CIRCULATING TUMOR CELLS AS DIAGNOSTIC TOOLS AND THERAPEUTIC TARGETS

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CIRCULATING TUMOR CELLS AS DIAGNOSTIC TOOLS AND THERAPEUTIC TARGETS

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Circulating tumor cells have been a major area of focus for cancer metastasis research for the last few decades, and while the majority of progress has mainly centered on the isolation and enumeration of CTC for prognostic purposes, researchers are beginning to explore additional uses for CTC in the clinical setting. While it is well known that CTC counts correlate with prognosis, CTC counts are also being explored as a marker for response to therapy. CTC enumerated before and after surgical procedures are shedding light on the advantages of minimally invasive techniques.

CTC characterization has also increased in the past decade. When CTC were first reliably isolated, the majority of work focused solely on EpCAM-expressing cells found within the blood of patients with late stage cancer. Today, EpCAM-negative CTC and CTC expressing mesenchymal markers are under great scrutiny for a potentially increased metastatic potential. Moreover, CTC that differ in protein and gene expression to the primary tumor have been found elucidating prospective issues with drug targets. CTC have been isolated and identified in patients with local tumors or even pre-cancerous lesions.

Here, we present a diagnostic method that uses CTC to prescreen for drug susceptibility. This method is then used to show primary CTC sensitivity to a novel TRAIL-based immunotherapeutic targeted towards CTC. Peripheral blood samples are shown to contain circulating stromal cells believed to migrate alongside CTC and

likely alter their survival under shear conditions as well as their drug susceptibility. We culminate these studies by conducting the first efficacy study of EST liposomes in an immunocompetent breast cancer tumor resection murine model.

BIOGRAPHICAL SKETCH

Jocelyn Marshall was born in Kent, OH on October 4, 1988. She attended Ohio University where she earned a Bachelor's of Science in Chemical Engineering. During her tenure at Ohio University, she worked under Monica Burdick investigating the involvement of glycosphingolipids (GSL) on head and neck squamous cell carcinoma (HNSCC) adhesion to inflamed endothelium. She continued her studies at Ohio University earning a Master's of Science in Biomedical Engineering where she showed that CD44 proteins on the surface of HNSCC cells sterically interfered with GSL-mediated adhesion. At Cornell University, under the tutelage of Michael King, Jocelyn characterized primary circulating tumor cells (CTC) from cancer patient blood, finding evidence of TRAIL sensitivity of CTC and presence of cancer-associated fibroblasts within the blood. Jocelyn received the prestigious National Science Foundation Graduate Research Fellowship and was named Outstanding PhD Teaching Assistant in Biomedical Engineering for the 2015-2016 academic year. After leaving Cornell, Jocelyn plans to continue her career as a medical science liaison on the East Coast.

To my family for their constant love and support without whom none of this would have ever been possible

We are Marshall

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Capture and Targeting of Circulating Tumor Cells

Portions of this chapter were adapted from:

Marshall JR, King MR. Selectin-Mediated Targeting of Circulating Tumor Cells for Isolation and Treatment. In: Circulating Tumor Cells [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc; 2016 [cited 2016 Oct 20]. p. 267–86.

1.1 Introduction

Circulating tumor cells (CTCs) have provided researchers with a wealth of information about metastatic cancer. However, the exact mechanisms of metastasis through the bloodstream are not yet definitively known. Mounting evidence indicates that CTCs utilize the same pathway through which immune cells are recruited to sites of inflammation. This pathway involves a variety of adhesion molecules, including those in the selectin family. In this chapter, we discuss how these adhesion molecules, namely E-selectin, can be used to recapitulate the early steps of CTC adhesion *in vitro* to achieve capture and characterization of CTCs from patient blood samples. We also discuss how this interaction may be exploited to target and kill CTCs *in vivo*.

1.1.1 Selectin Adhesion

Cell-cell adhesion is necessary for the maintenance of tissue homeostasis as well as communication between cells. Because of their importance, cell adhesion molecules have been the focus of extensive research for decades. In the late 1980s, an important group of adhesion proteins, called selectins, were discovered on different cells in the vasculature. L-selectin was found on leukocyte membranes; P-selectin was found in the granules of platelets, though it was also discovered to be expressed by the vascular endothelium; E-selectin was shown to be expressed by inflamed endothelium. All three of these proteins facilitate immune cell interactions, including leukocyte recruitment to areas of inflammation [1–4]. The selectins were first discovered independently using antibodies that inhibited different adhesion interactions, but it was quickly discovered that the proteins were related and belonged in the same protein

family. Not only are the selectins found within a 300 base pair section of chromosome 1 [5], but also they have very similar structures. Each selectin protein contains a lectin domain, an EGF (epidermal growth factor) repeat domain, a series of complement regulatory-like regions, and transmembrane and cytoplasmic domains. Where the proteins differ is in the number of complement regulatory-like regions; L-selectin having 2, P-selectin 9, while E-selectin has 6 [6].

Once the sequence and structure of the selectin molecules were discovered, focus transitioned to the function of the molecules. One of the main functions of these proteins is to mediate leukocyte recruitment to areas of inflammation, an important process for both the innate and adaptive immune systems [7–9]. It is known that this pathway has a number of discrete steps: tethering and rolling, activation, firm adhesion, and extravasation (Figure 1.1) [10]. The selectin family is important in mediating this process, in particular the tethering and rolling steps, while integrins have been shown to mediate the fast-to-slow rolling transition as well as firm adhesion [8,9,11]. Soon after their discovery, the selectins were shown to bind to sialylfucosylated ligands, sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a). While these are the minimum binding structures for the selectins, these carbohydrate groups are usually found on a carrier backbone of either a glycoprotein or glycolipid [12,13]. P-selectin glycoprotein ligand-1 (PSGL-1) is one of the most extensive selectin ligands studied, and it is one of the few ligands that has been shown to bind to all three selectins [14].

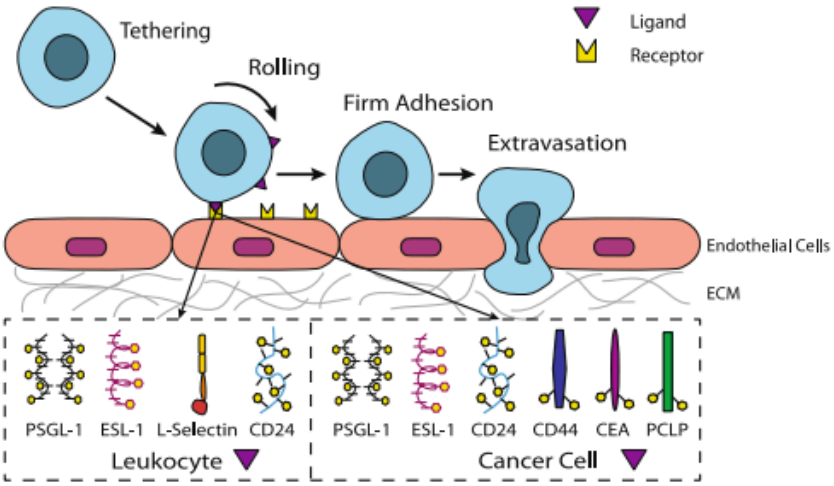

Figure 1.1. Leukocyte Adhesion Cascade, from [10]. Figure reprinted with permission.

The selectins, namely E-selectin, are also important at mediating the hematogenous metastasis of many types of cancer. It is believed that cancer cells use a similar mechanism to leukocyte adhesion to migrate to distant sites in the body. This phenomenon was first studied in detail for colon cancer cells [15–21] but since then different types of cancer cells have been shown to adhere to E-selectin including, but not limited to prostate [22–24], breast [25–30], lung [31], and pancreatic cancer cells [32–35]. The importance of these adhesive interactions in metastasis was solidified further when it was discovered that the expression sLe^x and sLe^a, two important selectin ligands, correlated with survival rate and metastasis [36–41]. Moreover, reagents blocking certain glycosylation processes have been shown to inhibit metastasis in several *in vivo* studies [42].

Cancer metastasis differs from leukocyte recruitment to areas of inflammation in some key ways. First, E-selectin is the major selectin implicated in cancer metastasis, while leukocyte recruitment uses a combination of interactions involving all three selectins. Colon, gastric, and lung cancer cell lines have been shown to bind to E-selectin, but not to P-selectin [17,43]. However, P- and L-selectin have been shown to influence cancer metastasis *in vivo*, as P-/L-deficiency in mice inhibits metastasis [11,13], though it may be that these selectins influence the adhesion of platelets and immune cells that enable cancer adhesion to endothelium rather than adhering to the cancer cells directly [11,13]. Secondly, sLe^a is found on a number of different cancer types in abundance, whereas leukocytes express low to no sLe^a. Takada, et al. showed that some cancer cell lines rely solely on sLe^a for adhesion to human umbilical vein endothelial cells (HUVECs), while others rely heavily on sLe^x [44]. Also, in a colon cancer xenograft study, sLe^a-expressing cells were shown to generate larger tumors when compared to their non-expressing counterparts [41]. The implication is that while cancer cells and leukocytes share a number of selectin ligands, such as PSGL-1, some cancers express novel ligands, which could serve as a target for anti-cancer therapies. One example is hemapoetic cell E-selectin/L-selectin ligand (HCELL), a CD44 glycoform, which on cancer cells is expressed on O-glycans, rather than N-glycans as on hemapoietic progenitor cells, thus demonstrating that HCELL is expressed as the variant form of CD44 in cancer [45].

Cancer-related inflammation has been the subject of much research, especially relating to metastasis. E-selectin is generally only expressed on cytokine-stimulated endothelial cells. Some cancers have been shown to secrete inflammatory cytokines

such as Il-1 β . The Hakomori lab showed that while not all cancer cells have the ability to secrete these cytokines, the interaction of cancer cells with platelets could induce this secretory ability, thereby promoting inflammation [46]. Moreover, tumors are infiltrated by a variety of immune cells, which may contribute to cancer-related inflammation [47]. It should also be noted that E-selectin is constitutively expressed on the skin and bone marrow endothelium [48].

1.1.2 Circulating Tumor Cells

CTCs are epithelial cells that have dissociated from the primary tumor and entered the blood stream. CTCs were first discovered in 1869 in the blood of cancer patient during an autopsy [49]. However, CTCs did not become a major subject of cancer research until two decades ago. Most work has centered around the detection and isolation of CTCs from blood samples of cancer patients, though in recent years research has expanded to the identification of important mutations present in CTCs, EMT (epithelial-mesenchymal transition) status of CTCs, and the correlation of CTC count with drug efficacy [50–59]. CTCs have been identified in the blood of patients with breast [50,53,60–65], lung [66], pancreatic [67], prostate [52,68,69], head and neck [51], colorectal [70–72], renal [73,74], gastric [75,76], and bladder cancers [77,78], among others [79–82]. Many studies have shown in both animal models and human studies that CTC count correlates with prognosis, suggesting that a higher CTC count is generally indicative of a worse survival rate and greater disease progression [51,61,63,83–88].

There is no single definitive approach for isolating or identifying CTCs from patient blood. The problem with isolating CTCs is their rareness in the blood, with CTC counts on the order of 1 per billion blood cells [89]. CTCs can be enriched by density centrifugation, which removes the majority of red blood cells, neutrophils, platelets, and plasma. However, the remaining leukocytes still greatly outnumber the CTCs; therefore, density centrifugation is usually used in conjunction with another isolation technique. Once CTCs are isolated, their identity must be confirmed. This is usually done by PCR or fluorescence microscopy [90]. A cell is considered a CTC if it meets the following criteria: a misshapen nucleus, positive for epithelial cell adhesion molecule (EpCAM) or cytokeratin (CK) expression, and negative for CD45, a leukocyte marker [91]. However, recent work has shown that CTCs are heterogeneous and one identification method may not work to identify all CTC subtypes [71,82]. Only one isolation technique, CellSearch, has been FDA-approved for the enumeration of patient CTC counts in prostate and breast cancer. CellSearch uses anti-EpCAM antibody-coated magnetic beads to capture CTCs from blood samples and then a strong magnet is used to precipitate the aggregates out of solution. The cells attached to the beads are then stained for CK, CD45, and DAPI, a nuclear stain. Samples are processed through a specialized detector that detects and enumerates positively-identified, intact CTCs [90]. Other isolation techniques have been developed using similar approaches. For example, the Chalmers lab, among others, uses magnetic bead technology to isolate CTCs via negative-selection. In their process, magnetic beads are coated with anti-CD45 antibodies that capture contaminating leukocytes. This process allows for the identification of both EpCAM-

positive and EpCAM-negative CTCs [51]. Nagrath et al. created a microfluidic "CTC-chip" that applies anti-EpCAM antibodies to microfabricated posts. A blood sample is applied to the chip, where fluid flow produces collisions between the CTCs and the posts [92]. The same group extended this idea by designing a chip without posts but with a herringbone pattern etched onto a glass substrate. Anti-EpCAM antibody was then applied to the whole chip. This allows for a greater capture area, and the herringbone etching increases the turbidity of the flow, thereby increasing CTCs collisions with the chip surface [93].

It is believed that CTCs facilitate metastasis by hijacking the process that leukocytes use when responding to inflammation signals. This involves the adhesion of cells to the vascular endothelium mediated by adhesion molecules such as E-selectin. Our group has employed this concept to develop a physiologic mimic microtube device that uses E-selectin adhesion to recruit CTCs to the surface and facilitate firm adhesion via antibody binding [81]. In the next section, the development and use of this technology is further detailed.

1.2 CTC Capture by E-selectin

E-selectin's use as a capture agent started about a decade ago when the Liepmann lab developed a cell separation device that uses E-selectin to concentrate cell solutions. In this microfluidic device, microfabricated posts are coated in recombinant human E-selectin (rhES) and cell solution is pumped through, allowing cells to be captured on the surface of each post. Two post geometries were tested: square pillars and slender, offset pillars. The offset pillars were shown to capture more cells initially, but the

square pillars enriched the cell solution better due to slower rolling speeds and less cell detachment. This device was not tested for CTC capture specifically, but in theory could be applied for that purpose [94]. In addition, Kim et al. developed a biomimetic chip to capture blood cells [95]. Rather than target one selectin interaction, they coated their chip with E-selectin, ICAM-1, and VCAM for the purpose of targeting all three selectins. However, they found that E-selectin was necessary for capture [95].

CTCs from patients with prostate cancer were only recently shown to adhere to microtubes functionalized with rhES [96], but work to create a capture device using immobilized selectin started years earlier. Much of the work, in fact, was pioneered using a haematopoietic stem cell (HSPC) model. In this device, microrenathane tubes coated in P-selectin enriched HSPC samples seven-fold with an average purity of about 30% [97]. While this process represented a major advance, further work showed that by roughening the surface area of the tube with nanomaterials, one could greatly increase the efficiency of the device. For example, coating the tube with colloidal silica prior to protein absorption increased cell capture [98]. Capture was further increased with halloysite coatings; halloysite is naturally occurring aluminosilicate nanoparticles that range from 500-1200 nm in length and 40-200 nm in diameter. Combined hallyosite and P-selectin surfaces captured both KG1a and COLO205 cells at increased rates compared to tubes with P-selectin alone [99]. This technology was subsequently adapted for CTC isolation.

To capture CTCs, a length of microrenathane (MRE) tubing is first coated with poly-L-lysine to endow the tube with a positively charged surface that will facilitate the adsorption of the halloysite nanotubes. The halloysite coating is allowed to cure

overnight. Protein G is then added to the tube followed by a mixture of rhES and anti-EpCAM antibody. Protein G binds to the F_c region of both the rhES and anti-EpCAM antibody allowing for consistent molecular orientation within the tube interior [100]. The technology was optimized and characterized using a leukemia cell line prior to testing with patient samples. This approach typically yields a capture efficiency of 50% and CTC purities of >50% [81].

Primary buffy coat samples were processed through tubes without halloysite ("smooth tubes"), halloysite-coated tubes, and using CellSearch. For each method, half a tube of blood (3.75 mL) was processed and the resulting normalized cell counts were calculated and compared (Figure 1.2). For nearly all cancer blood samples, both the smooth tubes and halloysite-coated tubes captured more cells than Cell Search. Halloysite-coated tubes show generally higher CTC purities than the smooth tubes, due to the inability of leukocytes to spread and firmly attach on halloysite [81].

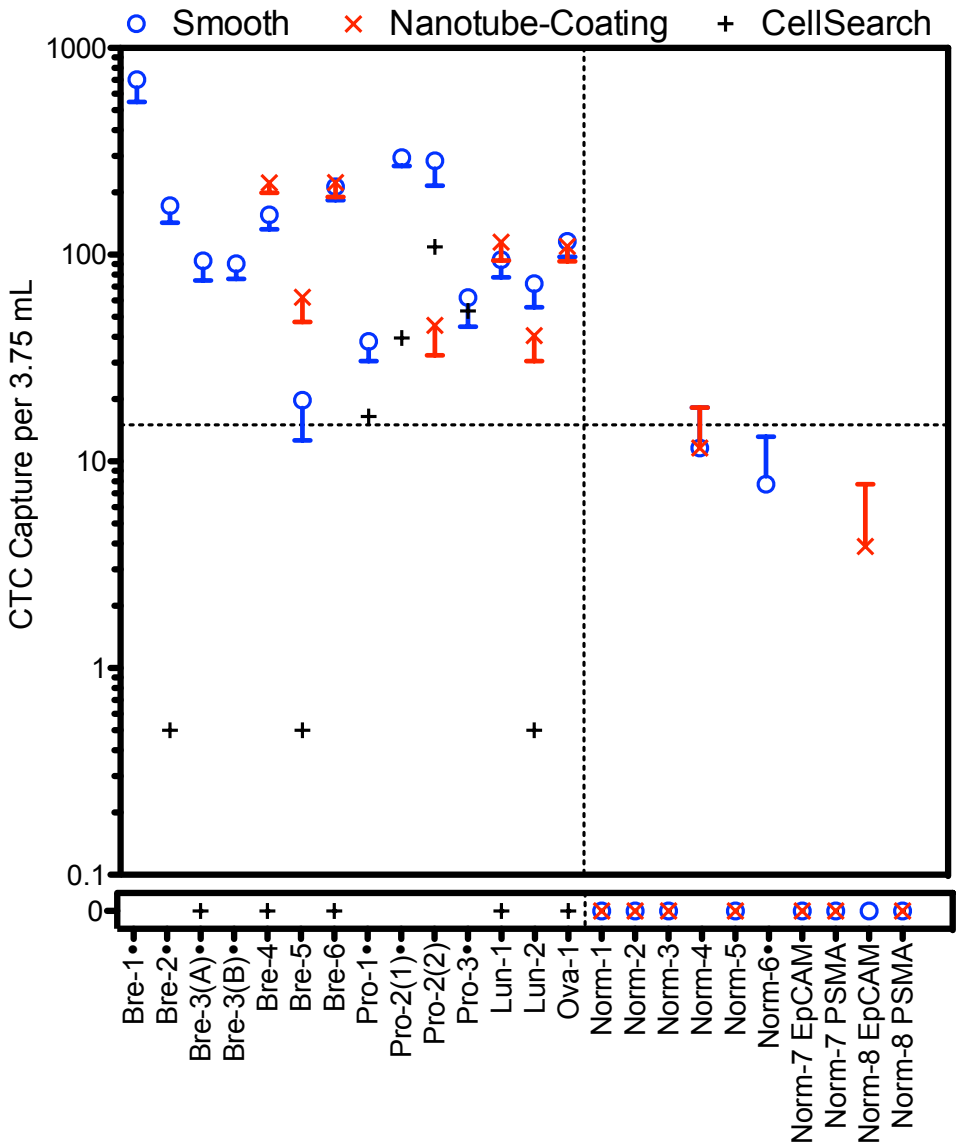

Figure 1.2. Number of CTCs captured from the blood of cancer patients, along with the results of samples collected from healthy patients. Smooth tube device, halloysite-coated device, and CellSearch methods were compared in parallel experiments, data from [81].

1.3 Applications for E-selectin in Cancer Diagnosis and Treatment

While CTC counts have primarily been used as a marker of disease progression, CTCs hold much potential for targeted, personalized therapy development. In fact, in the study that led to FDA-approval of CellSearch, CTC counts were correlated not just with disease progression, but also with response to treatment. Patients with >4 CTCs per sample were noted to have responded poorly to treatment [101]. Two other studies in prostate cancer also used CTCs count to track drug efficacy[102,103]. In the study by Reid et al., 63% of patients showed a CTC count decrease by half after treatment [102].

1.3.1 E-selectin capture for drug efficacy testing

Our lab has exploited knowledge of E-selectin adhesion to cancer cells to explore the potential for a broadly applicable diagnostic test for the development of personalized treatment regimes. We hypothesized that by employing the same device as described in the previous section, one could use CTC samples to test for drug sensitivity prior to administration. CTCs represent an advantageous medium for drug testing because one CTC sample can contain multiple different subsets of cancer cells believed to reflect the heterogeneous nature of the primary tumor [104] and because of their demonstrated correlation with metastasis, which accounts for 90% of all cancer-related deaths [105].

In previous work, a simple procedure to test for drug efficacy using CTC count was created. Each blood sample was split into multiple aliquots, leaving one aliquot as an untreated reference control. Blood samples were treated with chemotherapeutic drugs

and incubated overnight. The drug concentrations used in the study were chosen based on the peak plasma concentration determined by pharmacokinetic studies. After incubation, CTCs were isolated and enumerated. A reduction in CTC count, when compared to the reference control, was concluded to indicate drug sensitivity [106]. The method was first characterized using a cell line model that consisted of spiking a known number of prostate or breast cancer cells into the blood of a healthy donor. All of the cell lines tested were found to be sensitive to each drug in media and in blood, and the trend in cell count reduction followed the same pattern for both conditions. Following characterization, this method was tested on the blood from 7 cancer patients (3 breast, 2 prostate, 1 renal, 1 colon). Each patient's response to the drugs varied, with 3 patients responding to both drugs, 3 were sensitive to one drug, and 1 showed no sensitivity. More detailed results of the primary sample experiments can be seen in Figure 1.3 [106].

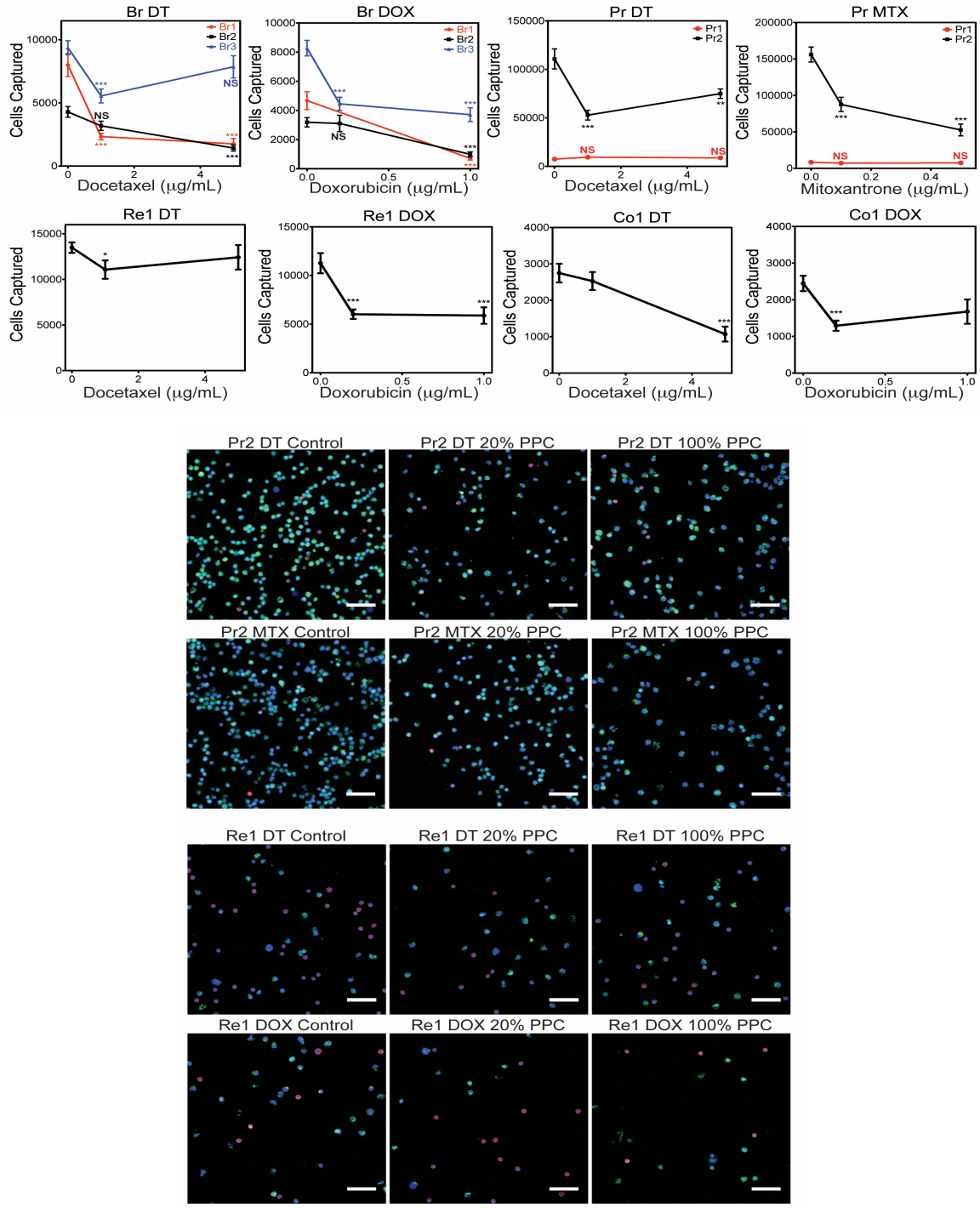

Figure 1.3. Patient samples were collected from three breast cancer patients (Br1, Br2, and Br3), two prostate cancer patients (Pr1 and Pr2), one renal and one colon cancer patient (Re1 and Co1, respectively). Each tube of whole blood was split into three aliquots and treated with vehicle control, 20% peak plasma concentration (PPC), or 100% PPC of the appropriate drug. (Top) CTC counts of patient samples treated with chemotherapeutic drugs. (Bottom) Example micrographs of 2 patient samples. Blue- DAPI, Green- EpCAM, Red- CD45. Patients showed varied responses to the drugs as shown in the line graphs. Pr2 shows significant response to both drugs, which can be seen in the top set of fluorescent images as a reduction in the number of green cells in the treatment images compared the control. Re1 shows no sensitivity to docetaxel, but is sensitive doxorubicin, which is observed in the lower set of fluorescent images as no reduction in green cells and a reduction in the number of green cells, respectively. DT = docetaxel, DOX = doxorubicin, MTX = mitoxantrone. Error bars represent standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; scale bar=50 um, from A.D. Hughes, J.R. Marshall, E. Keller, J.D. Powderly, B.T. Greene, M.R. King, Differential drug responses of circulating tumor cells within patient blood, Cancer Letters. (2013), [106]. Figure reprinted with permission.

There are several advantages to this system. First, it is a straightforward procedure that can be performed on standard laboratory equipment. Secondl, the method can be used with any CTC isolation device, for instance, CellSearch or CTC-Chip. Moreover, the technique supports the testing of intravenous drugs or combinations of therapeutics [106].

1.3.2 E-selectin for Use in Targeted Cancer Therapy

The fact that E-selectin can be used to recruit CTCs to surfaces suggests that it may also be useful in targeted therapeutics. Previous attempts have been made to create cancer drugs that exploit E-selectin adhesion including drugs targeting glycosylation, selectin ligands, but none of these formulations have reached the clinic [13,42]. Unlike previous technologies, we have explored the use of E-selectin as a targeting agent and employed a secondary protein, TNF-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in CTCs [107]. TRAIL is a protein expressed by cells of the immune system, and the receptors for TRAIL, termed death receptors, have been shown to be upregulated on the surface of many types of cancer cells [108]. By employing the combination of TRAIL and E-selectin, this therapeutic increases its targeting of cancer cells and decreases its cytotoxicity toward healthy cells.

Huang and King showed that selectin molecules have the ability to target cells by using P-selectin-coated liposomes to deliver siRNA to HL60 cells. Liposomes were absorbed onto the surface of MRE tubes and HL60 cells rolled across the surface, and the encapsulated siRNA was successfully delivered to the cells to induce a knockdown

of the elastase gene (ELA2) to less than half of the original expression level [109]. E-selectin has also served as a successful targeting protein for liposomes. Rh-ES was conjugated to the surface of liposomes encapsulating doxorubicin and used to treat cancer cells *in vitro.* E-selectin was successful in targeting cancer cells as shown by liposomes adhering to nearly 100% of cells. Under static conditions, the targeted liposomes experienced a similar kill rate to normal liposomal doxorubicin, but killed fewer cells than soluble doxorubicin. To study the effect of shear on the targeting and killing of cancer cells by E-selectin functionalized liposomes, they were adsorbed onto the surface of MRE tubes and cells were perfused through the tubes. Under dynamic conditions, E-selectin functionalized liposomes killed significantly more cells than identical liposomes containing no doxorubicin, although the kill rate was less than that of soluble doxorubicin. When a halloysite coating was added to the MRE tubing before liposome adsorption, however, the number of apoptotic cells increased greatly and the observed difference in the kill rates of the functionalized liposomes and soluble doxorubicin was lower [110].

In a study by Rana et al. the dual power of TRAIL and E-selectin was observed. MRE tubing was coated with a combination of rhES and TRAIL and COLO205 cells were flowed through the device for 1-2 hours. While 1 hour of flow did not produce significant apoptosis, after 2 hours less than 40% of COLO-205 cells remained viable. Pretreatment with 1 mM aspirin significantly increased the effectiveness of the surface, bringing the kill rate of 1-hour of rolling to that of the 2-hour treatment without aspirin[111].

The work culminated in the development of E-selectin/TRAIL-functionalized liposomes that target CTCs within the bloodstream. Dual-functionalized liposomes were shown to kill significantly more cancer cells than naked liposomes, E-selectin only liposomes, or TRAIL only liposomes. The liposomes also bound to human leukocytes *in vitro* without causing apoptosis. The cancer cell killing effect was greatly enhanced when cells were in the whole blood environment, with less than 5% of cells remaining viable. It was shown in this study that the liposomes are capable of not only killing cancer cells directly, but more importantly also attach to leukocytes and remain available to bind to cancer cells and induce death. This dual action enhances the utility of the therapy by increasing the circulation time of the therapy. One limitation of previous TRAIL therapies has been its short circulation time (15-30 minutes) and rapid clearance. Interestingly, E-selectin liposomes without therapeutic were found to cause CTCs to be retained in the circulation *in vivo* rather than adhering to the vascular endothelium by competitive inhibition. This was shown in a mouse model where COLO205 cells were injected via the tail vein, followed by liposome therapy, control liposomes, buffer, or soluble TRAIL. The CTCs were collected via cardiac puncture and enumerated after 2-3 hours in culture. Mice treated with E-selectin/TRAIL liposomes had the fewest remaining CTCs after treatment (Figure 1.4). In addition, the ES/TRAIL liposome treatment decreased the number of cells that lodged in the lungs of the mice. This study shows the importance and potential for E-selectin as a CTC targeting molecule [107].

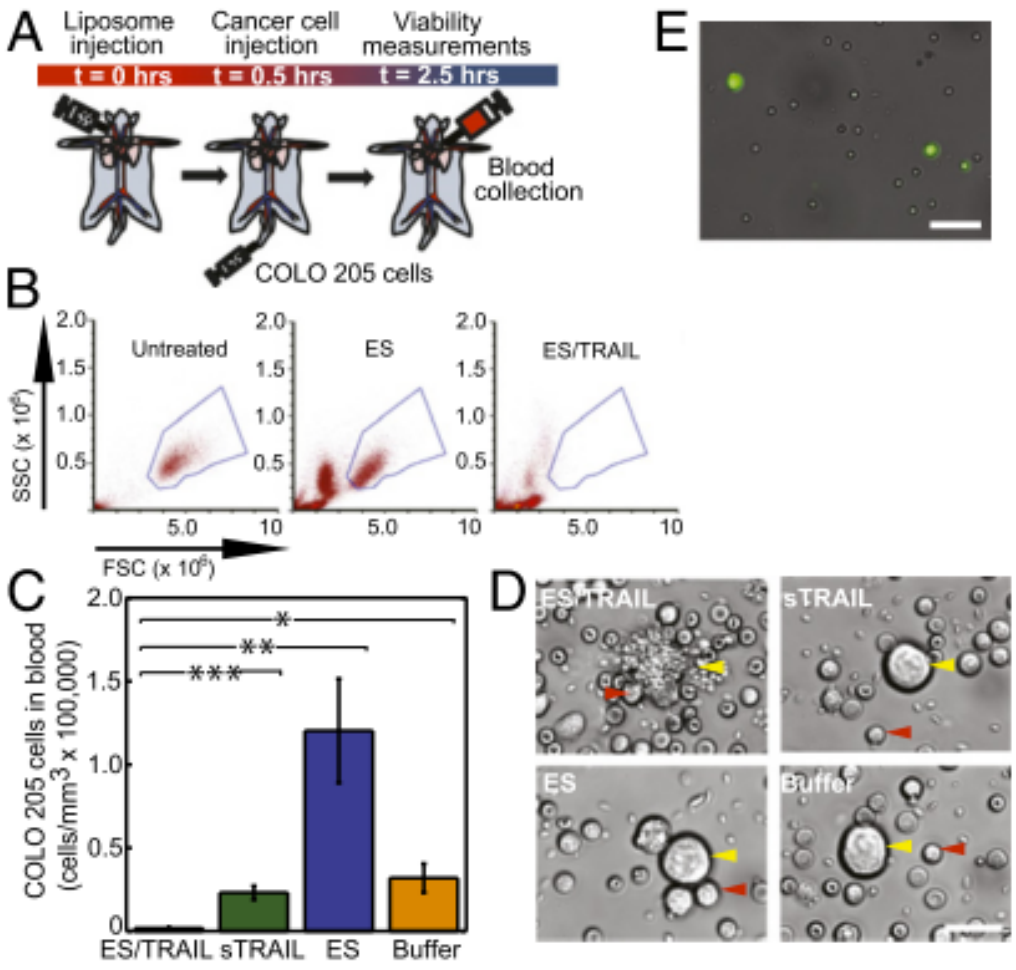

Figure 1.4. E-selectin/TRAIL liposomes eliminate CTC *in* vivo. A) Schematic of procedure for *in vivo* liposome experiments. B) Flow cytometry of COLO205 cells *in vitro* (L), recovered from cardiac puncture of ES-liposome treated mice (C), recovered from ES/TRAIL-liposome treated mice (R). SSL: side-scattered light; FSC: forward-scattered light. C) Number of viable cells recovered from blood of mice compared by treatments. n=3 for all samples. Bars represent the mean ± SD in each treatment group. *P < 0.01, **P < 0.001, ***P < 0.0001 (one-way ANOVA with Tukey posttest). D) Representative micrographs of cells recovered from mouse blood. Scale bar = 20 μm. E) Leukocytes functionalized with fluorescent ES/TRAIL liposomes recovered during cardiac puncture. Scale bar = 50 μm. ES/TRAIL liposomes bound to leukocytes in the circulation of mice and successfully killed COLO205 cells in the circulation of mice, from M.J. Mitchell, E. Wayne, K. Rana, C.B. Schaffer, M.R. King, TRAIL-coated leukocytes that kill cancer cells in the circulation., Proceedings of the National Academy of Sciences of the United States of America. 111 (2014) 930–5, [107]. Figure reprinted with permission.

1.4 Conclusions

Ever since the selectin family of adhesion molecules was discovered in the late 1980s, research has looked at the importance of this molecule in immune and cancer interactions. Recent work has revealed new ways that E-selectin adhesion can be used to detect and kill CTCs (Figure 1.5). It has been demonstrated that E-selectin, in combination with other specific cancer antibodies, can be used to capture CTCs from the blood of patients to better characterize a patient's prognosis and also to potentially test living tumor cells for the most effective treatment option. Going further, E-selectin has been used as a targeting molecule for cancer treatment. By combining the targeting of E-selectin with the killing and targeting of TRAIL, CTCs in mice were killed in the bloodstream and the number of embedded cells in the lungs was decreased significantly. E-selectin has great potential in the realm of cancer treatment and its full utility is just now being fully appreciated.

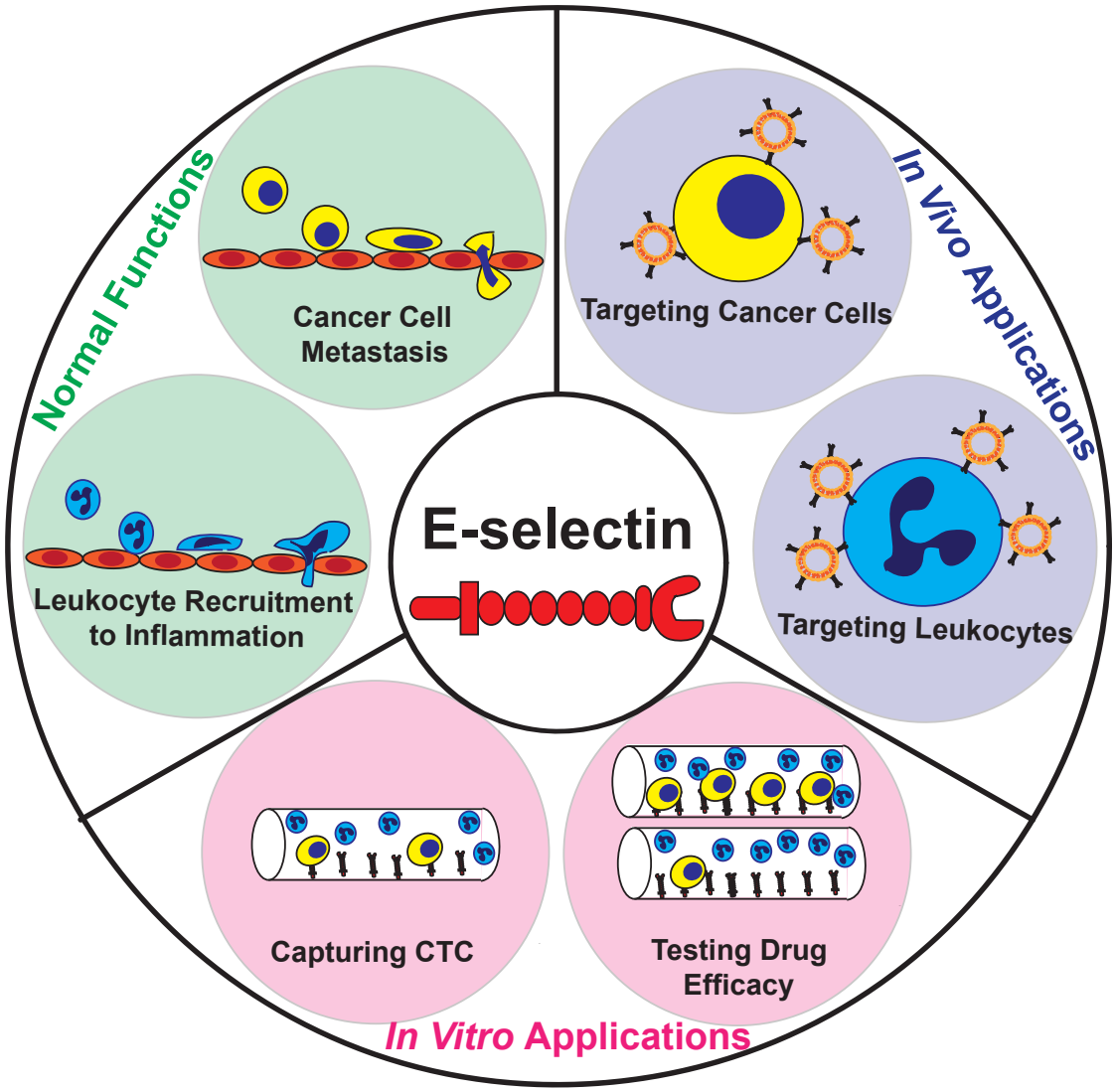

Figure 1.5. Functions and uses for E-selectin in cancer treatment.

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Chapter 2

Patient-Specific Drug Efficacy Analysis on Circulating Tumor Cells Captured from Peripheral Blood

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2.1 Abstract

Personalized medicine holds great promise for cancer treatment, with the potential to address challenges associated with drug resistance and interpatient variability. Circulating tumor cells (CTC) can be useful for screening cancer drugs as they may reflect the severity and heterogeneity of primary tumors. Here we present a platform for rapidly evaluating individualized drug susceptibility. Treatment efficacy is evaluated directly in blood, employing a relevant environment for drug administration, and assessed by comparison of CTC counts in treated and control samples. Multiple drugs at varying concentrations are evaluated simultaneously to predict an appropriate therapy for individual patients.

Keywords: personalized medicine, circulating tumor cell, chemotherapy, drug resistance

2.2. Introduction

It is increasingly apparent that the most effective treatment for a cancer patient is a personalized approach based on predictive criteria for that individual. Traditional practice to achieve this goal has been to identify predictors of sensitivity or resistance in malignant cells. For example, it has been shown that panitumumab can be an effective therapy for colorectal cancer patients, but only in patients without KRAS mutation, which renders the treatment ineffective [1]. Thus, patients are screened for mutated KRAS prior to pantumumab treatment. Patients with non-small-cell lung

cancer are evaluated for specifically mutated EGFR prior to being placed on gefitinib [2]. However, for more general chemotherapeutics, such as taxanes, no single mutation or marker has been identified that will serve as a reliable predictor of patient response. Chemotherapeutic resistance, both intrinsic and acquired, is a significant problem and is believed to result in failure in more than 90% of patients with metastatic disease [3]. In an attempt to determine patient-specific sensitivity to cytotoxic and cytostatic agents, studies have been conducted wherein tumor cells are biopsied and treated *ex vivo*. Unfortunately no significant benefit has been found in these types of assays because sensitivity *ex vivo* does not necessarily translate to a similar response *in vivo* [4]. This is likely due in part to spatial heterogeneity within tumors and the fact that biopsies only sample a small section of a tumor [5; 6], and in part a consequence of the environment in which the cells are treated [7].

In recent years, much interest has been focused on circulating tumor cells (CTC) [8]. Many studies have found that CTC appear early in the disease, and their prevalence in blood correlates with disease severity [9; 10; 11; 12]. Clinicians are beginning to view CTC isolated from blood draws as a 'fluid biopsy,' something of a snapshot of the current state of a dynamic tumor, and CTC are believed to reflect in some way the breadth of tumor heterogeneity [13]. Indeed, the case has been made that CTC are the relevant cancer cell subpopulation to target for therapy based on the fact that 90% of cancer deaths are due to metastasis [14]. In addition, the circulatory system, within which cancer cells are termed CTC, is the primary route of metastasis [15]. As such, CTC are being investigated on a patient-to-patient level for

characterization purposes, such as epithelial-to-mesenchymal (EMT) state [16] and detection of surface markers that correlate with specific drug response [17].

We recently reported a technique for the isolation of CTC from patient blood in a relatively simple device using off-the-shelf components [18; 19]. The device is modeled on an inflamed postcapillary venule and is functionalized with recombinant human E-selectin to rapidly bind flowing cells and anti-EpCAM antibodies to firmly adhere cancer cells. It has been suggested that E-selectin plays a role in metastasis, specifically in the extravasation of metastatic cells [20; 21; 22]. In this paper we present a technique to rapidly screen patient samples for sensitivity to multiple chemotherapeutics in a relevant setting. To accomplish this, blood samples from a patient diagnosed with metastatic cancer are split into multiple aliquots with chemotherapeutics introduced at clinically relevant dosages to treat the CTC *in situ*. Subsequently, the CTC are isolated from the paired aliquots and enumerated. Reductions in CTC count are interpreted as drug sensitivities. This assumption was validated using drug-sensitive cell lines spiked into normal whole blood. It is concluded that one may successfully detect significant CTC count reductions using this approach, providing a platform upon which to make informed therapeutic decisions. This technique has the potential for additional use as a companion tool to detect acquired resistance throughout treatment.

2.3 Materials and Methods

2.3.1 Cell culture

BT20 and PC3 cells were purchased from the American Type Culture Collection (ATCC). BT20 cells were grown in Eagle's Modified Medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin-streptomycin (Lonza, Basel, Switzerland). PC3 cells were grown in RPMI 1640 media (VWR, Randor, PA) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C and 5% CO_2.

2.3.2 Antibodies and reagents

Anti-EpCAM (clone 158210), anti-EpCAM-FITC (clone 158206) antibodies and recombinant human-E-selectin were purchased from R&D Systems (Minneapolis, MN). Anti-CD45-APC (clone HI30) antibody and Annexin V-APC kit were obtained from BD Biosciences (San Jose, CA). Anti-EpCAM-FITC (clone HEA-125) was obtained from Miltenyi Biotec (Auburn, CA). Halloysite nanotubes were a gift from NaturalNano (Rochester, NY). Ficoll-Paque was purchased from GE Healthcare (Waukesha, WI). Erythrocyte lysis buffer was obtained from Qaigen (Germantown, MD). Docetaxel, mitoxantrone, and calcium carbonate were purchased from Sigma Aldrich (St. Louis, MO). Doxorubicin was purchased from Sellek-Pfizer (Houston, TX). ViaCount Viability Kit was purchased from Millipore (Billerica, MA). Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), PBS supplemented with calcium and magnesium, and trypsin were purchased from Life Technologies (Grand Island, NY). Paraformaldehyde was acquired from Electron Micropscopy Sciences (Hatfield, PA). DAPI was obtained from Vector Laboratories (Burlingame, CA). Bovine serum albumin (BSA) was purchased from Sigma Aldrich (St. Louis,

MO). Dimethyl sulfoxide (DMSO) was obtained from Avantor Performance Materials Inc. (Center Valley, PA).

2.3.3 Spiking of cancer cell line cells into blood

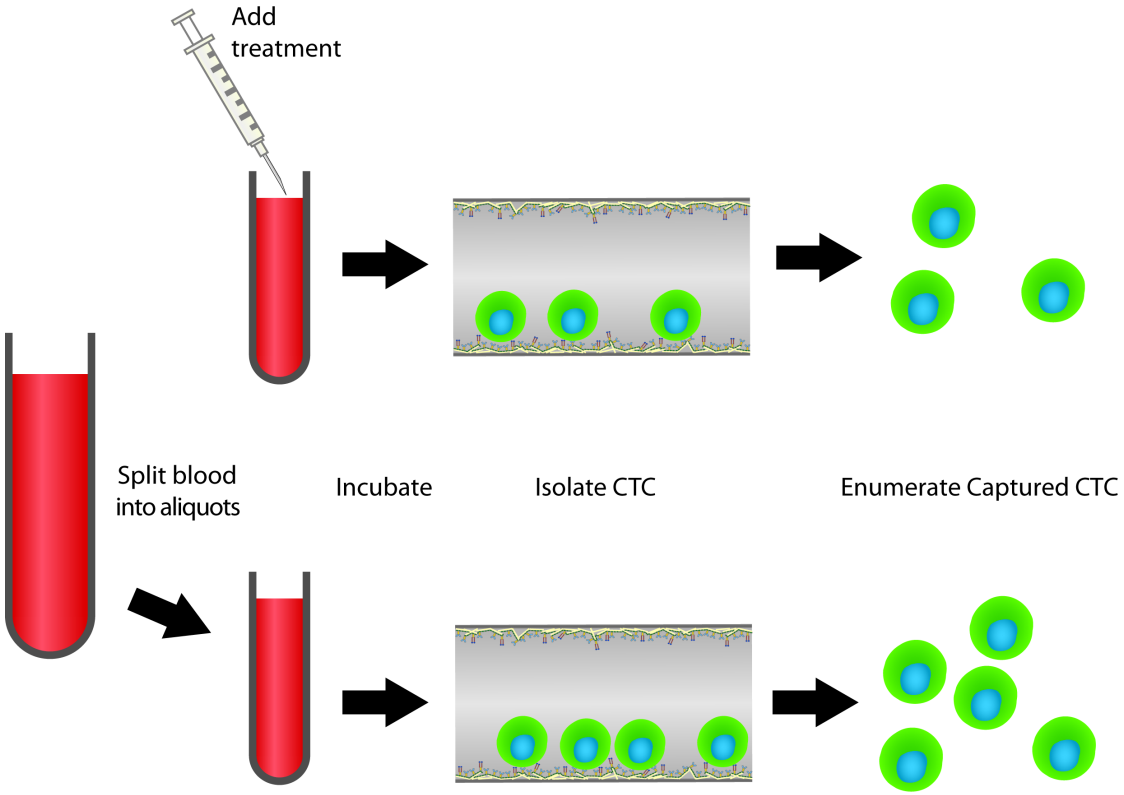

Figure 2.1 Schematic of CTC analysis protocol.

Cancer cell line cells were spiked in blood, treated with chemotherapeutic drugs, and then isolated. This process is depicted schematically in Fig. 2.1. Peripheral blood was drawn from healthy volunteers after informed consent and transferred to 8 mL polystyrene round-bottomed tubes (BD Biosciences) in which the interior lumen had been blocked with 3% BSA for 1 h at room temperature. 50,000 breast cancer (BT20) or prostate cancer (PC3) cells were added to 5 mL of blood. The spiked blood was then treated with vehicle control (dimethylsulfoxide, DMSO) or one of three drug dosages based on published pharmacokinetic data (20% of peak plasma concentration (PPC), 100% PPC, and 300% PPC). Breast cancer spiked blood was treated with docetaxel (1 ug/mL, 5 ug/mL, 15 ug/mL) or doxorubicin (0.2 ug/mL, 1 ug/mL, 3 ug/mL); prostate cancer spiked blood was treated with docetaxel or mitoxantrone (0.1 ug/mL, 0.5 ug/mL, 1.5 ug/mL). Peak plasma concentrations were determined by previous pharmacokinetic studies [18-20]. Samples were incubated for 24 h at 37°C on a BioRad UltraRocker rocking platform (Hercules, CA).

2.3.4 Preparation of selectin-functionalized microtubes

Selectin-functionalized microtubes for cancer cell isolation were prepared as previously described [18,19]. Microrenathane tubing was washed with ethanol and distilled water, then coated with poly-L-lysine (1:250) and 6.6 wt% halloysite nanotubes. The tubes were subsequently washed with distilled water and allowed to cure overnight at RT. The halloysite-coated microtubes were then perfused with 20 ug/mL Protein-G and allowed to incubate for 2 h at RT. A solution of 10 ug/mL E-selectin-Fc chimera and 50 ug/mL anti-EpCAM antibody was then pulled into the

microtubes. The tubes incubated with this solution for 2 h at RT. The tubes were blocked with 5% milk for 1 h at RT.

2.3.5 Cell isolation and enumeration from spiked blood

Buffy coat was extracted from spiked blood using a Ficoll density centrifugation as previously described [18]. Briefly, buffy coat was washed in HBSS and any remaining red blood cells were lysed with erythrocyte lysis buffer for 10 min at room temperature (RT). Cells were washed with HBSS and resuspended in 2 mL of flow buffer. Flow buffer was prepared by saturating PBS containing Ca^{2+} and Mg^{2+} with $CaCO_3$, followed by sterile filtration through a 0.2 μm PFTE syringe filter (Millipore). Cells were perfused through the selectin-functionalized microtube device a shear stress of 2 dyn/cm^2. After flow, the microtube devices were washed with cell-free flow buffer, and adherent cells were removed from the tube by introducing trypsin for 10 min at RT. The recovered cells were plated onto glass bottom petri dishes (Grenier Bioone, Frickenhausen, Germany) and allowed to recover in media supplemented with 30% FBS for 4 h.

Cells were fixed in 4% paraformaldehyde for 45 min at RT. Plates were incubated with anti-EpCAM antibody conjugated to FITC diluted 1:100 in PBS for 1 h at RT followed by incubation with anti-CD45-APC antibody diluted 1:100 for 45 min at RT. DAPI was added and the plates were imaged using an Olympus IX81 fluorescence microscope (Center Valley, PA) or Zeiss LSM710 confocal microscope (Oberkochen, Germany) within the Life Science Core Facility at Cornell University. Cell counts were based on EpCAM and CD45 expression, nucleus size and shape, and cell size

and morphology. A CTC was taken as any cell that met the following requirements: greater than 8 um in size, nonsymmetrical nucleus, positive for EpCAM, negative for CD45. Fluorescent micrographs were taken at 20 randomly selected locations within each well, and total cell counts estimated based on the total well area [18].

Processed cells that were not captured in the tube were collected, washed with PBS, and incubated with anti-EpCAM-FITC (clone 158206) for 1 h at RT. Stained cells were subsequently washed and stained with annexin-V and propidium iodide according to manufacturer instructions. Quantification was carried out using a Millipore Guava Easycyte flow cytometer.

2.3.6 Patient sample isolation

Two tubes of peripheral whole blood (7.5 mL per tube) was collected from patients diagnosed with stage IV cancer by BioCytics Inc. at Carolina BioOncology Institute, PLLC, after informed consent. Samples were analyzed from 3 breast cancer patients (Br1 through Br3), 2 prostate cancer patients (Pr1 and Pr2), one renal cancer patient (Re1), and one colon cancer patient (Co1). Samples were shipped overnight to Cornell University where they were split into 3 2.5 mL samples and treated with vehicle control, subclinical (20% PPC), or clinical dosages (100% PPC) of drug. Drugs were selected based on the cancer type. Prostate samples were treated with docetaxel and mitoxantrone; breast, colon, and renal samples were treated with docetaxel and doxorubicin. Samples were processed and enumerated in the precise manner as in cell spiking experiments as described above.

2.3.7 EpCAM expression following drug treatment

In order to determine whether the reduction in captured cells was from cell death or loss of adhesion ability, the expression of EpCAM was measured after drug treatment. BT20 and PC3 cells were plated on 24 well plates. Cells were treated with the same dosages and drugs as in blood spiking experiments. The plates were incubated for 4 h at 37°C. The cells were released from the plates with trypsin and then allowed to recover for 1 h at 37°C. Cells were stained with a 1:100 dilution of anti-EpCAM conjugated with FITC (clone HEA-125) for 30 minutes on ice. Cells were washed twice with buffer and analyzed using a flow cytometer.

2.3.8 Cell viability

Cells were plated on 24 well plates and treated with drug at the same concentrations used in the isolation studies for 24 h at 37°C. Cells were released from the plate with trypsin and washed with buffer. Cells were then diluted 1:10 in ViaCount viability reagent and incubated for 10 min at RT, according to the manufacturer instructions. The samples were then processed on a flow cytometer using built-in ViaCount software.

2.3.9 Statistics

All graphical error bars represent standard error of the mean. Significance was determined by performing an unpaired two-tailed t-test with $\alpha=0.05$ in GraphPad Prism.

2.4. Results

2.4.1 BT20 and PC3 cells showed dose dependent susceptibility to chemotherapeutic drugs in vitro.

Chemotherapeutic drugs of interest (docetaxel, doxorubicin, mitoxantrone) were tested for their efficacy *in vitro* prior to testing the drugs *in situ* in whole blood (Fig. 2). Data are expressed as the number of viable cells relative to the untreated sample. BT20 showed dose-dependence, and this effect reached a plateau at ~50% viability with docetaxel. A similar effect occurred with docetaxel on PC3. Extended dose dependence was seen with doxorubicin and BT20 as well as with mitoxantrone and PC3, where viability was reduced to 3 and 1.5%, respectively.

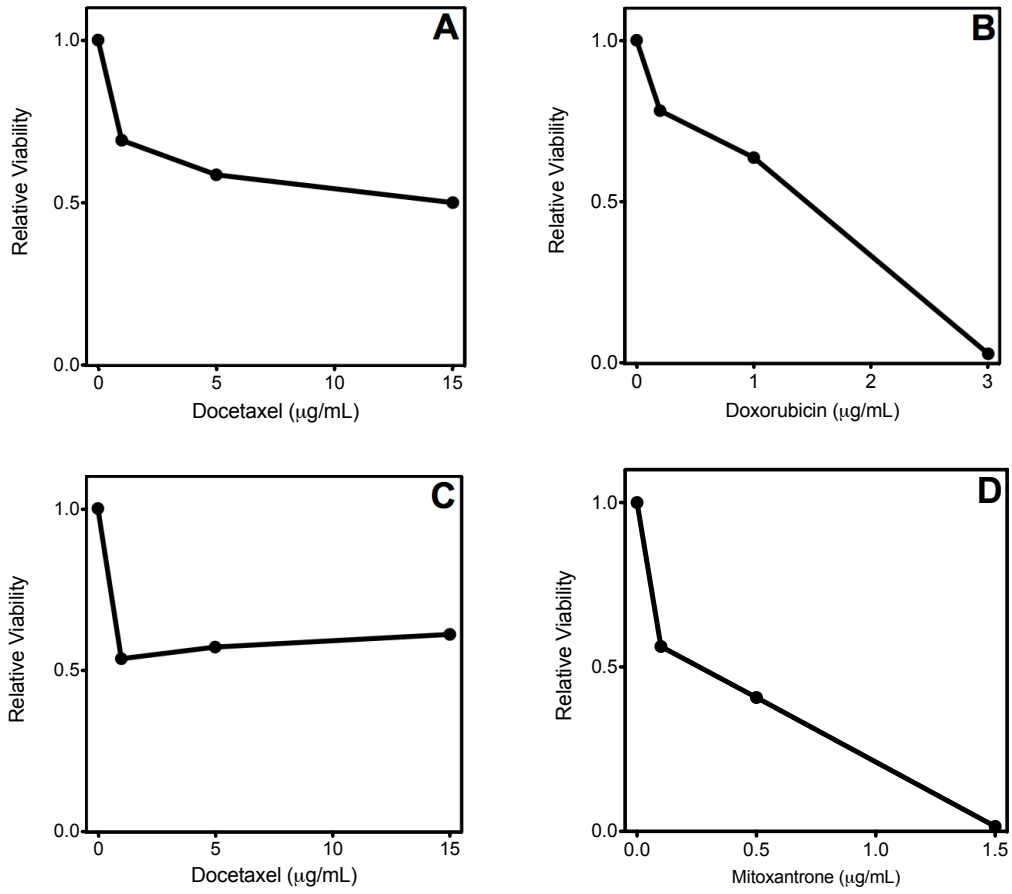

Figure 2.2. Breast and prostate cancer cell lines are sensitive to docetaxel, doxorubicin, and mitoxantrone in vitro. Results are presented as the ratio of viable cells following 24 h of drug administration to the number of viable cells in the control sample. (A) BT20 cells treated with docetaxel. (B) BT20 cells treated with doxorubicin. (C) PC3 cells treated with docetaxel. (D) PC3 cells treated with mitoxantrone. Figures are representative of two independent experiments.

2.4.2 BT20 and PC3 cells showed drug dependent susceptibility to chemotherapeutic drugs in whole blood.

50,000 BT20 or PC3 cells were spiked into whole blood, treated with appropriate chemotherapeutic drug, and isolated as described above. The clinical dosage of each drug was taken to be the maximum plasma concentration determined by previous pharmacokinetic studies [18-20]. BT20 cells were treated with docetaxel (1 ug/mL, 5 ug/mL, 15 ug/mL) and doxorubicin (0.2 ug/mL, 1 ug/mL, 3 ug/mL). Cell counts of

BT20 treated with docetaxel were reduced to 70.2±5.4% (mean±SEM), 43.9±2.7%, and 47.7±4.1% of the untreated sample. When treated with doxorubicin, cell counts decreased to 83.9±3.8%, 59.9±3.0%, and 52.4±2.5%, with respect to the untreated control. PC3 cells were treated with docetaxel and mitoxantrone (0.1 ug/mL, 0.5 ug/mL, 1.5 ug/mL). Docetaxel treatment of PC3 cells reduced the cell count to 86.3±7.1%, 41.7±4.4%, and 60.3±6.2 of control, while mitoxantrone treatment counts were 86.1±8.0%, 54.7±5.9%, and 54.5±6.2% of the untreated control (Fig. 2.3, Table 2.1).

To confirm that the uncaptured cells were indeed rendered not viable rather than just non-adhesive, the cells from the syringe that did not stick to the tube were stained with annexin-V and propidium iodide. No significant number of viable EpCAM-positive cells were observed in any of the samples studied (data not shown).

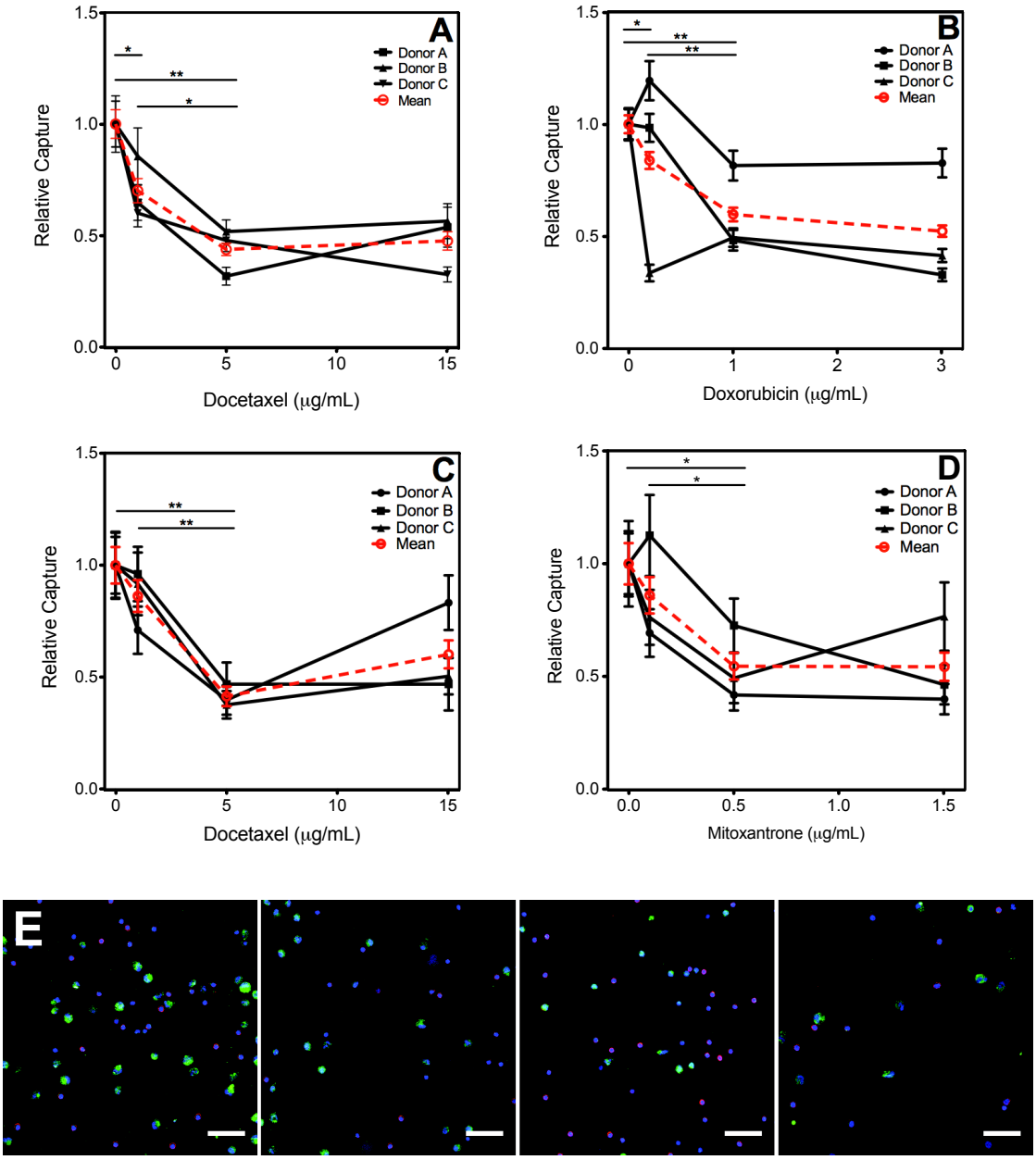

Figure 2.3. Breast and prostate cancer cell lines were spiked into 5 mL whole blood and treated with various doses of drugs. Following 24 h incubation, cancer cells were isolated from the blood and enumerated. Results are presented with individual donor data represented by black lines and the mean capture by a red dotted line. (A) BT20 cells treated with docetaxel. (B) BT20 cells treated with doxorubicin. (C) PC3 cells treated with docetaxel. (D) PC3 cells treated with mitoxantrone. (E) Representative micrographs of PC3 cells captured from blood samples treated with docetaxel. Cells were stained for EpCAM (green), CD45 (red), and nucleus (DAPI). Error bars represent standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; scale bar = 50 um

Table 2.1. Experimental data detailing the number of cells captured from cell spiking in whole blood. PPC = 5 ug/mL docetaxel (DT), 1 ug/mL doxorubicin (DOX), and 0.5 ug/mL mitoxantrone (MTX).

Cell Line	Treatment	Donor	Control	20% PPC	100% PPC	300% PPC
BT20	DT	A	1522 ± 136	987 ± 81	487 ± 42	820 ± 114
BT20	DT	B	2597 ± 186	2222 ± 289	1347 ± 97	1469 ± 173
BT20	DT	C	1906 ± 139	1147 ± 82	914 ± 67	624 ± 45
BT20	DOX	A	2276 ± 116	2717 ± 142	1857 ± 118	1883 ± 110
BT20	DOX	B	2539 ± 121	2499 ± 103	1228 ± 100	837 ± 59
BT20	DOX	C	1521 ± 72	514 ± 50	754 ± 51	632 ± 33
PC3	DT	A	1251 ± 112	890 ± 105	500 ± 68	1043 ± 120
PC3	DT	B	4260 ± 437	4095 ± 300	2006 ± 355	2006 ± 455
PC3	DT	C	3992 ± 422	3662 ± 400	1512 ± 184	2020 ± 241
PC3	MTX	A	6019 ± 572	4178 ± 494	2529 ± 332	2419 ± 332
PC3	MTX	B	2419 ± 247	2721 ± 332	1759 ± 224	1127 ± 176
PC3	MTX	C	7778 ± 1039	5937 ± 518	3848 ± 690	5964 ± 861

2.4.3 Chemotherapeutic drug treatment of BT20 and PC3 cells did not cause loss of EpCAM expression.

It was investigated whether the reduction of isolated cells as a result of drug treatment was due to drug efficacy or to simply loss of adhesion markers. To address this, the

surface expression of EpCAM was tested following drug treatment by flow cytometry (Fig. 2.4). No significant change in surface EpCAM expression was seen post-treatment for any of the drug concentrations. This suggests that the reduced cell counts in drug treated samples were due to reduction in the number of viable cells rather than a loss of adhesion affinity per se.

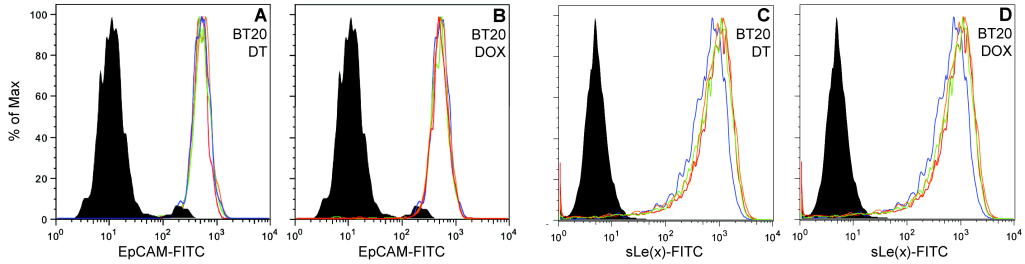

Figure 2.4. EpCAM expression of BT20 and PC3 did not change following treatment with docetaxel, doxorubicin, or mitoxantrone, as evaluated by flow cytometry. Data is presented in histograms wherein the black shaded region represents isotype control, the blue line is the control untreated sample, the red line is the 20% PPC, the orange line is 100% PPC, and the green line is 300% PPC. (A) BT20 cells treated with docetaxel. (B) BT20 cells treated with doxorubicin. (C) PC3 cells treated with docetaxel. (D) PC3 cells treated with mitoxantrone. Figures are representative of two independent experiments.

2.3.4 Primary cancer blood samples show heterogeneous susceptibility to chemotherapeutic drugs.

To investigate the relevance of this platform for clinical use, we tested primary blood samples from 7 cancer patients (3 breast, 2 prostate, 1 colon, 1 renal). Subclinical and clinical dosages were tested. Breast, colon, and renal

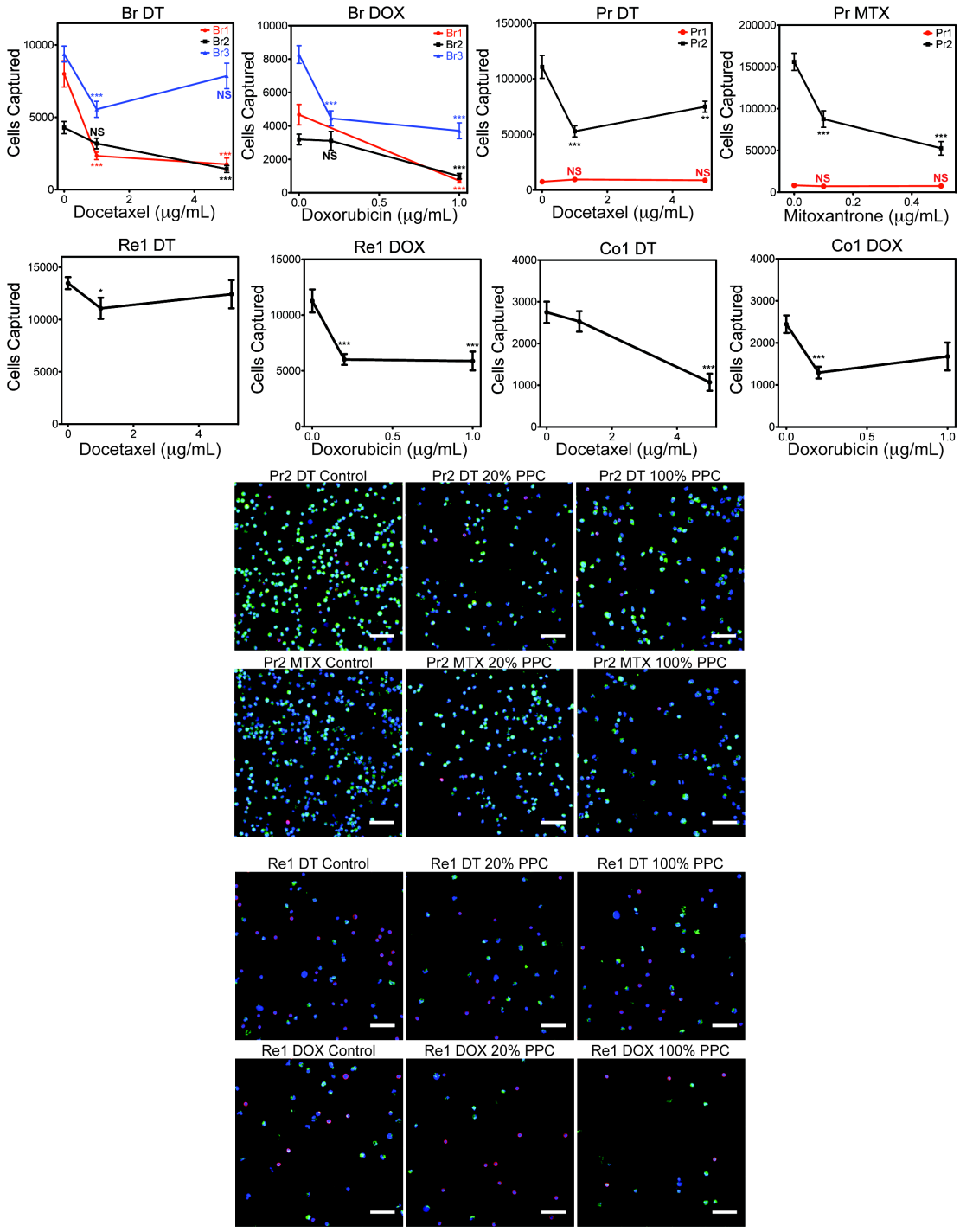

Figure 2.5. Peripheral blood from patient samples showed heterogeneous sensitivity to chemotherapeutics. Patient samples were collected from three breast cancer patients (Br1, Br2, and Br3), two prostate cancer patients (Pr1 and Pr2), one renal and one colon cancer patient (Re1 and Co1, respectively). Each tube of whole blood was split into three aliquots and treated with vehicle control, 20% PPC, or 100% PPC of the appropriate drug. DT = docetaxel, DOX = doxorubicin, MTX = mitoxantrone.

blood samples were treated with docetaxel and doxorubicin, while prostate cancer blood was treated with docetaxel and mitoxantrone (Fig 2.5; Table 2.2.). Overall, drug susceptibility for at least one of the drugs tested in 6 of 7 patients was detected. 3 patients were susceptible to only one of the drugs tested (Co1, Re1, Br3) while 3 were susceptible to both (Br1, Br2, Pr2). The remaining patient (Pr1) was not susceptible to either drug tested.

Table 2.2. Experimental data the number of cells captured from cancer patient blood samples. PPC = 5 ug/mL docetaxel (DT), 1 ug/mL doxorubicin (DOX), and 0.5 ug/mL mitoxantrone (MTX).

Donor	Treatment	Control	20% PPC	100% PPC
Br1	DT	7998 ± 905	2336 ± 255	1759 ± 423
Br1	DOX	4672 ± 609	-- --	715 ± 120
Br2	DT	4288 ± 423	3188 ± 365	1429 ± 241
Br2	DOX	3188 ± 318	3106 ± 563	989 ± 167
Br3	DT	9372 ± 550	5552 ± 550	7861 ± 879
Br3	DOX	8273 ± 533	4453 ± 449	3710 ± 473
Pr1	DT	7476 ± 524	9455 ± 1093	8768 ± 1070
Pr1	MTX	8300 ± 603	7146 ± 722	7531 ± 886
Pr2	DT	110765 ± 10336	52826 ± 5043	74952 ± 4918
Pr2	MTX	156087 ± 10276	87622 ± 9860	52606 ± 8115
Re1	DT	13486 ± 576	11076 ± 1011	12423 ± 1345
Re1	DOX	11269 ± 1034	6019 ± 486	5882 ± 843
Co1	DT	2749 ± 255	2529 ± 247	1072 ± 205
Co1	DOX	2446 ± 209	1292 ± 140	1677 ± 332

2.4. Discussion

In this paper a novel platform is presented for the prediction of cancer drug efficacy on a patient-to-patient basis, in a manner suitable for pre-screening prior to systemic administration. This platform was first characterized by spiking breast and prostate

cancer cell lines at known quantities into healthy blood, creating model samples of blood containing cancer cells with well-defined susceptibilities. Based on studies of drug efficacy on these cell lines in media (Fig. 2.2), we were able to recapitulate the therapeutic effect in whole blood (Fig. 2.3). It is interesting to note that the effect of doxorubicin and mitoxantrone at their highest dosages was to eliminate nearly all cancer cells in media, however in whole blood there was no significant increase in cell elimination in response to the the clinical dosage. The observed limit of efficacy to about 50% viability is likely due to various factors present in the milieu of whole blood. This underscores another advantage of our system, specifically that drug efficacy may be tested in the same environment in which it is actually administered. The dose dependence of treatment observed also demonstrates that it may be possible to identify patients that would respond to subclinical dosages at a level of efficiency equal to the maximum dosage, ameliorating detrimental side effects associated with chemotherapeutic toxicity.

A high degree of spiked cancer cell death was observed following incubation of blood samples for 24 h on a rocker. This is most likely due in part to the fact that the test tubes were thoroughly blocked with BSA and the motion of the blood from the rocker prohibited cell adhesion, contributing to in cell death via anoikis [23; 24]. Further cell death is likely the result of inhospitable factors within the whole blood collected from healthy volunteers, which would explain the relatively high degree of variability between donors (Table 2.1). Nonetheless, we were able to detect a therapeutic reduction in cell number, which is significant due to the fact that all comparative samples were matched. This is not expected to be the case for clinical samples since

the cancer cells are not foreign transplants from a different donor but native to the patient, and, additionally, primary cancer cells have been shown to avoid anoikis by various mechanisms [24; 25], and can escape immune activity [26; 27; 28].

Significant quantities of CTC were detected in blood samples of 7 patients diagnosed with metastatic cancer (Fig. 2.5). Of these, 6 showed a marked reduction in CTC count following enumeration, and of these 6 samples, three of them showed CTC reduction in response to one of the two drugs tested. The fact that 3 out of 7 of the samples responded differently to different drugs when treated in an identical manner otherwise suggests that these results are in fact due to sensitivities and resistances of the CTC. It is possible that in this case the differential sensitivities could be due to patients' previous treatment protocols. While all of the patient samples analyzed showed distinctly high CTC counts, one patient had exceptionally high counts: Pr2. It is interesting to note that Pr2, which showed much higher CTC counts than Pr1, also had a much higher PSA level of 2,149 ng/mL compared to 643 ng/mL for Pr1. It remains to be seen if our technique provides a true predictor of therapeutic response of primary tumor and metastatic lesions. Nevertheless, the assay is intended to be carried out in a relevant biological environment rather than an engineered environment, which may be a necessary step to development of a successful predictive clinical tool.

Clinical trials have been performed and more are in progress that monitor CTC count throughout the treatment of different cohorts of patients. It has been shown that CTC count is a reliable predictor of response and relapse [29; 30]. The combination of these clinical trial findings with the suggestion that CTC may be the most deadly subpopulation of cancer cells (in that they propagate metastasis) makes CTC a

particularly promising substrate for the development of personalized medicine determination in the clinic [31]. The assay developed here has the potential to be used in a number of ways. Patient cohorts could be selected based on drug resistance pre-screening. Alternatively, acquired resistance to chemotherapeutics can be monitored throughout the progress of clinical trials. Furthermore, as we have shown here for the administration of docetaxel and doxorubicin to renal and colon cancers, this platform allows for rapid screening of drugs approved for some cancers but remaining to be evaluated for others. This is particularly useful considering recent observations by the Cancer Genome Atlas Research Network that cancers from different tissues can have strikingly similar genetic signatures [32; 33].

In conclusion, we have developed a novel platform for screening drug efficacies of chemotherapeutics using CTC enumeration as a diagnostic output as a predictor for drug resistance in individual patients. BT20 and PC3 cells were spiked into whole blood and treated with the purpose of validating this technique. The assay is carried out in a rapid procedure that, in a clinical setting, could predict a patient's sensitivity in a single day. Two doses of two therapeutic agents simultaneously; scale up to test more drugs is limited only by the quantity of blood that can be drawn from a patient and the required sample volume per test. Additionally, this technique is not limited to the isolation technique used in this paper; it can be adapted to any chip-based or magnetic bead-based CTC isolation method.

Acknowledgements

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Chapter 3

Primary Circulating Tumor Cells Isolated from Peripheral Blood Demonstrate Sensitivity to Novel TRAIL Liposomal Therapeutic

3.1 Introduction

Cancer metastasis accounts for the majority of all cancer related fatalities (1). While therapies against primary tumors have progressed significantly in the last few decades, therapies to treat or prevent metastasis are lacking.

Metastasis occurs when cells from the primary tumor break off from the primary tumor and migrate to the vasculature. Cells then intravasate into a vessel, where they then become circulating tumor cells (CTC). CTCs travel through the bloodstream where they interact with immune cells and the endothelium. When they encounter the correct proteins on the endothelium, they are able to roll and adhere, extravasating out of the bloodstream, to a distant site(2).

Circulating tumor cells are thought to reflect the complexity and breadth of primary tumors, even more than traditional biopsies. For this reason, CTCs tend to be considered liquid biopsies. While currently CTCs are only used clinically to determine prognosis for many cancer types (3–7), CTCs hold significant potential as both diagnostic and treatment targets.

Studies have shown that CTC can be helpful in tracking or determining a patient's response to therapy. For example, Nolé *et al* showed that breast cancer patients with CTC counts higher than 5 before and after chemotherapy had worse prognosis than those with counts of less than 5 (8). The DETECT study aimed to use CTC status and genetic markers to direct personalized treatment (9). Additionally, the Haber group showed that cultured CTC could be used to test drug sensitivity (10).

We previously reported on a novel broad application diagnostic test using CTC enumeration to test for drug sensitivity. In this test, drugs are added directly to whole blood and CTC are enumerated and compared to a control sample. A reduction in CTC count is considered a show of sensitivity (11).

While progress has been made on the diagnostic use of CTC, still very few drugs have been created to target CTC. We recently described a novel liposomal therapeutic targeting CTC. The drug is comprised of a phosphatidyl choline (PC)-based liposome functionalized with two proteins, E-selectin, a natural adhesion protein found on the surface of activated endothelium, and TNF-related apoptosis inducing ligand (TRAIL) (12). TRAIL is an apoptotic protein constitutively expressed by a variety of cells, including various immune cells. Studies have shown that tumor cells have increased susceptibility to TRAIL and that the majority of normal cells show no sensitivity (13). In one study, TRAIL was shown to have a cytotoxic effect on 16 different cancer cell lines, yet exhibit no obvious signs of toxicity when injected into primates (14). Additionally, blockage of TRAIL in a mouse tumor model increased the occurrence of liver metastases (15).

The one drawback of TRAIL therapies is the short half-life of soluble TRAIL; TRAIL only remains in the body for approximately 30 minutes. (14) However, we found that when functionalized to a liposome, the half-life of the therapy is much longer. In a pharmacokinetic study in mice, we showed the presence of EST liposomes 72 hours after injection (16). Additionally, EST liposomes were shown to eradicate over 99% of CTC after tail vein injection in nude mice. Mice treated with EST liposomes also showed presence of apoptotic cancer cells within the lungs (12). In a prostate model of

metastasis, EST liposome treatment was shown to block widespread metastasis, as well as decreased the CTC count and the size of the primary tumor (16).

While the EST liposome therapy shows great promise *in vitro* and *in vivo* against cancer cell lines, the efficacy of the therapy on primary CTC has not been proven. Here, we use our previously described method to test drug efficacy to determine the sensitivity of human primary CTC to EST liposomes. We show that treatment of whole blood with EST liposomes decreases the number of viable CTC.

3.2 Methods

3.2.1 Cell Culture

SW620 and Colo205 colon cancer cells were obtained from the American Type Culture Collection (ATCC). Colo205 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). SW620 cells were cultured in Lebowitz's-15 media supplemented with 10% FBS and 1% PS. All cells were grown under humid conditions in 5% CO_2 at 37°C.

3.2.2 Liposome Formation and Functionalization

Unilamellar liposomes were created as described previously (12). L-α-lysophosphatidylcholine (Egg PC), egg sphingomyelin (Egg SM), ovine wool cholesterol (Chol) and 1,2 dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl](nickel salt) were combine at weight ratios 50%:30%:10%:10% and held under vacuum for 18 h to create thin lipid film. The film was then hydrated with liposome buffer (150 mM NaCl, 10mM Hepes, and 1 mM

$MgCl_2$ in water) to create multilamellar liposomes. To create unilamellar liposomes of the desired size (100nm), liposomes were subjected to 10 freeze thaw cycles followed by two subsequent rounds of extrusion at 60°C through polycarbonate membranes with 200 nm and 100 nm pore size, respectively. Fresh liposomes were then incubated with recombinant human ES and TRAIL at final concentrations of 71.43 nM and 250 nM respectively at 37°C for 30 minutes. ES and TRAIL both contained a his-tag and were able to bind to the Ni-NTA lipid on the surface of the liposome. Liposomes were stored at 4°C overnight and used within two weeks to decrease the chance of oxidation of lipids.

3.2.3 Cell Spiking Treatment

100,000 Colo205 cells were spiked into 4 mL aliquots of blood from healthy human donors. Cells were incorporated for 15 min on a rotator. Blood aliquots were then treated with 20-50 μL/mL liposome buffer, E-selectin/TRAIL liposomes (EST), or 1 μg/mL soluble recombinant human TRAIL (sTRAIL). Treated blood samples were placed on a rotator at room temperature (RT) overnight.

3.2.4 Primary blood treatment

8 mL blood samples were obtained from stage IV cancer patients at Guthrie Medical Center or Rochester University. Blood was split into 4 mL aliquots and placed into tubes pre-blocked with 3% bovine serum albumin (BSA). Blood was then treated with 50 μL/mL liposome buffer or EST liposomes. Blood was incubated overnight on rotator at RT.

3.2.5 Circulating tumor cell isolation

Whole blood was carefully layered onto Ficoll-Pacque and centrifuged at 2000xg for 15 min with no brake. Buffy coat was extracted using a Pasteur pipet and washed with Hank's Buffered Saline Solution (HBSS-) with no Ca^{2+}/Mg^{2+}. Residual erthyrocytes were lysed with erythrocyte lysis buffer for 10 min and subsequently washed with HBSS-. Resulting buffy coat was then resuspended in 80 μL per 10^7 cells of MACS buffer (PBS with 0.5% BSA, 2mM EDTA). 20 μL per 10^7 cells of anti-CD45 magnetic beads (Miltentyi Biotec) was added and solution was incubated for 15 min at 4°C. Magnetic cell solution was washed with additional MACS buffer then resuspended in 500 μL fresh buffer and loaded into a primed magnetic MS column. Once the cell solution was completely through the column, the column was rinsed 3 times with MACS buffer. The solution that came through the column is centrifuged at 300xg for 8-10 min. The cell pellet was fixed in 4% paraformaldehyde for 20 min at RT. After washing CTC were deposited onto glass slides using Cytospin at 1000 RPM for 4 min.

3.2.6 CTC Labeling and Imaging

CTC on cytospun slides were first labeled with PI for 15 min at RT to identify any apoptotic cells. Slides were washed with phosphate buffered saline (PBS) 3 times. Anti-cytokeratin-FITC at 1:10 dilution in 0.5% Triton-X was added to the slides for 15 min at RT. Slides were washed with PBS 3 times and then allowed to dry. Mounting media with DAPI was added to the slides and coverslips were placed. Slides were

imaged using inverted Zeiss 880 confocal microscope in the Cornell Imaging Core Facility using 32 times magnification. Viable and apoptotic CTC were enumerated based on staining profiles. Viable CTC were determined to be cells with large nuclei, PI-, CK+. Apoptotic CTC were considered cells with large nuclei, PI+, CK+.

3.3 Results

3.3.1 SW620 cell count was reduced by treatment with EST liposomes in whole blood

10,000 SW620 colon cancer cells were labeled with CellTracker Green and spiked into whole blood from healthy donors. Blood was treated with 20 μL/mL liposome buffer (vehicle control) or EST liposomes. The cell count in the treated sample was reduced by 85% when compared to the vehicle control, showing that the EST liposomes are effective at killing the SW620 cells (Figure 3.1).

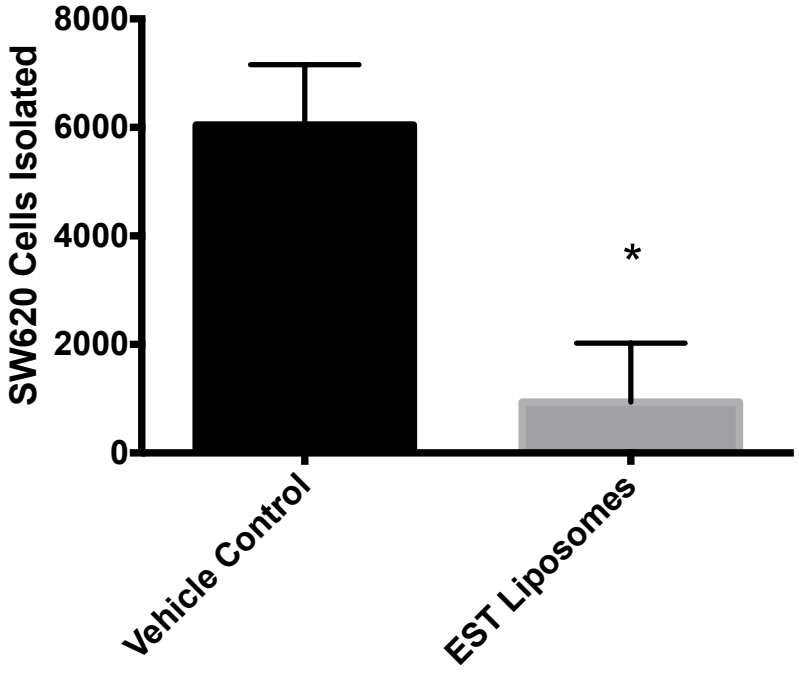

Figure 3.1. SW620 cells are sensitive to EST liposomes in whole blood. 10,000 SW620 cells were spiked into healthy whole blood and treated with EST liposomes. The number of cells recovered after treatment is shown. Error bars designate the standard deviation. Significance was determined using an unpaired, two-tailed t-test where * represents $p<0.05$.

3.3.2 Patient Demographics

Patients were selected based on cancer stage. All patients were suffering from Stage IV solid tumors and being treated at Guthrie Medical Center or Rochester University.

Specific demographics for each patient can be seen in Table 3.1.

Table 3.1. Cancer Patient Demographics

Patient Number	Gender	Age	Primary Location
097	M	41	Cholangiocarcinoma
121*	M	69	Prostate
123*	F	46	Breast
125	M	67	Renal
127*	F	61	Pancreatic
128*	M	53	Colon
129*	M	37	Melanoma
130	M	63	Prostate
131	F	55	Breast
132	F	86	Pancreatic
202			Prostate

*Excluded due to no viable CTC in control sample

3.3.3 Primary CTC are sensitive to EST liposome treatment

Blood was collected from 11 patients with stage IV cancer during routine visits at Guthrie Medical Center or Rochester University. The blood was split into two aliquots and one was treated with EST liposomes while the other served as the vehicle control. After overnight treatment, CTC were isolated using CD45-positive cell depletion. CTC enumeration was conducted based on positive cytokeratin staining. Viability was assessed based on PI exclusion. 6 of11 patients had viable CTC in control sample and were therefore evaluated for EST liposome sensitivity. All six patients saw a greater than 50% reduction in viable CTC with treatment (Figure 3.2, Table 3.2). Counts are shown as average ± standard error of the mean. Patient 097 showed the most sensitivity with 3756.8±560.8 viable cells in the vehicle control reduced to 11.1 ± 11.1 viable cells in the treated sample (Figure 3.3).

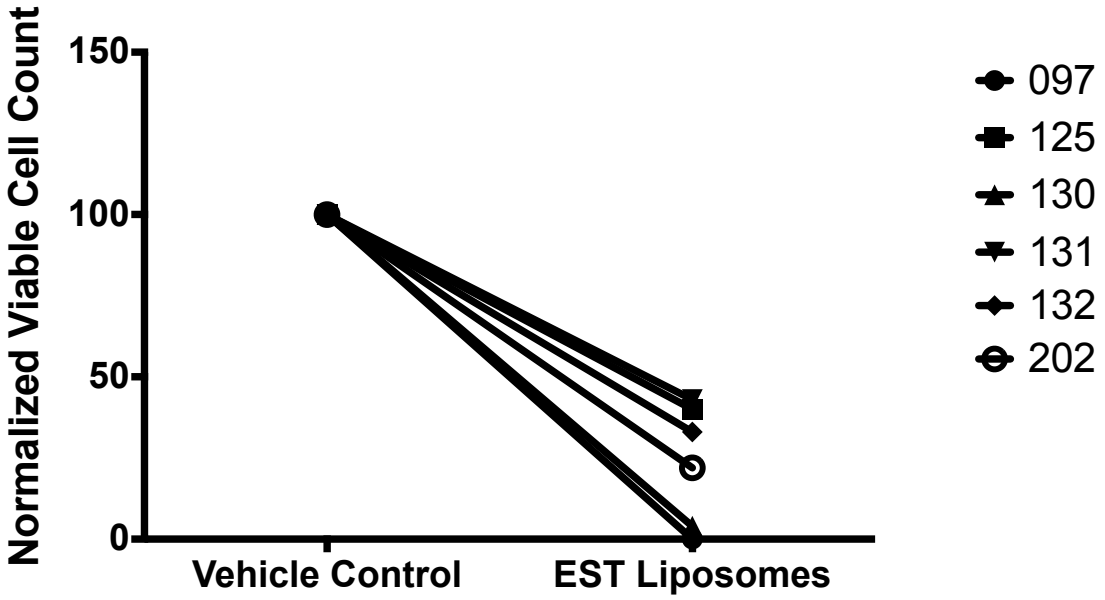

Figure 3.2. EST Liposome treatment reduces primary human CTC count in patient cancer blood. Whole blood from Stage IV cancer patients was treated with 50 µL/mL EST liposomes overnight. Resulting CTC counts are shown.

Table 3.2. Cell Counts and Viability from Patient Blood EST Treatment

Patient Number	Vehicle Control		EST liposomes		%Reduction Viable Cells
	Total CTC	% Viable	Total CTC	% Viable	
097	3939.3±583.6	95	1474.5±257.0	1	99.7
125	22.3±12.0	100	8.9±5.9	100	60.0
130	111.3±18.5	90	120.2±20.7	3	96.3
131	100.2±22.0	85	37.1±9.2	100	56.5
132	2741.2±449.5	99	1713.7±171.7	52	67.2
202	40.0±15.5	100	8.9±5.9	100	77.8

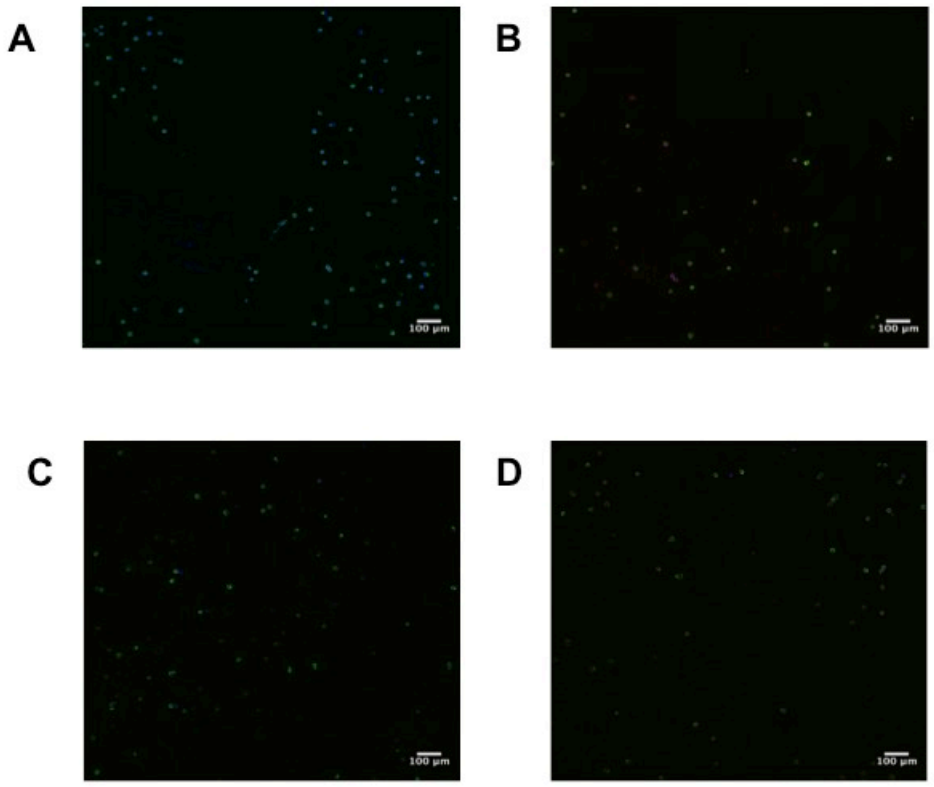

Figure 3.3. Example micrographs from patient CTC enumeration. (A) Blood from patient 097 treated with vehicle control, (B) 097 EST liposomes, (C) 132 vehicle control, (D) 132 EST liposomes. Colors correspond to cytokeratin (green) staining, PI (red), and DAPI (blue). Scale bars are 100 µm.

3.4 Discussion

In this study, we showed that EST liposomes are effective for treating primary human CTC. Whole blood from stage IV cancer patients treated with EST liposomes had over 50% less CTC than blood treated with a vehicle control. This is the first study where EST liposomes have been used on primary patient cells.

The ability for cancer cells to evade the immune system is one of the pivotal hallmarks of cancer (17). In the past decade, researchers have worked to develop therapeutics that reverse this action, thereby sensitizing cancer cells to immune action. This is done

by increasing the natural cytotoxicity of immune cells or by decreasing immune suppression by tumor cells (18). The therapeutic presented in this study presents a third mechanism of action, introduction of additional cytotoxic proteins to the surface of immune cells. This therapy holds great potential due to the fact that it works within the bloodstream eliminating CTC. CTC are believed to be the cells that cause metastases, which in turn account for the vast majority of cancer fatalities. Therefore, if CTC are targeted and eliminated, it should decrease the occurrence of metastases downstream. Additionally, previous *in vivo* work shows that the liposomal therapy caused apoptosis of lung metastases and decreased the size of primary tumors, suggesting that the functionalized leukocytes can migrate to tumor and metastatic sites (12).

TRAIL is uniquely suited as a cancer therapeutic due to its built-in targeting of cancer cells (13). In this study, we presented for the first time that primary CTC from patient blood are sensitive to TRAIL. TRAIL-based therapies to date have been limited in their clinical use due to low levels of therapeutic killing (19). While many cancer cell lines have been shown to be highly sensitive to TRAIL (14,20,21), primary cancers are inherently resistant or gain resistance over time (22,23). Because of this, significant work has been done to develop and discover sensitizing agents that can increase the efficiency of the anti-tumor effect of TRAIL therapies. It was discovered that this could be achieved through the use of common chemotherapeutics as well as natural, non-toxic compounds including curcumin and aspirin (21). We are currently exploring whether the use of low dose curcumin can increase the sensitivity of primary CTC to TRAIL liposomes. However, many worry that these sensitizers could also

increase the sensitivity of normal cells to the therapies (19). To overcome this, sensitizing agents can be encapsulated into nanoparticles, like the liposomes presented in this study. In fact, positive results have already been seen with TRAIL nanoparticles encapsulating doxorubicin (24) and paclitaxel.

Another limitation of recombinant TRAIL therapies is nonspecific aggregation of TRAIL molecules. Currently the recombinant TRAIL therapy used in clinic is dulanermin (19,25). As of 2014, dulanermin had been tested in two randomized control trials, showing no therapeutic advantage (26,27). It is believed this is due to the short half-life in the plasma as well as its inability to cluster death receptors, a necessary step for the formation of the DISC. Other recombinant TRAIL proteins, such as his-tagged TRAIL have been limited as well due to nonspecific aggregation of the TRAIL proteins (28,29). TRAIL is naturally expressed as a trimer molecule, which in turn facilitates the clustering of death receptors on the cancer cell allowing for the formation of the DISC. While his-tagged TRAIL is used in this study, the TRAIL is bound to the surface of a liposome using Ni-NTA chemistry and therefore unable to aggregate. Additionally, it is possible that the functionalization of many TRAIL molecules on the surface of the liposome is sufficient for DR clustering, though this has yet to be confirmed.

While recombinant TRAIL therapies hold great promise as anti-tumor agents, their success is currently limited by a number of factors. The TRAIL/E-selectin functionalized liposomes presented in this study have the potential to overcome a number of these limitations including avoidance of nonspecific aggregation and ability to encapsulate sensitizers. Additionally, the liposomes fall into the optimal size for

taking advantage of the EPR effect, which can increase TRAIL efficiency towards cancer cells. More work is needed to determine whether this therapy would be effective in the clinic, but the potential is evident.

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Chapter 4

Primary Whole Blood from Stage IV Tumor Patients Contains Circulating Cancer-Associated Fibroblasts (cCAF) that Influence CTC Survival Under High Shear Conditions

4.1 Introduction

Tumors are comprised of several different types of cells that serve various functions. Traditionally, most cancer research focused solely on epithelial tumor cells, but as more came to light about cancer progression and immune evasion, the importance of supporting cells came to be better understood. One cell type in particular that is of great importance to the progression and metastasis of cancer is the cancer-associated fibroblast (CAF).

CAF are defined by their spindle-like morphology as well as expression of several markers including alpha smooth muscle actin (SMA), fibroblast specific protein (FSP), and fibroblast activation protein (FAP) (1,2). Evidence suggests that CAF are derived from the transdifferentiation of several different cell types, resident fibroblasts, bone marrow derived progenitor cells, epithelial cells, and possibly endothelial cells. While CAF share many features with normal myofibroblasts involved in wound healing, CAF persist in the tumor and are not removed by apoptosis (3). This is mainly due to the presence of cancer cells, which secrete growth factors, and other cytokines that cause the transdifferentiation of CAF and help the phenotype persist (3).

Aside from assisting in preparing the metastatic niche, CAF have been implicated in a number of other aspects of cancer progression. Activated CAF secrete inflammatory signals that may induce EMT in cancer cells (4), as well as recruit immune cells to the tumor (5), and enhance angiogenesis (6,7). Studies have also shown that removal of CAF from tumor microenvironment improves tumor sensitivity to chemotherapeutics

(1). For example, breast cancer cells co-cultured with cancer-associated fibroblasts showed greater resistance to tamoxifen (8).

Due to their tumor promoting nature, many groups have worked to develop therapeutics targeting CAF (9). Some of the more promising drugs target signals involved in the proliferation of CAF including PDGF receptor (1). A anti-FAP antibody (sibrotuzumab) was shown to selectively target cancers and no off target organ uptake was shown in a preliminary clinical trial (10), though no anti-tumor effect was seen with this particular antibody therapy (11).

The Jain lab performed a study where they looked at whether stromal and supporting cells migrate alongside cancer cells (12). In this study, they injected fluorescent cancer cells into a GFP mouse and observed both cancer cells and GFP cells in the blood of the tumor-draining vein. Next, they transplanted a skin flap from a GFP mouse onto a non-GFP mouse and injected fluorescent cancer cells into the skin flap. Again, they found both cancer cells as well as GFP stromal cells in the tumor-draining vein. They also found GFP stromal cells in the metastases. This was the first study that showed evidence that cancer cells may bring their own "soil" with them (12).

Until recently, there was no evidence that this phenomenon also happened in humans. In 2015, Ao *et al* detected the presence of circulating CAF cells (cCAF) in the blood of 88% of metastatic breast cancer patients, but only 23% of patients with localized breast cancer, suggesting the presence of cCAF may be related to metastasis (13).

In this study, we confirm the presence of cCAF in whole blood from patients with stage IV cancer. We also show the first direct isolation of cCAF from whole blood.

Additionally, we demonstrate that the presence of cCAF in the blood may help protect CTC from death due to the high shear forces of blood flow.

4.2 Methods

4.2.1 Cell Culture

PC3 cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% peniciliin-streptomycin (PS). All cells were grown under humidified conditions at 37°C and 5% CO_2.

4.2.2 Circulating Cancer Associated Fibroblast (cCAF) Isolation

Blood from stage IV cancer patients was layered onto Ficoll-Pacque and centrifuged at 2000xg for 15 min with no brake. Buffy coat was removed with a Pasteur pipet and washed with Hank's buffer saline solution with no Ca^{2+}/Mg^{2+}. If necessary, red blood cells were lysed for 10 min at room temperature (RT) with erthyrocyte lysis buffer. Clean buffy coat was resuspended in 80 μL of pre-cooled MACS buffer (PBS supplemented with 0.5% BSA and 2mM EDTA). 20 μL anti-fibroblast beads from Miltenyi Biotech were added and solution was incubated for 30 min at room temperature. Cells were washed with additional MACS buffer then loaded into MS magnetic column. Cells were pulled through the column by gravity and when the column it was rinsed by MACS buffer 3 times. The column was then removed from the magnet, 2 mL MACS buffer added, and cells in the column eluted with a plunger. After pelleting by centrifugation, cells were fixed in 4% paraformaldehyde for 20 min

at RT. Then cells were resuspended in PBS and deposited onto glass slides by Cytospin.

4.2.3 Circulating tumor cell (CTC) Isolation

Blood from stage IV cancer patients was layered on Ficoll-Pacque and centrifuged for 15 min at 2000xg with no brake. Buffy coat layer was extracted using a Pasteur pipet and washed with HBSS. Erythrocytes were lysed with lysis buffer for 10 min and subsequently washed with HBSS. Buffy coat was resuspended in 80 μL of MACS buffer and 20 μL anti-CD45 magnetic beads for 15 min at 4°C. After washing with more MACS buffer, magnetic cell solution was added to MS column on magnet. Cells moved through column by gravity and column was subsequently washed 3X with MACS buffer. Cell and wash solution collection was spun down and pellet was fixed with 4% paraformaldehyde. Cells were placed onto slides using Cytospin at 1000 RPM for 4 min.

4.2.4 Fibroblast Staining and Imaging

Cells deposited onto cytospun slides were first incubated in anti-smooth muscle actin antibody diluted 1:10 in 0.5% Triton-X for 20 min at RT. After 3 washed with PBS, a secondary antibody solution consisting of 10 μg/mL goat anti-mouse IgG-Alexa 594 and 1:10 anti-cytokeratin-FITC diluted in 0.5% Triton-X was added to the slide and incubated for 20 min at RT in the dark. The slides were washed 3 additional times with PBS. Mounting media with DAPI was added to the slide and the slide was covered with a coverslip. Slides were stored at 4°C until imaging. Slides were imaged

in the Cornell Imaging Core Facility on a Zeiss inverted 880 confocal microscope with 40X magnification.

4.2.5 Primary Mouse CAF Isolation

Single cell suspension of mouse tumor cells was a gift from Cynthia Reinhart-King. Tumor cell suspension was counted and resuspended in 90 μL of MACS buffer for every 10^7 cells with 10 μL anti-mouse CD45 beads for every 10^7 cells. Solution was incubated for 15 min at 4°C. Cells were washed then passed through an LS column that was rinsed 3 times. The column run through was centrifuged at 300xg for 10 min and the resulting pellet was resuspended in 90 μL of MACS buffer for every 10^7 cells. 10 μL/10^7 cells of anti-mouse EpCAM beads were added and the solution was incubated for 15 min at 4°C. After washing the LS column separation was repeated. The flow through was pelleted and the cells were cultured in DMEM media supplemented with 4.5 g/L glucose, 10% FBS, and 1% PS. Presence of CAF was confirmed by flow cytometry.

4.2.6 Flow Cytometry

Cells at a concentration of 10^6/mL were incubated in an antibody solution (anti-rat IgG-PE, anti-mouse EpCAM-PE, anti-mouse CD45-PE, or anti-mouse PDGFRα-PE) for 30 minutes on ice. After 2-3 washes with PBS, cells were resuspended in PBS and analyzed using Millipore Guava EasyCyte flow cytometer.

4.2.7 Microshear Pulsing

Cancer cells in a primary tumor are exposed to relatively low fluid shear stress (FSS) conditions (on the order of 0.1 dyn/cm^2) exerted by interstitial flow (14). Once a CTC enters the vasculature, it is subjected to much higher shear conditions. Venous flow can have FSS of 1-6 dyn/cm^2, while arterial flow shows FSS of 10-70 dyn/cm^2 (15). Moreover, at certain areas of the vasculature, cells are exposed to very high levels of FSS (>1,000 dyn/cm^2) (16,17), particularly during blood flow through the heart, vessel bifurcations, and in vessels of large diameter, for 1-30 milliseconds at a time (18–20). In order to successfully metastasize, a CTC must navigate and survive these high FSS. It has previously been shown that malignant cells have an inherent resistance to FSS (21). So as to test whether the presence of CAF could influence this resistance, we exposed prostate cancer cells grown alone and in the presence of CAF cells to millisecond pulses of high shear flow using a previously described method (21). PC3 cells were labeled with Calcein-AM for 15 min at RT in the dark and washed 2 times with fresh media. Then, PC3 and mouse CAF cells were co-cultured for 48 hours under normal culture conditions at a ratio of 1:1. Cells were lifted and resuspended at 500,000 cells/mL. Once the number of PC3 cells had been determined in each sample using flow cytometry, the volume equivalent for 200,000 PC3 cells was placed into a syringe and pumped through a 30G-½ in needle. This process was repeated every 2 min, to mimic the physiological conditions of high shear exposure (21), for a total of 5-10 shear rounds. Cells were then plated in serum-free media overnight to allow for cell death to complete. The following day, the number of fluorescent PC3 cells remaining was counted using flow cytometry.

4.3 Results

4.3.1 CTC Fractions contain cancer-associated fibroblasts

CTC were isolated from primary human blood drawn from patients diagnosed with Stage IV cancer. Cells were placed into culture. Upon inspection of the cell cultures, two morphological states were observed. Images of the cultures can be seen in Figure 4.1. Red arrows show the rounded morphological state; black arrows indicate the elongated, spindle-like morphology. Based on the morphological characteristics, it was believed the long, spindle-like cells might be fibroblasts.

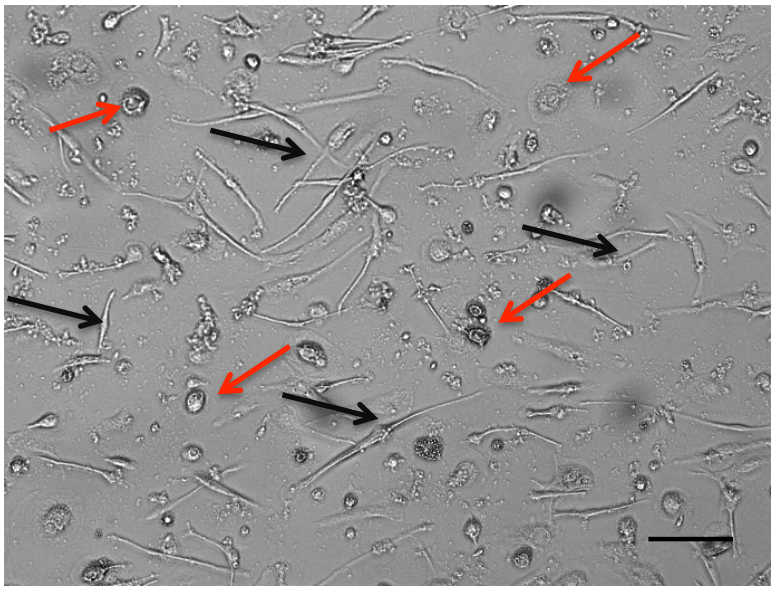

Figure 4.1. CTC cultures contain cells of two different morphological states. CTC isolated from stage IV cancer patients were cultured for several days. Phase contrast images were taken on a Zeiss IX81 microscope. Black arrows indicate cells with elongated, spindle-like morphology, red arrows indicate round morphology. Scale bar represents 25 μm.

To confirm the presence of fibroblasts in the CTC fractions, CTC isolated from cancer patients was stained for the presence of smooth muscle actin (SMA), a known cancer-associated fibroblast (CAF) marker, as well as cytokeratin (CK), a CTC marker. Cells

were imaged at high magnification using a confocal microscope. Both SMA- and CK-positive cells were seen in the patient CTC fractions. As shown in Figure 4.2, SMA-positive cells exist both as single cells as well as part of clusters.

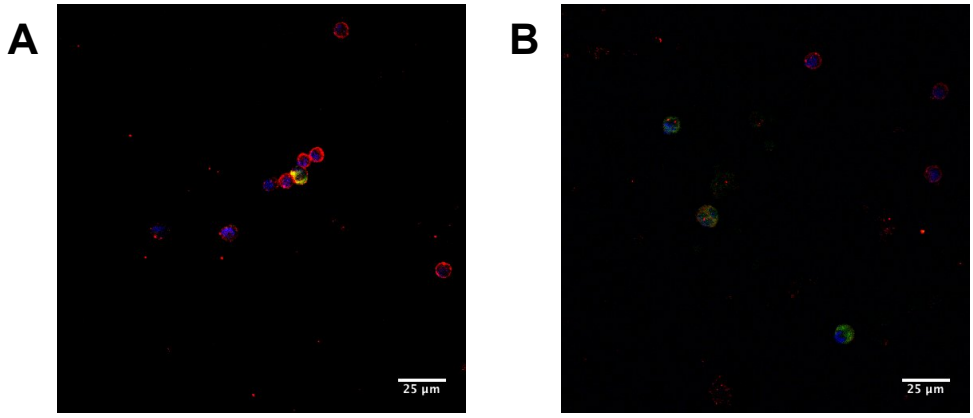

Figure 4.2. CTC fractions contain cells that are SMA+. CTC fractions from stage IV cancer patients were imaged for the presence of CAF cells. Cells were stained for SMA (red), CK (green), and DAPI (blue) and imaged using Zeiss i880 confocal microscope. CAF cells are indicated by SMA+ and DAPI+ nucleus, whereas CTC are indicated by CK+ and DAPI+ nucleus. Images shown are from (A) female breast cancer patient 123 and (B) male colon cancer patient 124. Scale bars are 25 µm.

CAFs (SMA+) and CTCs (CK+) were enumerated from multiple different patients in order to determine whether there is a pattern to the number of CAFs in patient blood. Individual counts ± standard error of the mean can be seen in Table 4.1. While it was determined there was no set pattern, there was a trend of much higher numbers of CAF cells than CTC for all patients.

Table 4.1. cCAF and CTC Counts from Primary Human Cancer Blood

Patient	Cancer Type	# CAF (SMA+)	# CTC (CK+)	CAF:CTC Ratio
88	Cervical	705.0±43.0	40.0±0.0	17.6
96	Prostate	105.7±15.7	25.0±8.1	4.2
123	Breast	1295.5±288.0	9.4±9.4	138.0
124	Colon	582.9±105.0	53.6±53.6	10.9

4.3.2 CAFs were directly isolated from the whole blood of cancer patients

In order to run genetic testing on cells, a relatively pure sample needs to be obtained. Until this point, all work to isolate and identify circulating CAF (cCAF) cells from human cancer patient blood has been indirect. We attempted to directly isolate CAFs from human blood using the MACS technology from MIltenyi biotech. Buffy coat fractions were isolated using density gradient centrifugation then labeled with anti-fibroblast MACS beads. Fibroblast cells were positively selected using a magnetic column and eluted. Cells isolated were stained for SMA and CK to determine the type of cell isolated. Figure 4.3 shows that cells isolated from human blood are SMA+ CAF cells.

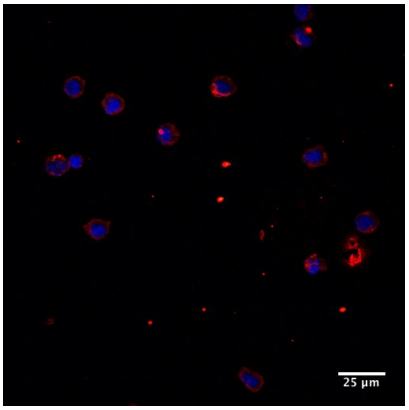

Figure 4.3. cCAF cells isolated from human cancer patient blood are SMA+. cCAF cells were directly isolated from whole blood of cancer patients, stained and imaged using Zeiss i880 confocal microscope. This figure shows a representative image from patient 126 (cholangiocarcinoma). SMA staining is shown in red, DAPI is shown in blue, while CK was stained in green. Scale bars are 25 µm.

4.3.3 CAF protect CTC from high shear forces

Anoikis resistance is a main tenet of circulating tumor cells and must occur in order cells to metastasize. We hypothesized that the presence of stromal cells may increase a CTC's chance of survival under these hostile conditions. In order to mimic the forces experienced by cells as they pass through the heart, CAF co-cultured with PC3 and PC3 cultured alone were exposed to a series of millisecond pulses of high shear force. As shown in Figure 4.4, the presence of CAF allowed for a higher retention of viability of the PC3 cells when compared to PC3 cells cultured and sheared alone.

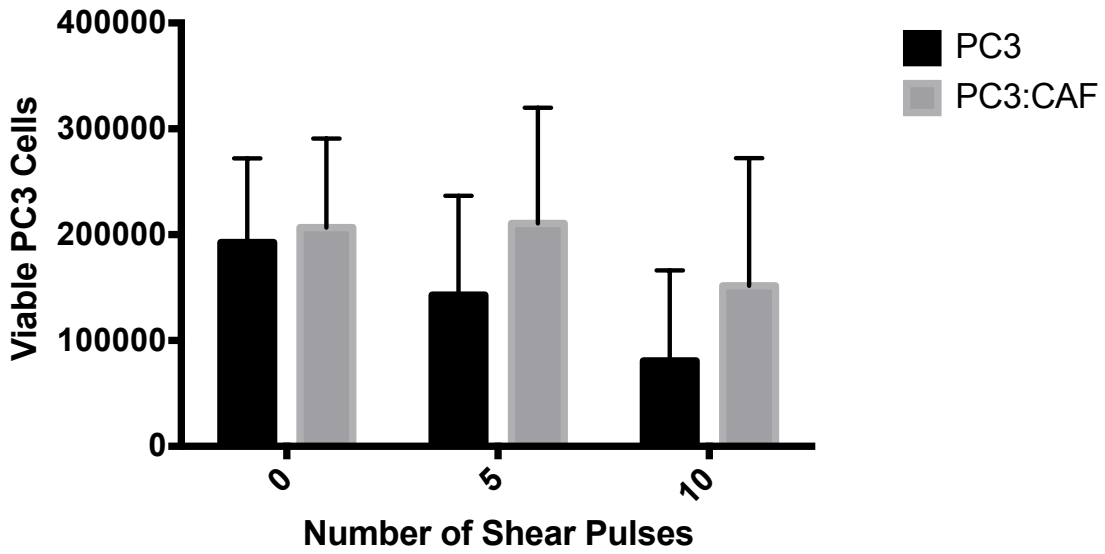

Figure 4.4. CAF protect cancer cells from shear-induced death. PC3:CAF co-cultures or PC3 cultured alone were subjected to millisecond pulses of 5290 dyn/cm^2. Viability after 24 hours was evaluated based on Calcein-AM staining of PC3 cells. Data is from n=3 experiments. No significant difference was seen in the samples based on a two-way ANOVA.

4.3.4 Soluble factors secreted from CAF cells not sufficient to protect cancer cells from high shear forces

In order to better determine the method of protection, PC3 cells grown in normal media and PC3 cells grown in conditioned media from CAF cultures were subjected to the same shear conditions as the co-cultured cells. As figure 4.5 shows, there is no significant difference between the viability of cells from the two different culture conditions.

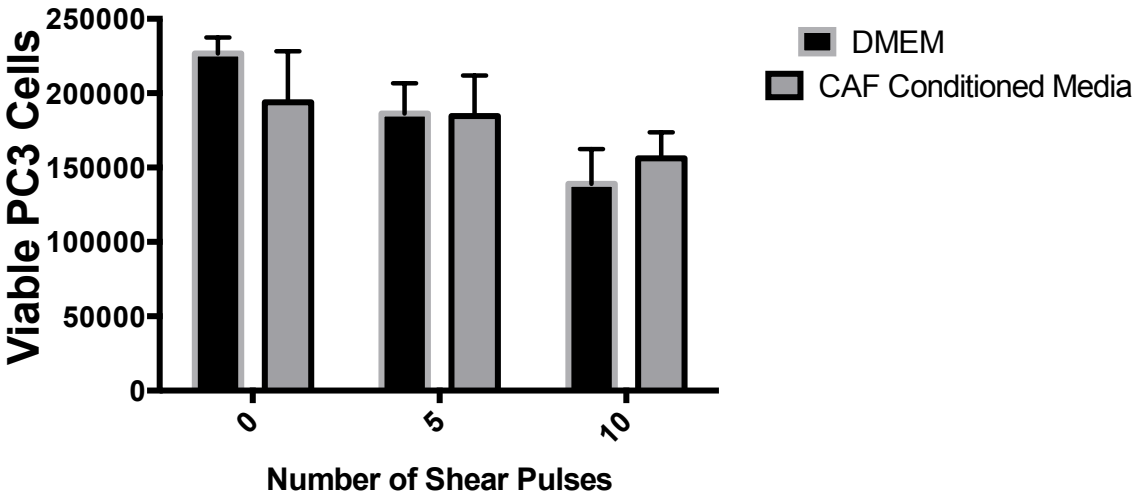

Figure 4.5. Conditioned media from CAF cells not sufficient for providing shear resistance of cancer cells. PC3 cells grown in normal media and grown in media conditioned by mouse CAF cells were subjected to a series of millisecond pulses at 5290 dyn/cm^2. Viability was evaluated after 24 hours based on Calcein-AM staining. Graph shows data from n=2 experiments.

4.4 Discussion

Cancer-associated fibroblasts have been greatly implicated in the progression and metastasis of cancer (22). CAFs are known to secrete various pro-invasive paracrine signals including TGF-β (23), stromal-derived factor 1 (SDF-1) (24-26), MMPs (19,20), and hepatocyte growth factor (HGF) (29). When exposed to these factors, cancer cells increased invasive phenotypes and underwent epithelial to mesenchymal transition (EMT) (23). Moreover, CAFs created tracks in ECM that allowed for cancer cell collective migration (30). Most of these studies have been conducted *in vitro* and the *in vivo* implications still need to be determined.

In this study, we show that CAFs not only promote the invasive phenotype in cancer cells, but also physically migrate alongside cancer cells. This was previously shown in mice (12), but until recently had not been confirmed in human samples (13).

Many studies of circulating tumor cells (CTC) have recently centered on the expression of EMT and stem cell markers by CTC (31-34). A study by Balasubramanian *et al* showed circulating cells that expressed the mesenchymal marker vimentin (33). CAFs are also known to express vimentin (35). It is possible that studies have incorrectly identified cCAFs as mesenchymal CTCs. Moreover, the role of mesenchymal CTC and cCAFs have not been elucidated and the isolation techniques described in this study would allow for direct analysis of cCAF cells. CAFs also promote drug resistance in cancer cells due to the induction of cell-adhesion mediated drug resistance (CAM-DR) (22). In this study, we showed that cCAFs can be found in clusters with CTC. This could potentially have important implications to the drug resistance of CTC. Additionally CAFs are known to express integrins and other adhesion molecules. CTC are believed to migrate via adhesive interactions with the endothelium, which involve integrin-based adhesive interactions (36). It is possible that the physical association of CAF and CTC cells within the bloodstream could provide an adhesive advantage and increase the probability of extravasation and thereby distant metastasis.

In this study, we present the first study of CAF:CTC interactions under high shear conditions. We have previously showed different properties of cancer cells can be modulated by shear stress (37,21). Due to the shear conditions to which CTC are exposed to, it is important to study these cells under the same conditions *in vitro*; however the vast majority of CTC studies are conducted under static conditions. The influence of CAFs on cancer progression and metastasis has been well characterized in the primary and metastatic sites. While there is more to learn about

these sites, very little is known about the importance of CAFs during metastatic transit. In this study, we present a novel isolation technique that successfully isolated cCAFs from patient blood. This technique could provide researchers with opportunities to study primary cCAFs both *in vitro* and *ex vivo*.

We also showed the possibility that cCAFs can influence CTC survival under harsh shear conditions. While more investigation is needed, this finding could have major implications on drug delivery and targeting. cCAFs could provide a novel therapeutic target that could help prevent and decrease the impact of metastasis.

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Chapter 5

Surgical Adjuvant Administration of EST Liposomes Do Not Provide Therapeutic Benefit in Breast Cancer Model

5.1 Introduction

Localized breast cancer is virtually curable with a five-year survival rate of 99%. However, the survival rate decreases to 25% when the cancer has metastasized distantly (1). Therefore the study of metastasis in breast cancer is of upmost importance. One of the most popular areas of translational metastasis research to date is circulating tumor cells (CTCs). CTCs are cells that break off from the primary tumor and disseminate into the vasculature in order to spread to distant parts of the body.

Hematogenous metastasis is generally accepted to occur in a stepwise fashion. First, subsets of cells mutate to allow for a migratory phenotype and begin to break through the basement membrane of the primary tumor. Next, cells make their way into the blood stream, or lymphatic system, where they circulate until they encounter the ideal environment. Here, they adhere and roll along the surface of the endothelium until they receive the correct cues to extravasate out of the vasculature. In the metastatic site, cells begin to multiply and create micrometastases that go on to become full blown distant metastases (2).

Only a very small percentage of CTC are able to complete the metastasis process and propogate metastases. Because of this phenomenon combined with the importance of metastasis in cancer mortality, significant resources have been invested into CTC research. While the first clinical evidence of CTC was discovered in a cadaever in the 19th century (3), it wasn't until the late 1990s/early 2000s that CTC became a significant research area. Many technologies have been developed for the detection

and enumeration of CTC including size-based filtration devices, microfluidic chambers, and magnetic separation techniques (4–11).

CTC have been shown to correlate with progression-free survival (PFS) and overall survival (OS) at levels of ≥5 CTC in late-stage breast cancer and ≥1 CTC in localized breast cancer (12). Similar trends have been shown in lung, colon, and prostate cancers (13–15). The correlation of CTC levels and the changes to CTC level in the course of treatment have also been studied, though with varying results (16,17).

Tumor resection remains the most widely used form of treatment for breast cancer. While the reduction in tumor burden is thought to decrease the flux and rate of CTC shedding (18,19), it has also been shown that the surgical process can introduce a bolus of CTC into the bloodstream, thereby accelerating the metastatic potential. In fact, a study done in a xenograft model showed that various diagnostic and therapeutic techniques commonly used for breast cancer treatment can in fact introduce additional CTC into the vasculature (20).

One of the main tenets of cancer that allows it to metastasize is its ability to evade the immune system (21). Recently, researchers have developed therapeutics, terms immunotherapies, that work to combat and overcome the gained immunoevasion of cancer cells. Currently there are two main types of immunotherapies on the market; one set works to decrease cancer-directed immunosuppression, and the other increases the cytotoxicity of immune cells against cancer (22,23).

In our lab, we developed the first immunotherapeutic drug targeting circulating tumor cells (CTC) within the bloodstream in hopes of preventing cancer metastasis (24). The drug consists of a bilayer liposome that has been functionalized with two proteins, E-

selectin, an adhesion protein found on the surface of inflamed endothelium, and TRAIL, TNF-related apoptosis inducing ligand. It binds to the surface of leukocytes in the blood, allowing the leukocytes to present the therapeutic protein from the drug to circulating tumor cells (CTC). The drug killed over 95% of COLO-205 or PC3 cells spiked into healthy donor whole blood. Additionally, when introduced into the circulation of mice, followed by an infusion of 2 million cancer cells, the liposomes reduced the number of cells to less than 2000. Moreover, a large increase of apoptotic cancer cells were found lodged in the lungs of mice of liposome-treated mice compared to those treated with buffer or soluble TRAIL alone (24). We also showed the efficacy of the liposomes in treating prostate cancer *in vivo*. In this orthotopic model, CTC count was over 90% lower in EST liposome treated mice compared to buffer control treated mice. Additionally, administration of EST liposomes reduced the primary tumor size as well as metastatic spread (25).

In this study, we explored whether minimal treatment with liposomes could prevent metastases in a tumor resection mouse model. Mice were given liposome treatment perisurgically as well as one week pre- and/or post-surgery. While the minimal treatment protocol was not enough to prevent metastases *in vivo*, this was the first study to show the lack of toxicity of EST liposomes in immunocompetent mice.

5.2 Materials and Methods

5.2.1 Cell Lines and Culture

4T1 and COLO-205 cell lines were purchased from the American Type Culture Collection (ATCC). Both were cultured in RPMI-1640 media supplemented with

10% fetal bovine serum and 10% penicillin-streptomycin under humidified conditions at 37°C and 5% CO_2.

5.2.2 Primary Tumor Establishment

8-wk-old female BALB/cJ mice weighing 19-23g were obtained from Jackson Laboratory. 50,000 4T1-luciferase cells were injected subcutaneously into the right abdominal mammary gland with a 30G needle. Animals were housed in pathogen free conditions at the Cornell University Transgenic Mouse Core Facility and injections were performed under sterile conditions. The Cornell University Institutional Animal Care and Use Committee approved all animal procedures.

5.2.3 Circulating Tumor Cell (CTC) Enumeration

Mice were kept alive until they died naturally or showed signs of distress, including immobility of extremities or greater than 20% loss of body weight. Mice showing signs of distress were euthanized using CO_2. Upon death, blood was collected from mice via cardiac puncture into heparinized tubes using heparinized syringes. Blood was diluted in Hank's Buffered Saline Solution (HBSS) and layered on top of 3 mL Histopaque 1077 (Sigma-Aldrich). Tubes were centrifuged at 2000xg for 15 minutes using no brake. The buffy coat was extracted and washed using HBSS twice. Residual red blood cells (RBC) were lysed using multi-species RBC lysis buffer at a dilution of 1:100 and washed immediately using HBSS. Cells were resuspended in serum-free growth media

and cultured in 37 °C and 5% CO_2 overnight on glass bottom plates. Cells were stained with anti-mouse EpCAM antibody for 1 hour at room temperature. Plates were washed and cells were subsequently stained with FITC-labeled secondary antibody. Images were captured of 30-40 randomly selected areas of the plate; EpCAM-positive cells were counted, averaged, and scaled for the entire area of the plate.

5.2.4 Bioluminescent Imaging

To track the progression of the primary tumor and metastatic spread of the breast cancer, mice were imaged using a Xenogen IVIS machine every 3-7 days. 150 mg of luciferin was injected intraperitoneally (IP) 10 minutes prior to imaging. Mice were anesthetized initially using 3.0% isoflurane until unconsicious. Mice were then moved into the imaging apparatus and anesthesia was maintained at 2.0% using a nose cone. Images were gathered using an exposure time of 1-5 seconds. Upon completion of imaging, mice were returned to cages.

5.2.5 Tumor Resection

At 14 days post-tumor cell injection, primary tumors were resected. Hair from the resection site was removed using Nair hair removal cream 24 h prior to surgery. On the day of surgery, mice were anesthesized with 3-5% isoflurane and kept under anesthesia with the use of a nose cone for the entirety of the procedure. Once the mouse was placed on the surgical table, the site was cleaned

with iodine and ethanol. An incision adjacent to the tumor was made with a 15C scalpel blade and the tumor was gently teased away from the skin with forceps and removed. If the tumor would not separate fully from the skin, additional skin was removed as well. Once the tumor was fully removed, the incision was closed using 7mm wound clips. Wound clips were removed after 10-14 days. Upon completion of the surgery, the mice were moved to a recovery cage with a warmer. Mice were kept under close observation for the next 72 h and received subcutaneous (SC) injections of 2 mg/kg ketoprofen immediately prior to surgery, 24 h post-surgery, and 48 h post-surgery. The Cornell University Institutional Animal Care and Use Committee approved all animal procedures.

5.2.6 Liposome Preparation

A lipid film of phosphatidyl chlone (PC), sphingomyelin, cholesterol, and DOGS-nickel NTA, (50%:30%:10%:10%) is prepared under vaccum for 18 h. The film was resaturated in buffer at 50°C then subjected to 10 freeze-thaw cycles. The homogenous lipid solution was then extruded through 200 nm filter 20-30 times, followed by a 100 nm filter 20-30 times. Resulting liposome solution was degassed under N_2 gas for up to 5 minutes followed by 20 min under vacuum. Liposomes were then sterilized by autoclave. The surface of sterile liposomes were functionalized with 250 nM recombinant human TRAIL and 71.4 nM recombinant human E-selectin for 15 minutes at 37°C using his-tag technology and then stored at 4°C.

5.2.7 Liposome Treatment

In order to test the efficacy of novel E-selectin/TRAIL (EST)-functionalized liposomes on the progression of mouse metastases, mice were injected with 120 µL of liposomes, liposome buffer, or soluble TRAIL (sTRAIL) through the tail vein. Mice were randomized into 5 groups based on intensity of BLI imaging on day 7 as well as by animal weight. Groups 1-3 were given four doses, beginning when the tumor is palpable (day 7), while groups 4 and 5 received three doses, beginning 24 h prior to surgery. The entire dosing schedule is shown below (Figure 5.1), where the blue line indicates tumor cell injection and the red line indicates tumor resection.

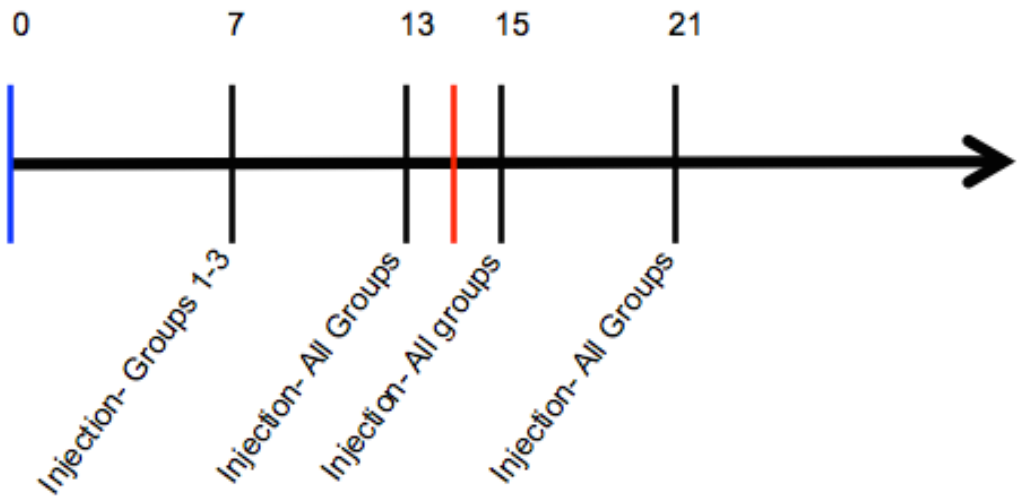


Figure 5.1. Treatment Schedule for Surgical Adjuvant Administration of EST Liposomes. Numbers indicate days post-injection. Blue line is representative of tumor cell injection and red line is indicative of tumor resection date. Black lines indicate liposome administration.

5.2.8 Weight

In order to monitor the health of the mice and the toxicity of the treatment, the mice were weighed at least once a week, usually coinciding with BLI imaging. As the health of the mice began to decline, the mice were weighed every 2-3 days.

5.2.9 Histology

Upon death or euthanasia, the mice were dissected and the lungs, kidneys, stomach, spleen, liver, as well as any additional metastatic nodules were removed and weighed. Organs were trimmed and fixed for 48 h in 4% paraformaldehyde. Thin sections of organs were made and stained for hemotoxin and eosin (H&E). The stained slides were evaluated by a veterinary pathologist to evaluate progression and state of the disease in each animal.

5.2.10 Liver Enzyme Measurement

Liver toxicity is a major problem for TRAIL therapies in clinic. We evaluated liver toxicity by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels within mouse plasma. AST and ALT levels were measured using BioVision colorimetric assay kits according to manufacturer's procedures.

5.3 Results

5.3.1 4T1 mouse model tumor progression and CTC onset time established

50,000 4T1-luciferase cells were injected subcutaneously into the mammary fat pad of 8-week BALB/cJ mice. At pre-determined time intervals, 5 mice were sacrificed and

blood was drawn and pooled. Buffy coat was isolated and stained for the presence of CTC. Additionally, the growth of tumors was tracked using bioluminescent imaging (BLI). CTC were observed after 7 days. Tumors were first palpable at 7-10 days and of resectable size around 14 days. Figure 5.2 shows a progression of tumor growth.

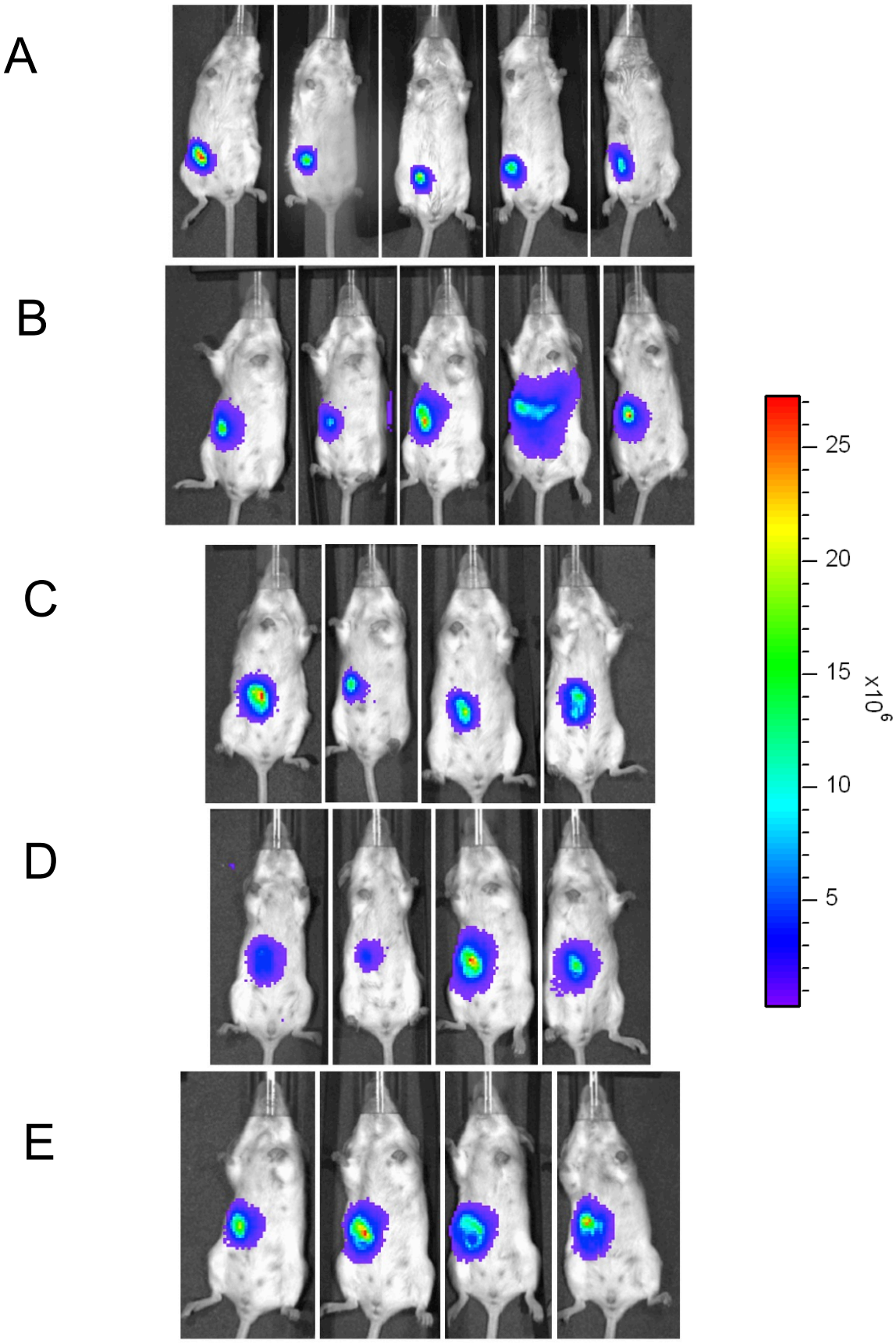


Figure 5.2. Tumor progression of subcutaneous injection of 4T1 cells in BALB/cJ mice. 50,000 4T1-luc cells were injected into mammary fat pad of 8-week BALB/cJ mice. Tumor progression was tracked using BLI. A) 7 days B) 10 days C) 14 days D) 17 days E) 21 days post-injection. Scale bar shows flux intensity in p/s/cm^2/sr.

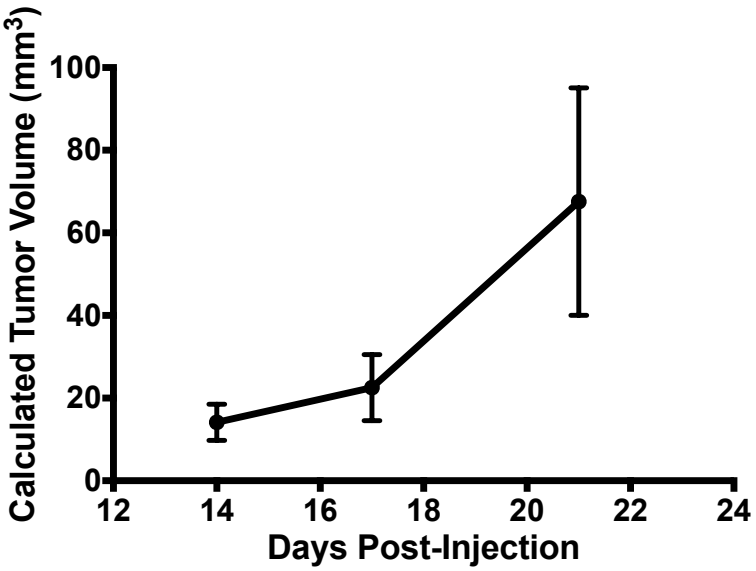


Figure 5.3. Tumor volume increased over time. Tumor length and width were measured using calipers. Tumor volume was calculated using Equation 5.1. Data represents average of n=4 mice. Error is error of the mean. Significance was tested using unpaired, two-tailed t-tests, but no significance was shown.

Tumor volume over time was calculated based on caliper measurements taken at time of imaging using the following equation:

$$Tumor\ vol\ (mm^3) = \frac{1}{2}(L * W^2) \quad (5.1)$$

Tumor volume calculations are shown in Figure 5.3. No signif icance between the time points was shown using unpaired two-tailed t-tests (Day 14-17 p=0.4; Day 14-21 p=0.15; Day 17-21 p=0.2), but the data did trend to greater tumor volumes over time. Upon sacrifice, blood was extracted from mice using cardiac puncture. Buffy coat was isolated and cultured for 7-10 days to allow for the death of leukocytes. Cells were then fixed and stained for EpCAM in order to detect CTC. CTC were detected in every sample. Representative images are shown in Figure 5.4.

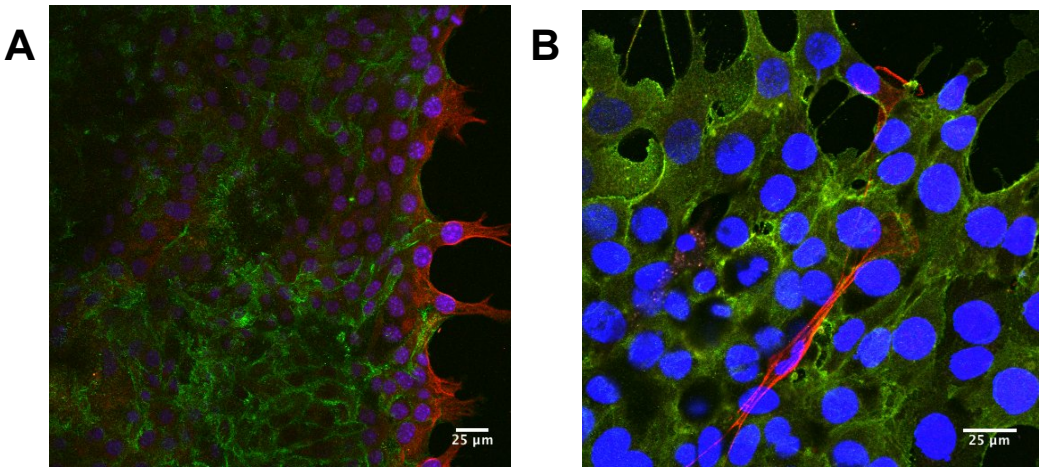


Figure 5.4. Mouse blood contains CTC and cCAF cells. Buffy coat isolated from mouse blood was stained for EpCAM (green), smooth muscle actin (red), and DAPI (blue). Slides were imaged using Zeiss i880 confocal microscope. Representative images from A) 7 days B) 13 days post-injection are shown. Scale bars are 25 µm.

5.3.2 Widespread metastases established in mice even following tumor resection

In order to determine whether mice grew metastases after tumor resection 50,000 4T1-luc cells were injected into the mammary fat pad of BALB/cJ mice and tumors were resected after 14 days. Tumor progression was tracked using BLI imaging (Figure 5.5). Additionally, tumors were weighed upon resection and survival time was recorded. Primary tumors weighed an average of 93.5±30.7 mg. Mice survived an average of 40.1±1.2 days post-injection (26.1±1.2 days post-resection).

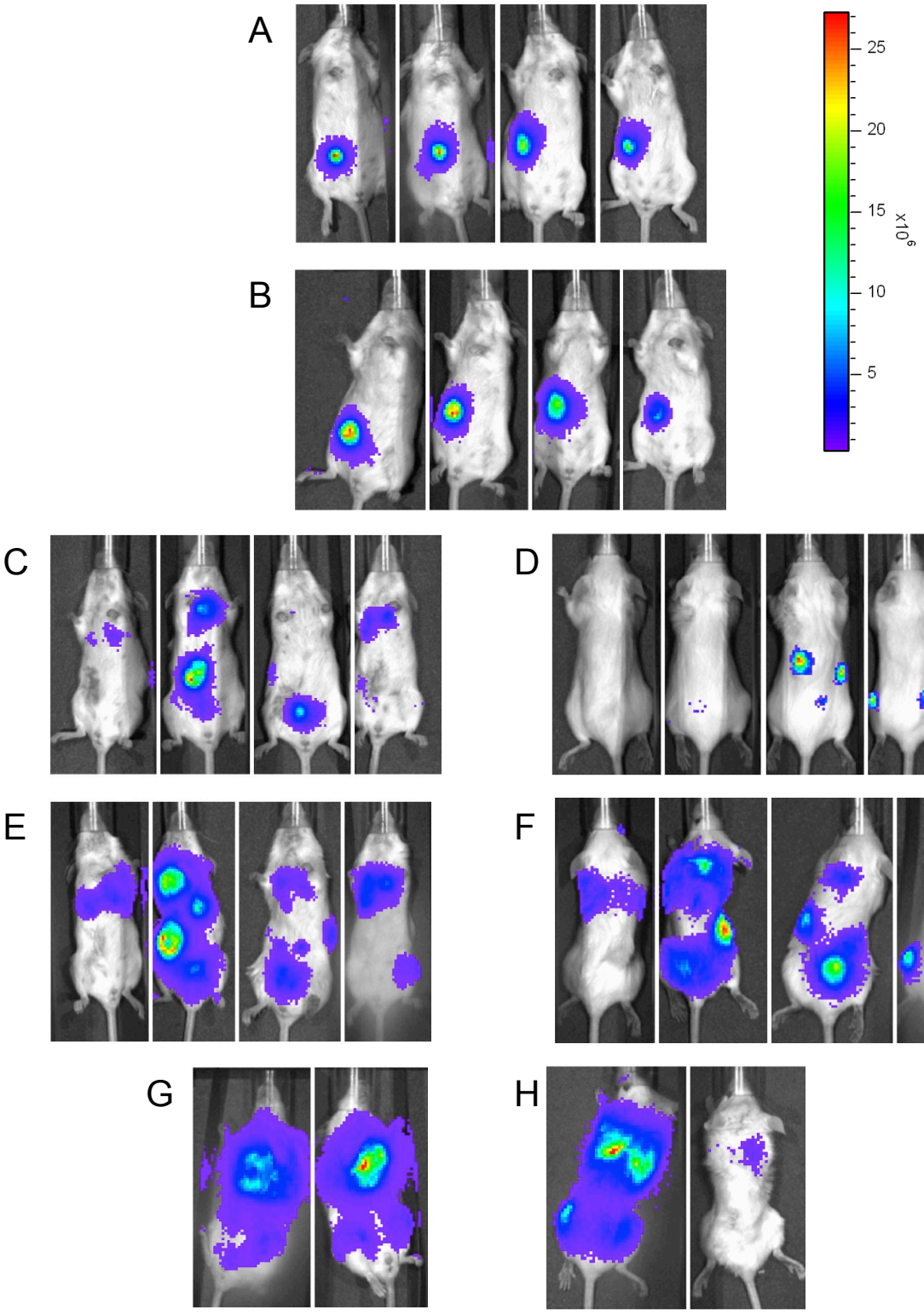


Figure 5.5. Metastases evident after tumor resection. 50,000 4T1-luc cells were injected subcutaneously into the mammary fat pad of BALB/cJ mice. Tumors were resected on day 14 and subsequent metastatic growth was observed by BLI. A) 7 days B) 14 days C) 28 days ventral view D) 28 days dorsal view E) 35 days ventral view F) 35 days dorsal view G) 41 days ventral view H) 41 days dorsal view. Scale bar shows flux intensity in p/s/cm^2/sr.

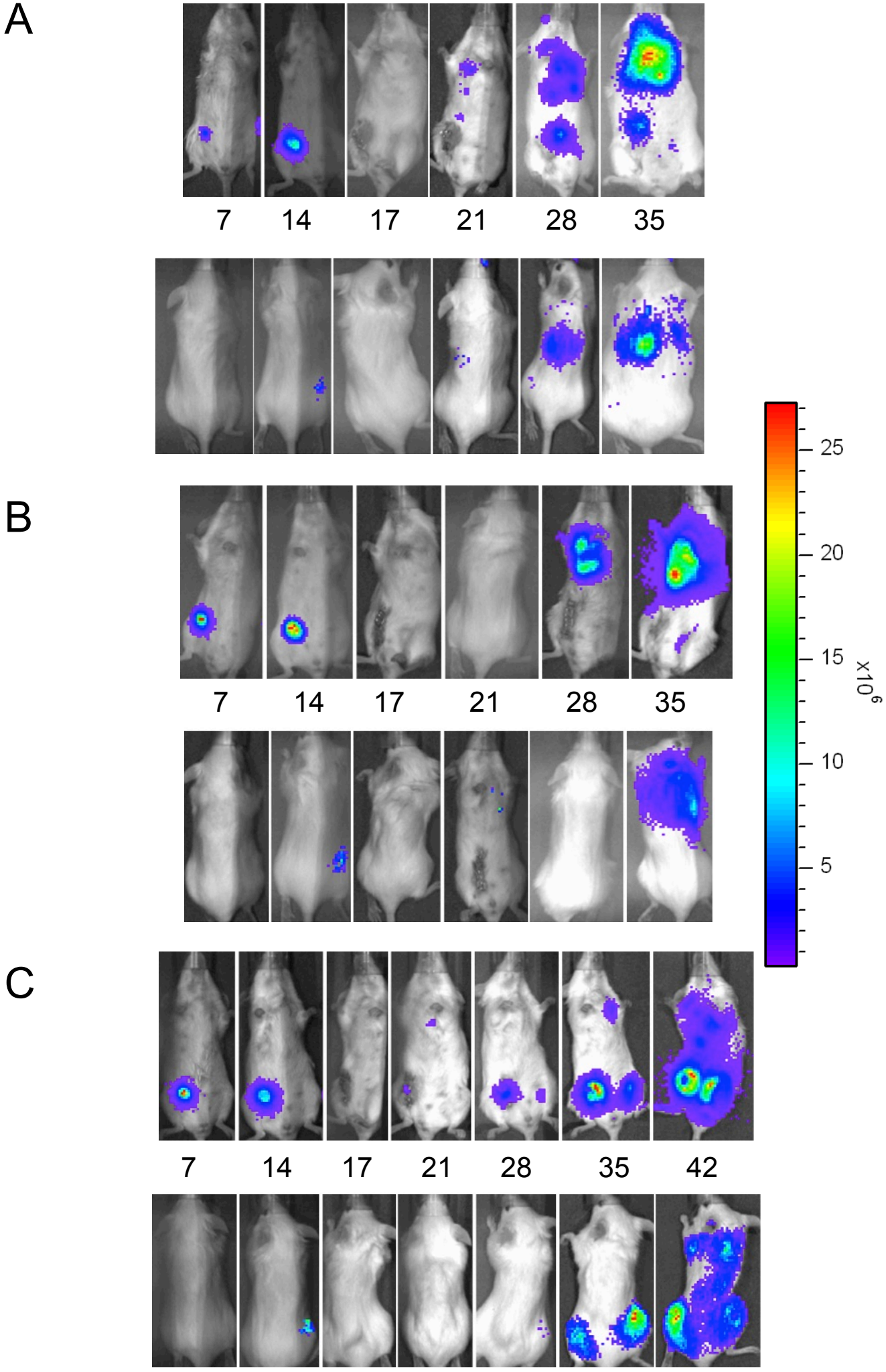
5.3.3 EST liposome treatment provided no survival benefit

Mice were injected with 50,000 4T1-luc cells and tumors were allowed to develop. Mice were divided into 5 groups and treated using the timeline described previously (Materials and Methods). Groups are shown in Table 5.1 below.

Table 5.1. Group Treatment types

Group	Treatment
1	Liposome Buffer
2	Early sTRAIL
3	Early EST Liposomes
4	Late sTRAIL
5	Late EST Liposomes

Tumors were resected after 14 days and subsequent tumor and metastases growth was tracked over time with BLI (Figure 5.6). The total flux from BLI images was calculated using Living Image software (Figure 5.7). At day 35, the BLI flux for the buffer group peaked and was shown to be significantly different than both EST liposome groups using two-way ANOVA. The endpoint analysis for this study was overall survival (OS). While there were some differences in BLI intensity, no difference was seen in the survival curves between the groups (Figure 5.8).


A
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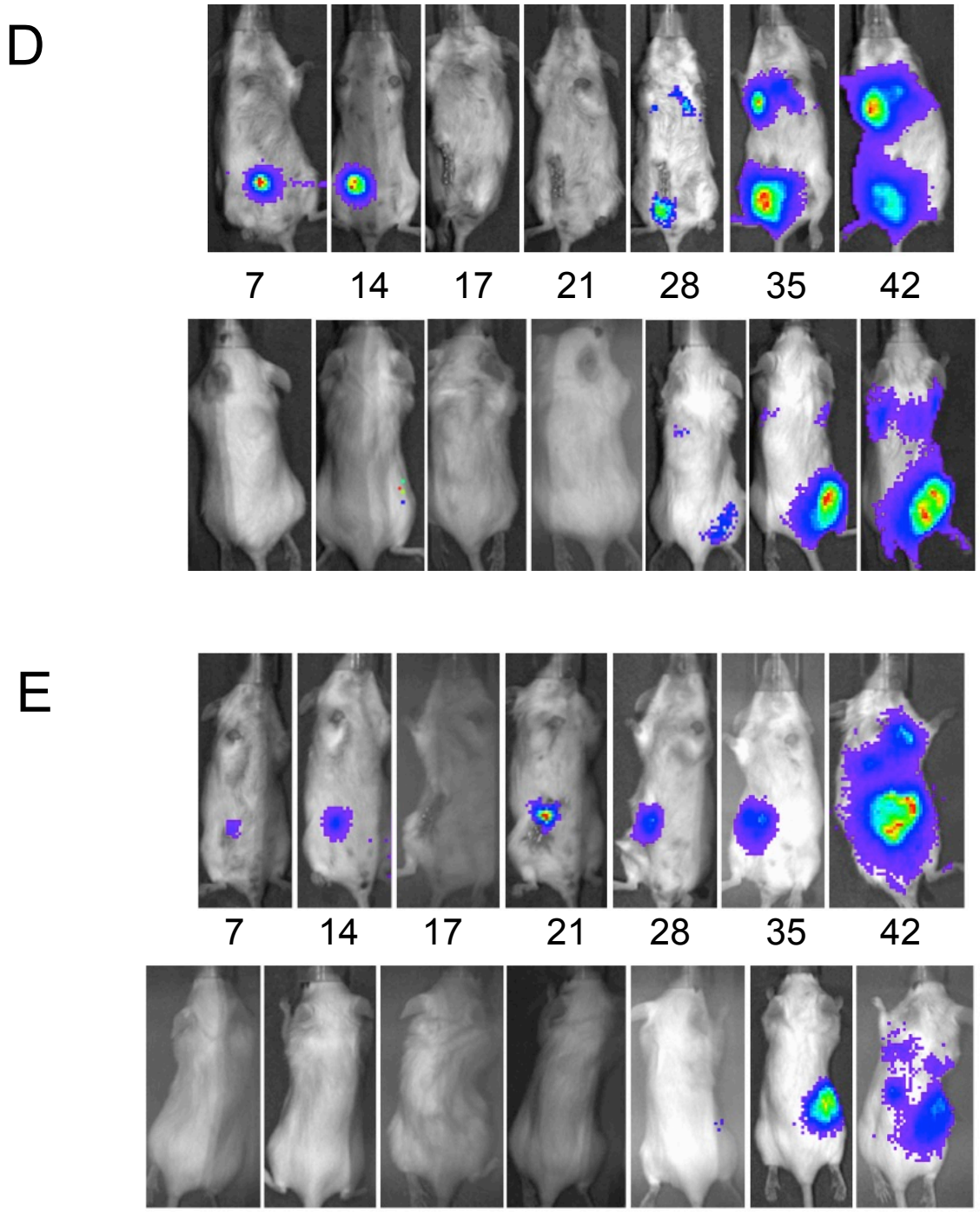

Figure 5.6. Tumor progression independent of treatment. The spread of metastases after tumor resection under different treatment conditions was tracked using BLI. Representative images from groups A) 1 B) 2 C) 3 D) 4 E) 5 are shown. Numbers indicate number of days post-injection when images were captured. Scale bar shows flux intensity in p/s/cm^2/sr.

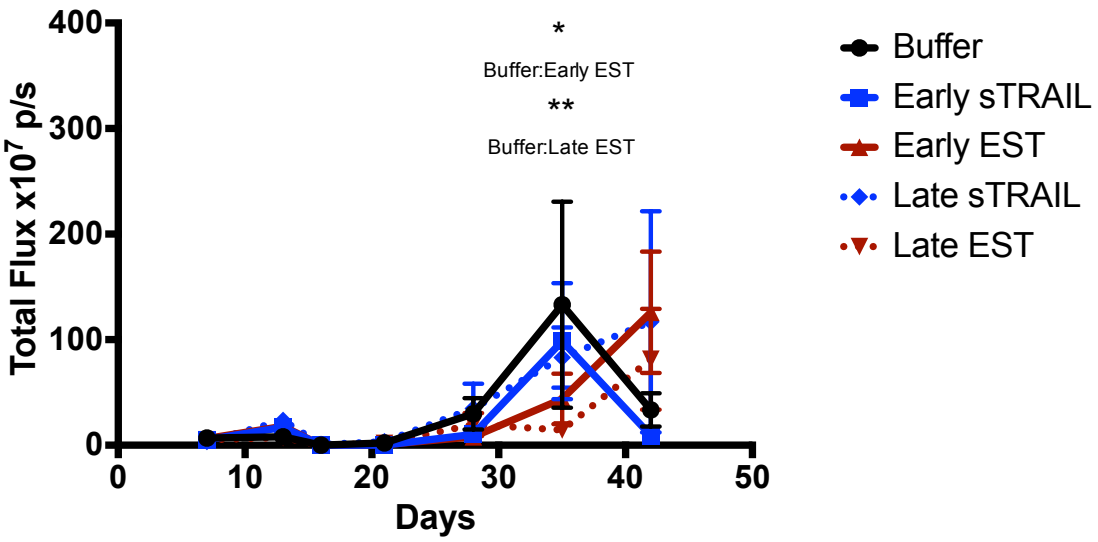

Figure 5.7. Quantitative analysis of BLI images. Total flux from BLI images of each mouse was calculated using Living Image software. The mean (n=6-8) was graphed and error is shown as standard error of the mean. Significance was determined using two-way ANOVA. * $p<0.05$, ** $p<0.01$

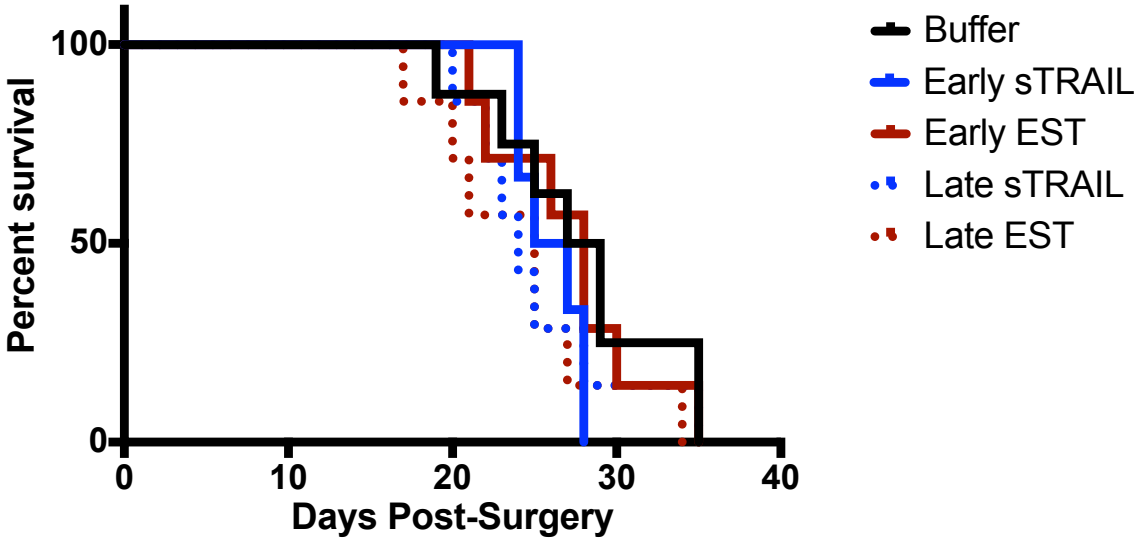

Figure 5.8. EST liposome treatment shows no survival advantage. Mice were closely monitored after tumor resection and adjuvant treatment. Day of death/euthanasia was recorded and survival curves are shown. There was no significant difference between the curves from any treatment.

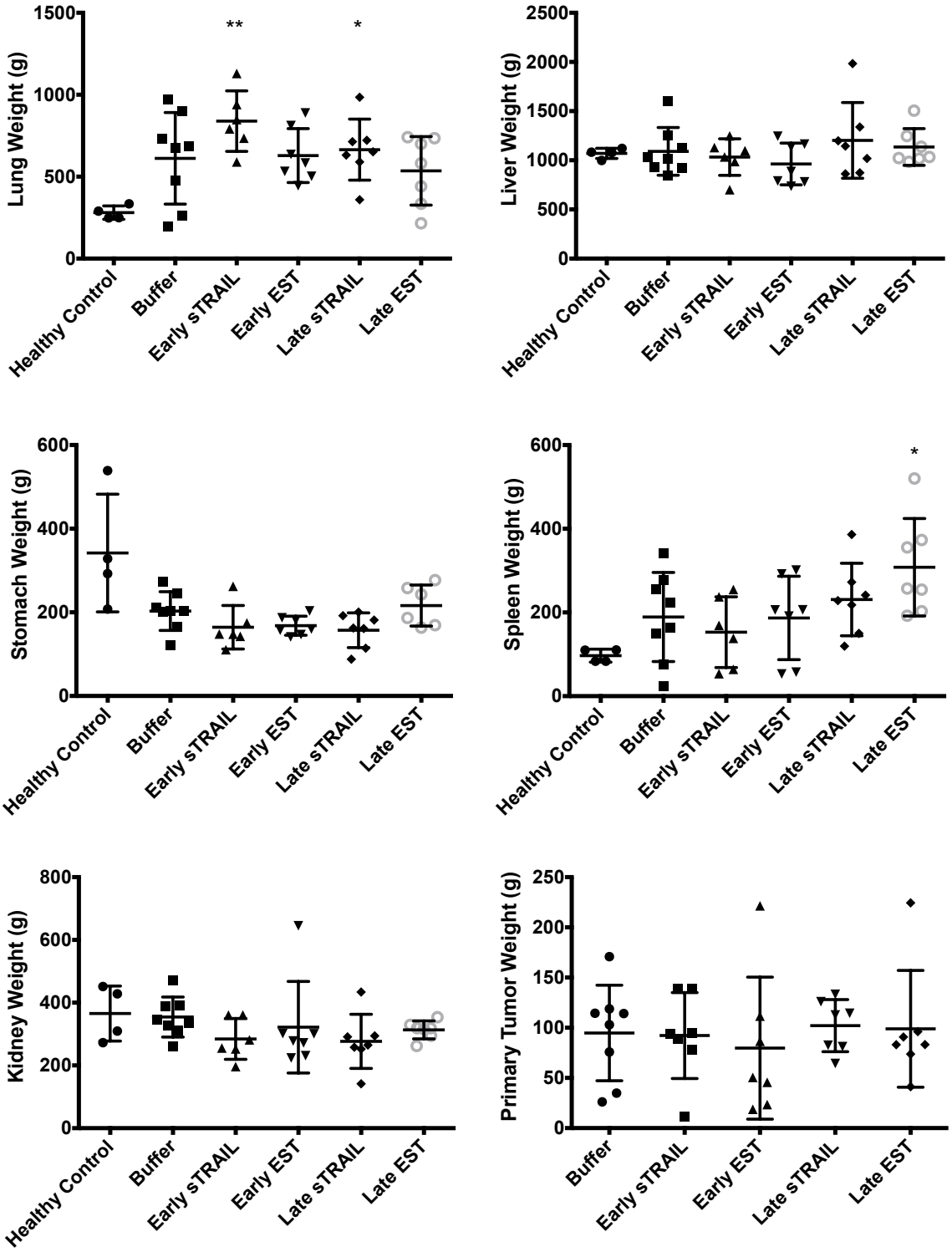

Figure 5.9. Organ weights showed some difference compared to healthy controls. Upon death/resection, organs were removed and weighed. Average weights were compared to healthy control mice that received no treatment or tumor cell injection. Significance was determined using a one-way ANOVA. * indicates $p< 0.05$, ** indicates $p<0.01$.

5.3.4 Injection of EST liposomes showed no toxicity

We previously showed that EST liposome treatment caused no detectable toxicity in immunocompromised mice (25). This is the first study to administer EST liposomes to immunocompetent mice. We hypothesized that we would also not detect any toxicity. BALB/cJ mice with no tumors were treated following the same treatment procedure as outlined above. One week after the last dose, mice were sacrificed and subjected to a number of toxicity tests. Organs were harvested and weighed. Organ weights showed no significant difference based on treatment (Figure 5.10). Blood plasma was isolated and tested for liver enzyme levels. Again, no significant difference was seen between treatment groups (Figure 5.11). Lastly, we followed the animal weight of mice in experimental groups for the same time period as toxicity study. While there was significant difference between the early EST liposome treatment group and the buffer treatment group, there was no difference between starting and ending weights of any of the treatment groups (Figure 5.12).

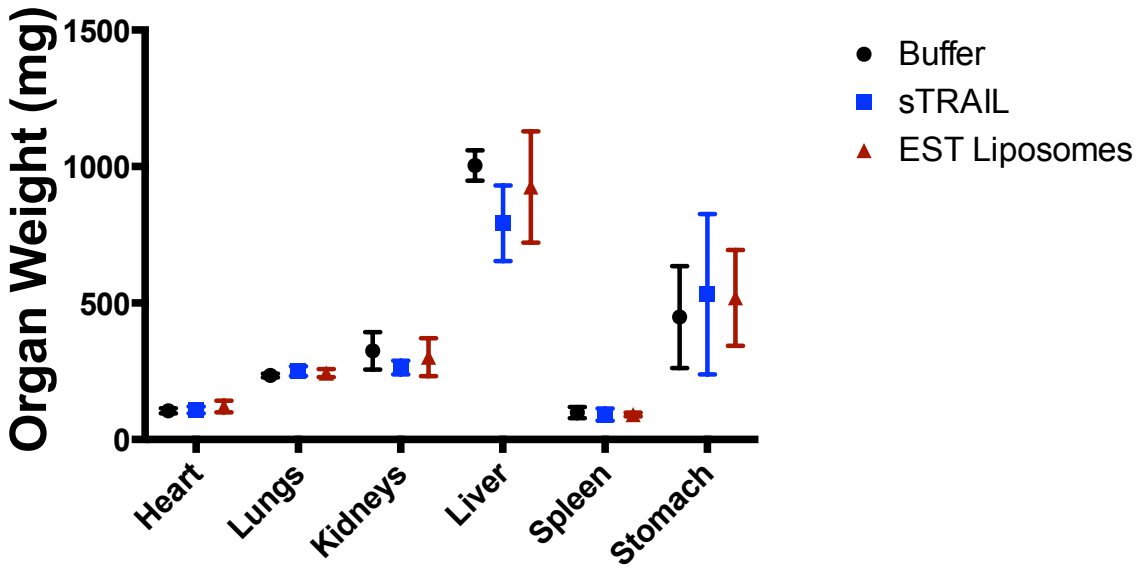

Figure 5.10. No significant difference in organ weights between treatment groups. Mice with no tumors were treated using same protocol as experimental groups. One week after final dose, mice were sacrificed and organs were harvested and weighed. Data shown is average ± standard deviation (n=2). Significance was determined using two-way ANOVA.

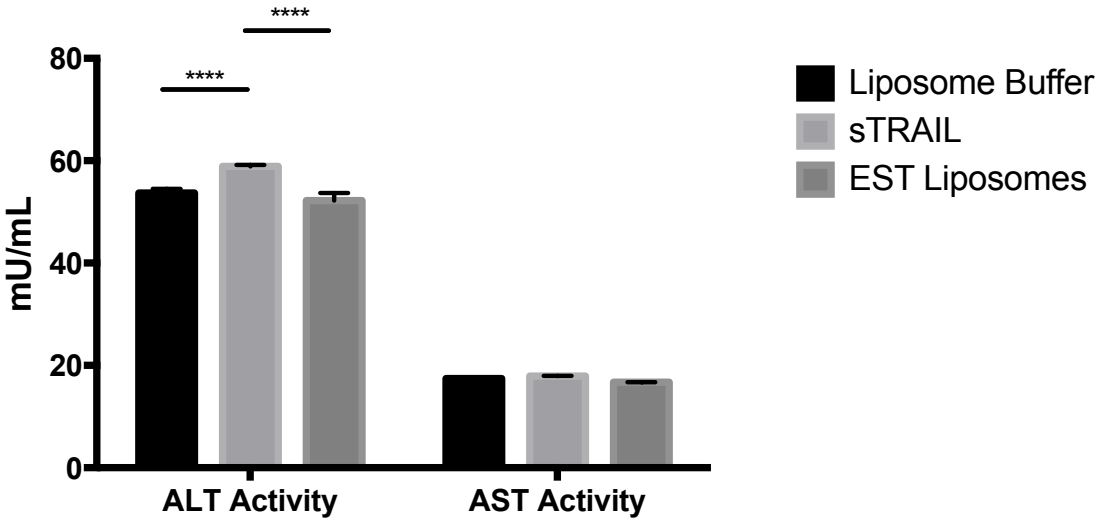

Figure 5.11. EST liposome treatment caused no significant difference in liver enzyme measurements. TRAIL is known to be toxic to hepatocytes. In order to determine liver health, plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in mice treated with liposome buffer, sTRAIL, or EST liposomes. sTRAIL was shown to have significantly higher levels of ALT than both buffer and liposomal treatment. AST levels did not change with any treatment. Data is representative of n=2-3 replicates with error bars of standard deviation. ****p<0.001.

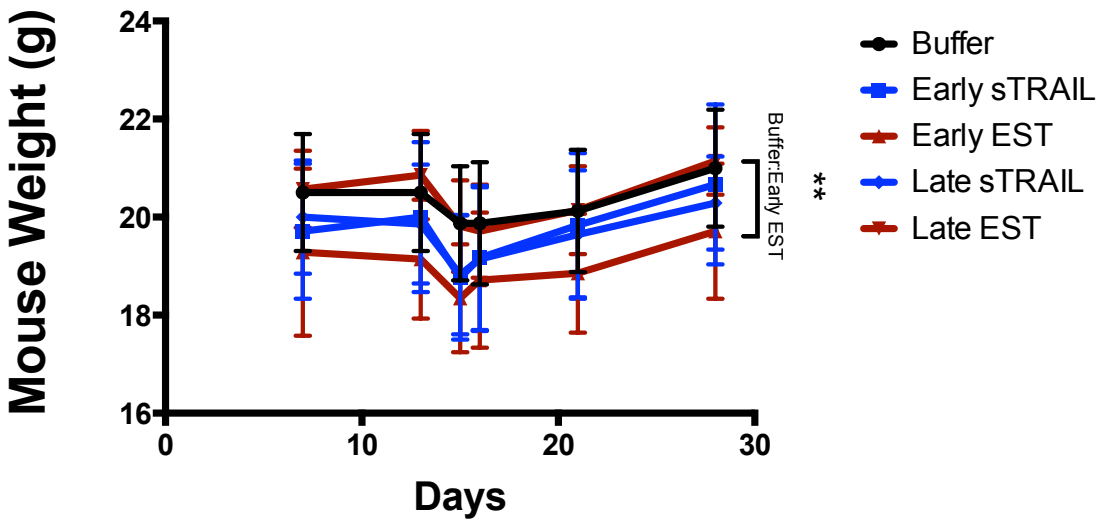

Figure 5.12. Animal weight stayed consistent over treatment. Animal weight was tracked over tumor growth/resection/treatment. Data shown is average of n=6-8 animals, with error represented by standard deviation. Significance was determined using two-way ANOVA where ** is $p<0.01$.

5.4 Discussion

Cancer metastasis accounts for 90% of all cancer fatalities (1), but the vast majority of cancer therapeutics target the primary tumor. We recently presented work on the first therapeutic that directly targets circulating tumor cells (24). The therapeutic works by adhering to circulating leukocytes that then interact with CTCs, bringing them in contact with TRAIL proteins on the surface of the drug. The liposomes eliminated over 95% of CTC *in vivo*. Here, we present the first study of EST liposomal treatment in combination with tumor resection.

Recombinant TRAIL therapies have been limited in the clinical setting due to hepatocyte toxicity caused by aggregation of TRAIL proteins. His-tagged TRAIL in particular has been shown to create supramolecule aggregates (26,27). In this study, we used recombinant TRAIL, but these EST liposomes presented no toxicity in immunocompetent mice reflected by no decrease in weight (Figure 5.12) or

discrepencies in organ weights (Figure 5.10). Moreover, there was no noticeable hepatocyte toxicity indicated by a lack of difference in liver enzyme levels between the control and treatment mice (Figure 5.11). The lack of toxicity is likely because the TRAIL molecules are bound to the liposomal membrane and therefore unable to aggregate. Other approaches to combat TRAIL toxicity include the creation of fusion proteins using small chain variable fragments (scFv) directed towards cancer cells or immune cells (28). This approach holds promise with our current strategy as well. We are currently exploring the creation of an E-selectin/TRAIL fusion protein. This would potentially minimize the variability of therapeutic from lot to lot. Additionally, the shelf life of the therapeutic may be increased due to the oxidation potential of liposomes.

While the therapeutic showed great promise on the toxicity side, the therapeutic advantage was not as pronounced. In this study, we explored how perisurgical administration of liposomes impacted the metastatic spread of breast cancer after tumor resection. EST liposomes did provide decreased metastatic burden at one time point when compared to mice treated with buffer control (Figures 5.6, 5.7), but EST liposome treatment also provided no survival benefit (Figure 5.8). This is the first *in vivo* study involving EST liposomes that showed no therapeutic benefit (24,25). It is believed that the lack of benefit may be due to the short administration timeline of therapy. When used to treat prostate cancer, the liposomes were administered every 3 days for a total of 6 weeks. We hypothesize that with a less minimal therapy schedule, therapeutic advantage may be seen in the tumor resection model.

EST liposomes may hold promise as a cancer therapeutic. This is the first therapeutic that directly targets CTC and has been shown to eliminate over 90% of CTC *in vivo* as well as decreased metastatic burden in a prostate xenograft model (25). Here, we showed that while minimal administration of EST liposomes perisurgically does not increase OS, liposomes do not cause toxicity in immunocompetent mice. More studies need to be conducted before the full clinical utility of EST liposomal therapy can be evaluated.

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Chapter 6

Conclusions and Future Directions

6.1 Concluding Remarks

Cancer research has been greatly improved in recent history by the adaptation of techniques, which reflect the real-life organization and heterogeneity of the disease. Instead of studying 2D cultures of purely cancerous epithelial cells, researchers now work in 3D orientations and incorporate a variety of supporting cells into cancer experiments. Most of this work though focuses primarily on primary tumors and solid metastases. Work on the transitional metastatic state, namely circulating tumor cells, is still in its infancy.

To date, CTC research has made great strides in identifying markers for subsets of CTC (1–5), relating prognosis to CTC count (6,7), and drawing conclusions on therapies and surgical techniques due to changes in CTC numbers (8–11). In Chapters 2 and 3, we added to this wealth of knowledge by introducing a unique broad application diagnostic for drug screening using peripheral blood treatment and subsequently showing that primary CTC can be killed by TRAIL-therapeutics. These studies also present some of the first studies of CTC *in situ*. Most CTC work is performed on isolated CTC, which may give false positives due to the harsh nature of isolation.

Just as the interaction between epithelial tumor cells and supporting cells, such as stromal and immune cells, is pivotal for the progression of primary tumors, in order for CTC to survive and successfully metastasize, they must interact with various cell types within the vasculature (12). Many hematogenous metastasis studies have explored the adhesive interactions between CTC and the endothelium (13–17). Recently, these adhesive events were confirmed using primary CTC from peripheral blood (18). The interaction between CTC and immune cells are also of upmost importance. We previously showed that this interaction can be used for therapeutic advantage by developing an immunotherapeutic that binds to the surface of leukocytes

and induces apoptosis upon immune cell:CTC binding (19). In Chapter 4, we introduce another CTC:supporting cell interaction that may play a role in metastasis. We showed that cancer-associated fibroblasts migrate with CTC in primary human blood. This work also introduced the first isolation technique to directly isolate cCAF from peripheral blood. Because of the heterogeneity of CAF in primary tumors (20), the direct study of cCAF is important to fully understanding their impact on metastasis and tumor progression. This isolation technique will allow for extensive characterization of the protein and gene signatures of cCAF.

6.2 Future Directions

The work presented in this paper introduced a number of new techniques for studying metastasis using circulating tumor cells. First, we introduced a novel diagnostic platform proposed to identify drug sensitivity prior to administration. The study presented here was merely a proof of concept. In order for the clinical utility of this technique to be confirmed, a matched study could be performed that would directly predict the drug sensitivity of patients, which could then be compared to the clinical response of the patients to the drug.

We also presented a technique to isolate cCAF from human blood. While it has extensively been shown that cCAF contribute to many areas of tumor progression including invasion, angiogenesis, immune evasion, and growth (21), the direct effect these cells have on cancer cells in the bloodstream has not been shown. Here we presented an initial study showing cCAF may protect CTC from high shear conditions. The direct mechanism of this action is currently being studied. Additionally, the effect

of cCAF presence on drug sensitivity under shear conditions, namely TRAIL sensitivity of CTC is also being studied.

cCAF presence may have a direct effect on the effectiveness of our EST liposomes as well. This will be explored in the future. Additionally, the functionalization of the liposomes could be altered to target cCAF using antibodies against fibroblast activation protein (FAP) or platelet derived growth factor-α/β.

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Appendix A

Commentary on Therapeutic Targeting of Circulating Tumor Cells

Portions of this appendix were adapted from:

Marshall JR, King MR. Therapeutic Targeting of Circulating Tumor Cells: An Important Problem That Deserves Careful Study. Cell Mol Bioeng [Internet]. NIH Public Access; 2015 Dec [cited 2016 Oct 20];8(4):527–9.

In cancer research, the discovery and study of circulating tumor cells (CTCs) has seemed to open a world of possibilities. We now have the potential to gain understanding of individual cases of metastatic cancer without invasive procedures. This area of research is, however, not without some basic pitfalls. In this commentary, we address some of these pitfalls by discussing two recent examples in the published literature and discuss ways to overcome their limitations in the hopes of educating those who may be entering the growing field of CTC research. Careful research design should always be followed to prevent incomplete or misleading studies from entering the literature, and thereby avoid setting back this burgeoning field.

Two recent CTC papers appearing in the journal PLOS ONE can serve as examples of preliminary studies lacking the necessary controls to yield meaningful results. In the first, Kim and Gaitas outline a creative application of photodynamic therapy (PDT) intended to prevent cancer metastasis, in which tubing could be attached to a patient's vasculature to divert blood into an external device where it would be exposed to LED illumination to induce cancer cell death [5]. The illumination, combined with a photosensitizer conjugated to an antibody to CD44, a cell adhesion protein found on many cancer cells, is intended to eliminate CTCs in the blood. This study attempts to examine the feasibility of this technique *in vitro*. Upon first reading the results of the paper might seem promising, with the vast majority of prostate cancer cells spiked into whole blood dying within an hour after treatment; however, the authors overlooked key aspects of treatment in blood that, when considered, significantly weaken the overall impact of the paper.

When critically evaluating this proposed therapy, one must first look at the feasibility of treating the entire blood volume in a realistic time frame. Limitations of throughput are a common issue in proposed CTC technologies. Most devices test a small volume of blood and while the information gleaned from this volume can be of scientific interest [4,10,14,15], the scale-up of such a device becomes more complicated. The scale-up of the PDT device to treat an adult blood volume (~5L) in this case does seem feasible, however. One may calculate that at a physiological blood velocity typical of diameter of the 1 mm internal diameter tubing used (~30 cm/s), 36 meters of tubing would be needed to achieve their prescribed 2 min of LED light exposure and thus the entire blood volume could be processed within about 6 hours. This amount of tubing would contain a blood volume of 28 mL, also feasible. A single or double layer of this tubing could, in theory, be arranged in a small bedside or even portable extracorporeal device. Unfortunately no analysis of this form was discussed in the 2015 paper presenting the technology[5].

Importantly, one must also consider the proposed therapy's effect on the normal blood cells, which would likewise also receive exposure to the photodynamic sensitizer and light source. This is most problematic issue with the proposed technology. The authors chose CD44 as the molecular target for the photosensitizer therapy. While CD44 expression is well documented in cancer [3,6,7,11], it is also a major adhesion molecule for leukocytes [8,18]. CD44 is also known as phagocytic glycoprotein-1, and is a receptor for hyaluronic acid. The implication of this, of course, is that while the photosensitizer may target CTCs, leukocytes will almost certainly become bound to the sensitizer and also killed during light treatment. Furthermore, even if a more appropriate antigen was

selected to better avoid off-target binding, the reactive oxygen species (ROS) generated by the therapy would still be likely to effect surrounding blood cells and accumulate within the plasma during the course of treatment. The authors state that the diffusion distance of ROS is small (~100 nm, reportedly) and that ROS generated on the surface of CTCs should not reach neighboring blood cells, and while this might hold true under static conditions, under dynamic blood flow conditions it is the much greater *convective* length scale that must be considered when estimating whether ROS generated in the vicinity of one cell surface may affect neighboring cells. The chaotic mixing of bulk blood flow nearly ensures that all cells within the peripheral blood flowing through the device will be equally exposed to extracellularly generated ROS. Remarkably, Kim and Gaitas demonstrated the ability to kill PC-3 cancer cells within spiked blood samples, but never measured whether the PDT approach was also killing blood cells at the same rate, a minimum requirement of any selective therapy designed to act in the bloodstream [5]. In our opinion, this important control measurement should be a necessary precursor to publication in a peer-reviewed research journal, and the absence of basic measurements such as these cast the overall conclusions into considerable doubt. The authors used the loss of fluorescence signal from PC-3 prostate cancer cells labeled with the live cell dye CellTracker as their sole measure of cell death, whereas an additional assay of apoptosis and necrosis such as Annexin V, TUNEL , or MTT is recommended.

Finally, whenever light is used to induce a cellular response, one must also be concerned with the effects of heating. This is particularly important in the blood since red blood cells will undergo lysis at temperatures above 45 degrees Celsius [2].

Anywhere from 60-95% of the energy of LEDs is converted to heat. This heat is generally believed to not be given off as infrared heat and therefore may not affect the sample directly [17]. However, there have been reports of IR heat production and resulting sample heating by LEDs, an effect that is dependent on the power used and the type of heat sink [12]. It may thus be possible to avoid this issue through careful engineering, but heating should still have been considered and tested in the paper (it wasn't). All of the PDT experiments of Kim and Gaitas were performed in a foil-lined Styrofoam insulated cooler, using sealed, static well plates and blood-filled tubing sealed on both ends. Despite the diagram of their Figure 1, no flow experiments were performed [5].

The majority of current CTC research focuses on the isolation and enumeration of cells captured from patient blood samples for analysis. While this work does not directly involve the toxicity of reagents on either cancer or blood cells, it nevertheless holds its own pitfalls and issues that, if are not properly addressed, can skew results and thereby lead to misleading conclusions.

One example of this is another paper by Gaitas and Kim published recently in PLOS ONE [1]. The authors present a method for isolating CTCs in a microtube device coated with antibody against epithelial cell adhesion molecule (EpCAM). The authors showed that they were able to capture 85% of EpCAM-expressing cancer cells suspended in buffer, and 44% of the cancer cells from whole blood spiked with cancer cells. While this method shares similarities with other EpCAM-antibody isolation approaches previously described in the literature [4,13,15,16], the basic idea presented is straightforward and would likely not encounter major challenges.

However, as with the previously discussed paper by Kim and Gaitas, some much needed control experiments and other considerations were neglected, seriously compromising the soundness of the study.

First, the device is presented as a dynamic microfluidic device intended to capture cells from the bloodstream of patients to achieve a therapeutic filtering of the blood, however the only experiments performed in the study were under static (i.e., no flow) conditions. It is likely that the addition of shear force would affect the capture efficiency of the device and this is something that should have been addressed to support the conclusions of the paper.

Second, the authors again failed to take the blood cells into consideration. In other CTC isolation studies, the capture purity as well as the capture efficiency is reported [4,9,14]. This is important for two reasons. First, if too many white blood cells adhere to the device, they will block the capture of further cancer cells, thereby decreasing the capture efficiency. This is especially important for the device discussed in the Gaitas and Kim paper, as they are suggesting that this technology could be used as a blood-filtering device for metastatic cancer patients. Such a device would process many more white blood cells than cancer cells, and over time the device would likely become less and less efficient due to nonspecific adhesion of leukocytes. The purity is equally important for post-isolation processing. Certain techniques that involve DNA and RNA analysis require very pure samples to detect cancer-associated mutations without excessive contamination from blood cell genetic material. For these reasons, the achieved purity is always reported for new CTC isolation devices.

We were quite surprised that Gaitas and Kim only reported the number of cancer cells captured by their antibody-coated tubing and did not attempt to assess the number of leukocytes captured, either qualitatively with a bright field or phase contrast micrograph to accompany the fluorescence image showing EpCAM+ capture, or quantitatively using a hemocytometer or flow cytometer. We are even more surprised that such a preliminary effort would be accepted for publication in a reputable journal such as PLOS ONE. Surely a study whose abstract's first sentence promises a "tube to selectively capture circulating tumor cells" should show some measure of selectively within its pages? Perhaps the bright field images showed a degree of indiscriminate cell capture which contradicted the stated goals of the study.

As the title of this commentary states, the development of new technology to study (and treat) circulating tumor cells is sorely needed, but it must be carried out with sufficient rigor to advance the field. Authors, reviewers, and editors should work together to avoid flooding the literature with hastily done, inadequate work that can act more to mislead and confuse rather than enlighten. We also hope that our commentary can serve a more productive purpose than simply singling out one laboratory that is new to the CTC field, by pointing out some of the common pitfalls and necessary considerations for researchers to be aware of. Overall, we are encouraged that the field of CTC research is growing and that more innovative ideas are being proposed, such as blood-filtering devices and new CTC-targeted therapies. These ideas are vital for the advancement of cancer metastasis research and treatment. By addressing common issues such as those presented in this commentary, we believe that CTC research can become a more important area of cancer investigation. We hope

those who are just entering the field can draw inspiration and advice from this commentary and we look forward to seeing what technologies the next wave of CTC research will bring.

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Appendix B

Commentary on Circulating Tumor Cells and Surgical Intervention

Portions of this appendix were adapted from:

Marshall JR, King MR. Surgical intervention and circulating tumor cell count: a commentary. Transl Cancer Res. 2016;5(1):S126–8.

Surgical resection of tumors is a common practice in breast, lung, melanoma, and many other cancers, and is known to extend life expectancy significantly. However, recurrence and metastasis are still frequently seen post-resection. Distant metastasis occurs when cells from the primary tumor enter the bloodstream, adhere to the endothelium, infiltrate a distant site and proliferate. The number of circulating tumor cells (CTCs) in the vasculature has been shown to correlate with patient survival and prognosis(1). CTC count perioperatively has been under investigation to determine whether surgical procedures introduce additional CTCs into the bloodstream. While this postsurgical CTC increase has been observed for various cancer types, many studies have shown that CTC counts normalize and often decrease after surgery(2). Still, the long-term effects on progression and survival of surgical release of CTCs have not been definitively determined. In this commentary, we discuss the prospect of minimizing surgical CTC increases using less invasive techniques as well as the need for more aggressive perioperative targeting of CTCs.

While the first CTCs were observed in the 1800s(3), the importance of CTC presence in cancer and its potential impact in cancer treatment have only recently been recognized. Early CTC research focused primarily on the isolation and enumeration of CTCs in different cancer types. Currently, studies have expanded to include the exploration of the use of CTCs in early diagnostic tests(4) as well as the development of anti-metastatic therapeutics(5–7).

One area of research that may have far reaching implications in cancer treatment is the relationship between surgical technique and CTC count. Many studies have shown

that common methods used for diagnosis (biopsy) and treatment (resection) of cancer can lead to bloodborne tumor cell dissemination. In one study, The Zharov lab showed that while mechanical palpation of breast tumors did not increase CTC counts in mice, tumor biopsy and resection did(8). Moreover, lung resection was shown to increase CTC count, where the presence of CTC clusters correlated with worse prognosis(9). Bayarria-Lara et al. show that CTC counts decrease one month after lung resection, though the presence of CTC after surgery was associated with early recurrence(10).

In a recent study published in *Investigative Urology*(11), Kauffman and associates investigated whether robotic assisted laparoscopic radical prostatectomy (RALRP) reduced CTC introduction in comparison to past studies conducted on open prostatectomy. They showed that RALRP did not significantly increase CTC numbers in patients, whereas past studies of open prostatectomy based on RT-PCR amplification of epithelial markers in blood were consistent with CTC increases. In the study, blood samples were drawn from 25 patients preoperatively as well as intraoperatively. Using EpCAM-positive selection, 48% of patients were shown to be CTC-positive preoperatively while 52% of patients were CTC-positive after surgery(11). Perioperative increases and decreases in CTC count were observed at the same frequency, and increases were found to never exceed 1 CTC per 8 mL blood(11). It is suggested that RALRP may hold an advantage to open prostatectomy due to the lack of CTC introduction(11).

Similar results have been obtained in studies focused on other cancer types. In esophageal cancer, minimally invasive esophagectomy showed lower intra- and post-operative CTC counts than open esophagectomy(12). Video-assisted lobectomy also yielded fewer CTCs than open thoracic surgery for the resection of lung cancer(13). However, the impact of the additional CTCs introduced is debated. Several reports have demonstrated a correlation between increased CTC numbers postoperatively and worse prognosis in lung, colon, and stomach cancers(14–16), while one study in pancreatic cancer shows no such relationship(17). In fact, reports show that the increase of CTC after surgical procedures normalizes over time, sometimes resulting in lower CTC counts than preoperatively(2,8,10,18). The eventual fate of these observed CTCs is of course unknown. Reports of this nature compel the need for further analysis of the correlation between surgical technique and cancer progression. In addition, methods to decrease CTC frequency during surgery should be investigated, including therapeutic agents to target CTCs.

Most methods for cancer treatment focus on the eradication and shrinking the primary tumor, even though 90% of cancer fatalities arise from metastasis. Recently, our group developed a therapeutic approach that directly targets CTCs(19). This nanomedicine construct is comprised of phosphatidylcholine liposomes conjugated with E-selectin, a natural endothelial adhesion molecule, as well as TNF-related apoptosis-inducing ligand (TRAIL), a pro-apoptotic ligand whose receptors are upregulated on many cancer cells. The drug acts by adhering to leukocytes or other immune cells within a patient's blood. These cells then interact with CTCs, inducing apoptosis through

TRAIL signaling(19). In pre-clinical studies, E-selectin/TRAIL liposomes were shown to significantly reduce CTC number in colon and prostate cancer models. When introduced into the bloodstream of mice containing colon cancer cells, the TRAIL liposomes decreased CTC count by over 90%(19). In an orthotopic prostate cancer model, CTC counts were found to be 94% lower in mice treated with ES/TRAIL liposomes compared to control mice(7). A therapeutic of this type could hold great promise as an adjuvant treatment when used perioperatively, by preventing the operative increase of CTCs and therefore any adverse downstream effects.

While CTC count surrounding surgical procedures has not been directly implicated in metastasis, it is hypothesized that the introduction of CTCs during surgery may promote cancer progression. This motivates further research to elucidate the correlation between type and timing of surgical intervention, and cancer progression. Moreover, since over 90% of cancer fatalities result from metastasis, a greater emphasis on treatments that target CTCs or disseminated tumor cells is also warranted. It is possible that by minimizing the surgically-induced CTC burst through minimally invasive surgical techniques, as well as by targeting CTCs perioperatively, we may one day decrease the occurrence of metastasis and achieve improved patient outcomes.

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