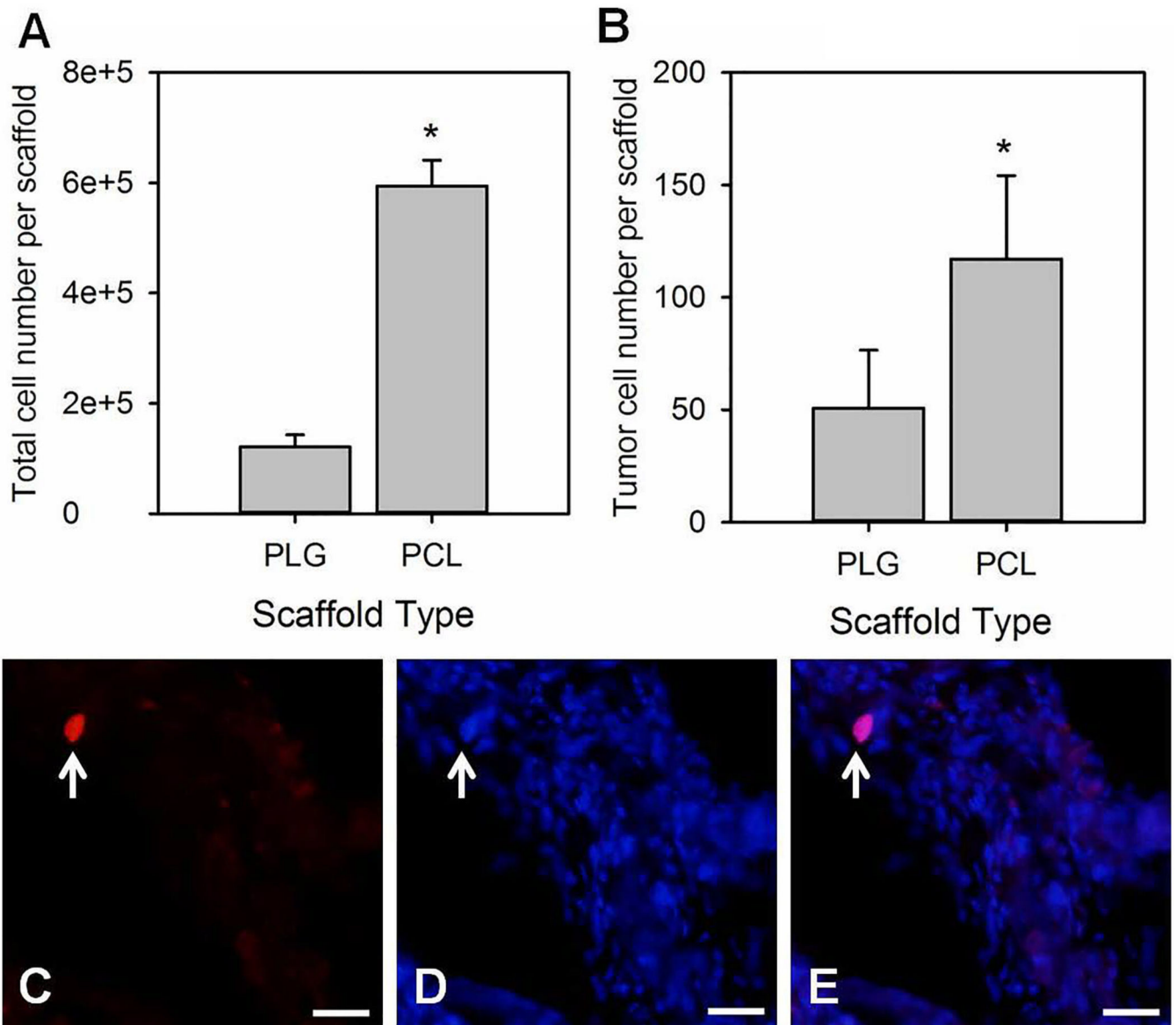


Figure 2. Micro-porous scaffolds implanted for 30 days prior to tumor inoculation recruit metastatic cells. Number of (A) total cells and (B) tumor cells (tdTomato+ cells) isolated from micro-porous PLG and PCL scaffolds at day 15 post tumor inoculation analyzed via flow cytometry (N = 10, *p < 0.01 as determined by t-test for analysis of total cell numbers and Wilcoxon rank-sum test for tumor cell numbers). Fluorescence image of a PCL scaffold section shows the presence of a tumor cell (indicated by white arrow) as identified using tdTomato (C) and DAPI (D) fluorescence and their co-localization (E). Scale bar indicates 20 μ m. Error bars denote s.e.m.

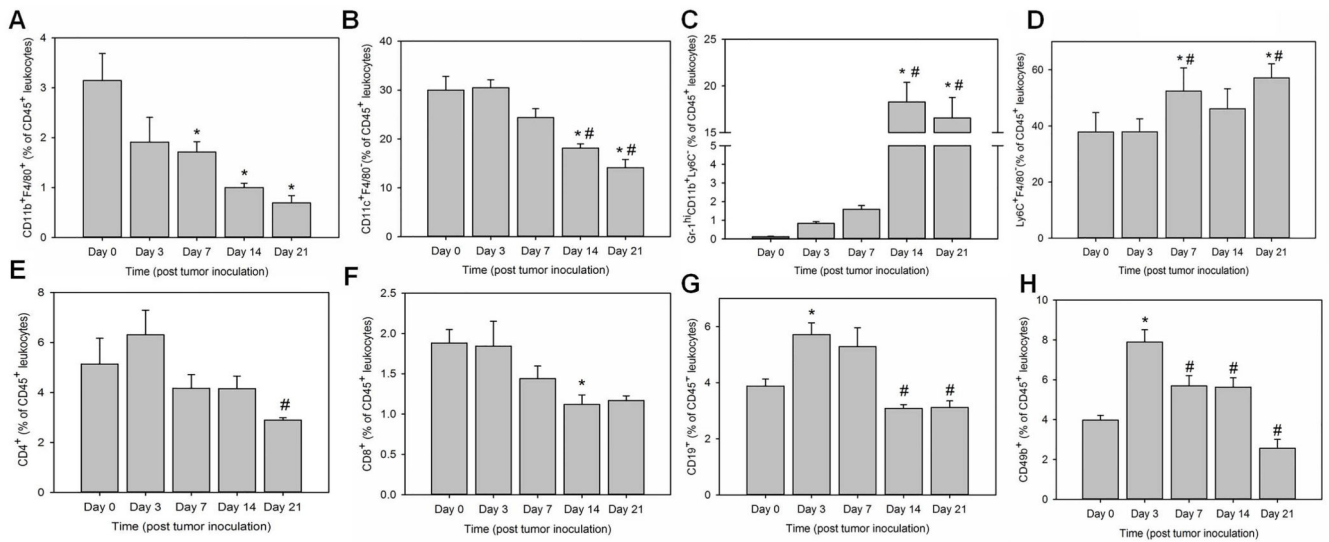


Figure 3.

Tumor progression influences dynamics of leukocyte populations at the PCL scaffold. Percentage of (A) CD11b⁺F4/80⁺ (B) CD11c⁺F4/80⁻ (C) Gr-1^{hi}CD11b⁺Ly6C⁻ (D) Ly6C⁺F4/80⁻ innate immune cell populations and percentage of (E) CD4⁺ (F) CD8⁺ (G) CD19⁺ and (H) CD49b⁺ adaptive immune cell populations in the total population of live CD45⁺ leukocytes at day 0, 3, 7, 14, and 21 post tumor inoculation (N = 8 for each time point examined, *p < 0.05 compared to day 0 and #p < 0.05 compared to day 3 as determined by Tukey-HSD test post ANOVA). Error bars denote s.e.m.

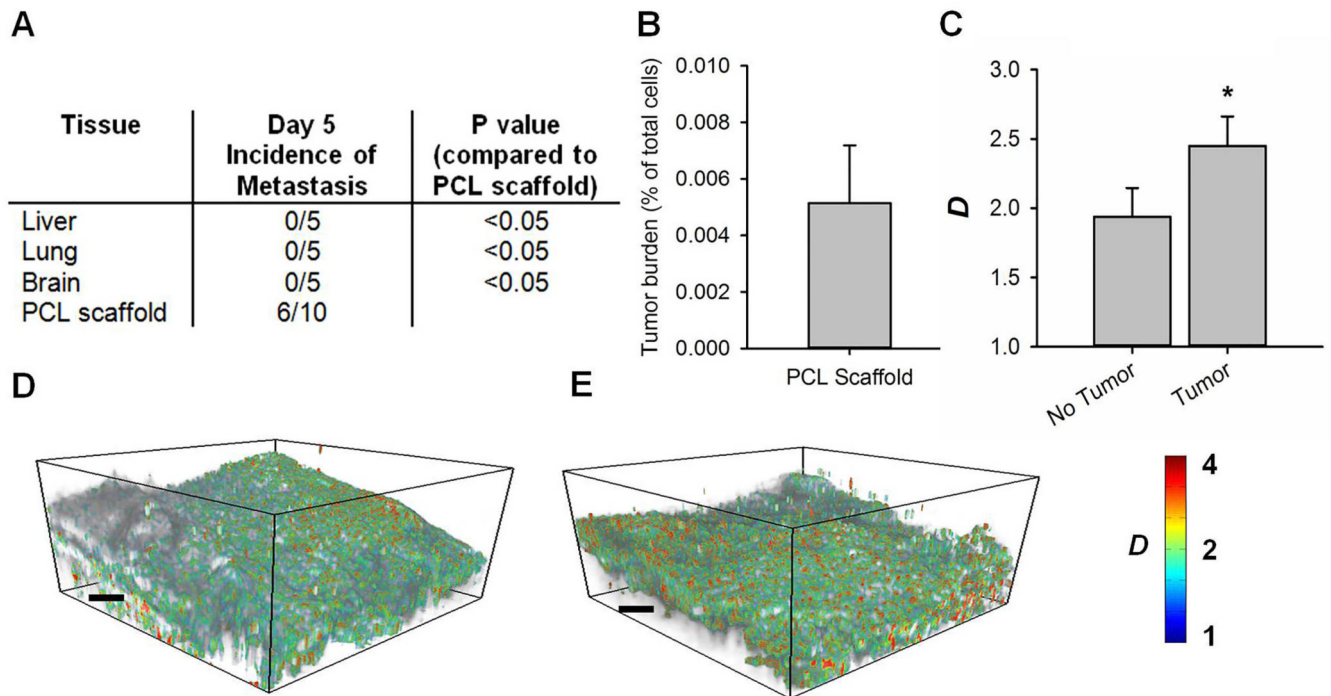


Figure 4. Micro-porous PCL scaffolds enable early detection of metastatic cells in a chronic model of scaffold implantation. (A) Number of mice with detectable tumor cells analyzed by flow cytometry in the lung, liver, and brain in a group of 5 mice at day 5 post tumor inoculation (N = 5 for lung, brain, and liver; N = 10 for PCL scaffolds, *p < 0.05 as determined using the Fisher's exact test). (B) Percentage of tdTomato+ tumor cells isolated from the PCL scaffold at day 5 post tumor inoculation analyzed via flow cytometry. (C) Average *D* value for PCL scaffolds isolated from tumor free and tumor bearing mice. Scaffolds from tumor bearing mice were isolated at day 5 post tumor inoculation. (N = 14 scaffolds for tumor free and N = 16 scaffolds for tumor bearing mice, *p < 0.05 as determined using the Wilcoxon rank-sum test). Representative three dimensional maps of *D* generated via ISOCT analysis of PCL scaffolds in tumor free (D) and tumor bearing mice (E). Scale bars indicate 200 μ m. Error bars denote s.e.m.

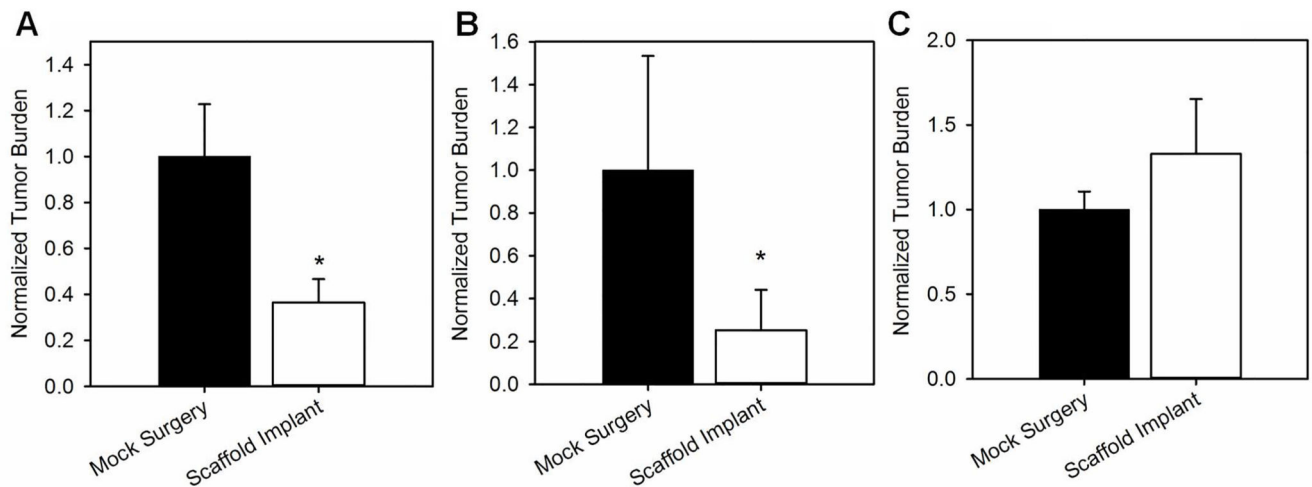


Figure 5.

Recruitment of 4T1 tumor cells to the PCL scaffold site reduces tumor burden in metastatic sites such as the liver and brain in a chronic model of scaffold implantation in BALB/c mice. Normalized average tumor burden in the (A) liver, (B) brain, and the (C) lung for the scaffold and mock surgery groups. The average burden in the mock group was set to 1 (N 6 for each group, * $p < 0.05$ compared to mock surgery as determined by the Wilcoxon rank-sum test). Tumor burden in the lung was identical in both groups. Error bars denote s.e.m.

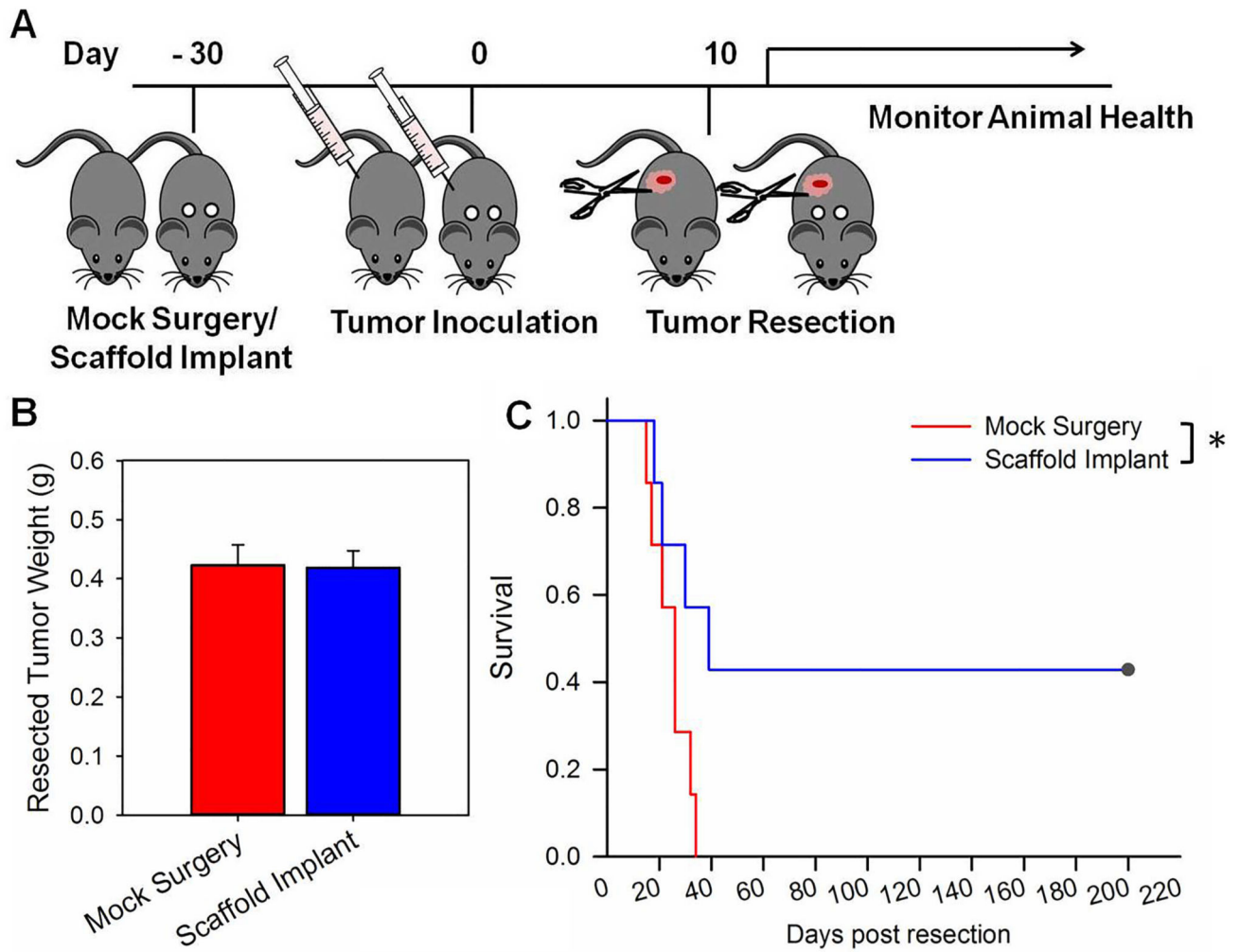


Figure 6. Micro-porous PCL scaffolds improve survival in a post-surgical model of breast cancer metastasis. (A) Schematic of experimental design to examine the influence of scaffold implant on survival (B) Average resected tumor weights for mock and scaffold group were identical, $p = 0.93$, t-test (C) Kaplan-Meier survival curve for mice undergoing mock surgery versus mice receiving a scaffold implant ($N = 7$ for each group, $*p < 0.05$ as determined using the Log rank test). Error bars denote s.e.m.

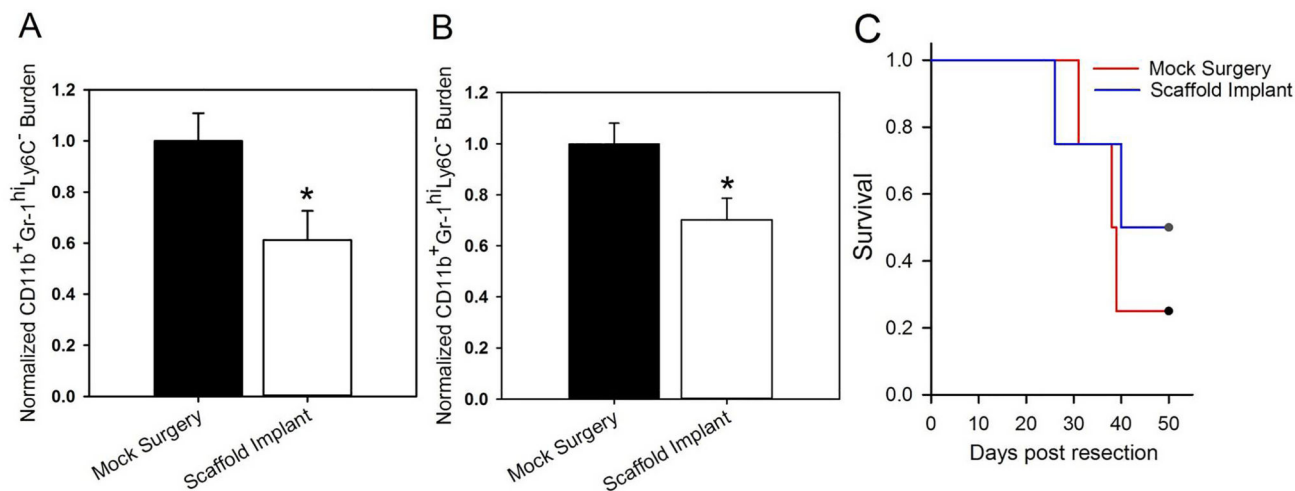


Figure 7.

Micro-porous PCL scaffolds reduce burden of CD11b⁺Gr-1^{hi}Ly6C⁻ cells in the (A) primary tumor and the (B) spleen in BALB/c mice. The percentage of CD11b⁺Gr-1^{hi}Ly6C⁻ cells in the CD45⁺ leukocyte population was examined at day 10 post tumor inoculation via flow cytometry and is reported as normalized burden. (N = 7 for mock surgery; N = 8 for scaffold implant, *p < 0.05 as determined using t-test). (C) Anti-Gr-1 depletion of CD11b⁺Gr-1^{hi}Ly6C⁻ cells enhances survival in both mock and scaffold groups and diminishes the survival advantage observed for PCL implanted mice (N = 4 per group). Error bars denote s.e.m.