



Circulating tumor cells (CTCs) from metastatic breast cancer patients linked to decreased immune function and response to treatment

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ABSTRACT

We aimed to examine the use of circulating tumor cells (CTCs) as an effective measure of treatment efficacy and immune system function in metastatic breast cancer patients. CTCs are believed to be indicators of residual disease and thus pose an increased risk of metastasis and poorer outcomes to those patients who are CTC-positive. We obtained peripheral blood samples from 45 patients previously diagnosed with metastatic disease originating in the breast. Using TLR agonists that bind TLR ligands and upregulate immune effects versus unstimulated cells, we calculated a percent specific lysis using chromium-51 assay to illustrate the functional abilities of patient natural killer (NK) cells. We found those with greater than 5 CTCs per 7.5 mL blood had significantly decreased responses by their immune cells when compared with those patients who had 5 CTCs or less. We furthermore found a correlation between disease progression and CTC-positive patients, indicating that those who have a positive test should be closely monitored by their clinician. CTCs represent an exciting new clinical opportunity that will ideally utilize their low invasiveness and quick turnaround time to best benefit clinical scenarios.

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Introduction

Human immunity is an elegant, powerful and complex system that often works in finely tuned mechanisms to protect the body against foreign invaders. However, there are some notable shortcomings of this system that we are still working to elucidate. One of these puzzlements is why some individuals seem to adequately mount an immune response when faced with aberrant or transformed cells and others do not, which can lead to tumor formation. Innate immunity can be defined by the general elimination of any element deemed foreign or alternately, any element not recognized as self (i.e. lacking self-markers). Adaptive immunity comprises antigen-specific immune responses that eliminate the pathogen and provide long-term effects in the form of effector memory cells (Cruse et al., 2004). Natural killer (NK) cells are an important component of immunity that mark any cell lacking the Major Histocompatibility Class (MHC) I marker, found on all nucleated cells, for destruction by programmed cell death (apoptosis). When functioning properly, NK cells protect the host against a vast repertoire of potential harmful agents, including cells having undergone cellular transformation in the process of forming a tumor.

One component of the innate immune system, Toll-like receptors, illustrates the multifaceted role the immune system may play in both protection and promotion of carcinogenesis (Basith et al., 2012; Huang et al.,

2008). It has been reported that mutations in TLR4 causes decreased responsiveness to bacterial pathogens, which can cause increased risk of infection and an increased risk of cancer (Karin et al., 2006; Theodoropoulos et al., 2012). Furthermore, different TLRs have been linked to both positive and negative effects on chemoresistance, tumor progression, and treatment efficacy (Fan and Malik, 2003; Kelly et al., 2006; Liao et al., 2012; Yang et al., 2010). Thus, TLRs have been considered as a possibility for blocking in certain cases to reduce effects of TLR signaling or the use of agonists to increase their signaling effects (Ridnour et al., 2013; So and Ouchi, 2010). TLRs represent a good example of the ability of the immune system to respond to different stimuli, whether their origins are bacterial, viral, or self (Kluwe et al., 2009; Li et al., 2010).

Tumorigenesis is a complex process in which accumulating genetic alterations transform normal cells into malignant ones. For tumor development to occur, the host immune system often loses important cell cycle regulators at the same time the tumor is gaining abilities such as angiogenesis, sustained growth factors, and evasion of apoptosis (Hanahan and Weinberg, 2000). The immune system protects the host from developing primary, nonviral cancer; however, it may also sculpt tumor immunogenicity due to the very fact that these cells have escaped actions by the immune system (Dunn et al., 2004).

Metastasis, or the distant spread of cancerous cells, remains the primary cause of death in cancer patients, which requires collaboration between tumor cells and the tumor microenvironment (Cristofanilli et al., 2004). This microenvironment includes fibroblasts, vascular cells, infiltrating leukocytes, and mesenchymal cells (Harmey et al., 2002). Microenvironments that promote tumor cell growth are rich in extracellular matrix remodeling proteases, have increased presence of pro-survival

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Table 1
Patient characteristics and progression status.

Characteristic	Positive	Negative	Unknown	Progression status
HR*	n = 29 (64.4%)	n = 11 (24.4%)	n = 5 (11.1%)	Progression
HER2*	n = 17 (37.8%)	n = 23 (51.1%)	n = 5 (11.1%)	n = 14 (31.1%)
Surgery	n = 41 (91.1%)	n = 4 (8.9%)	n = 0	Stable
Radiation	n = 19 (42.2%)	n = 26 (57.8%)	n = 0	n = 31 (68.9%)
Chemotherapy**	n = 34 (75.6%)	n = 11 (24.4%)	n = 0	

* Significant ($p < .05$) relationship between progression status and this group was found using the Chi-squared test.** Marginally significant ($.1 < p < .05$) relationship was found between progression status and this group using the Chi-squared test.

signals, and have increased pro-angiogenic signals that cause enhanced proliferation and invasive capacity (Chekhun, 2009; Glynn et al., 2010).

According to the most recent data available from the National Cancer Institute (NCI) (from the years 2008 to 2010), a woman's lifetime risk of being diagnosed with breast cancer is around 12% (or around 1 in 8 women). In those that develop a breast carcinoma, which constitutes the vast majority of breast tumors, distant metastasis beyond regional lymph nodes occurs in around 5% of cases. This represents a dire medical situation, with 5-year survival rates around 24% (NCI statistics, years 2003–2009). Certain factors known to adversely affect patient prognosis include: primary tumors greater than 1 cm, increased lymph node involvement, distant metastasis, increased tumor proliferation, overexpression of HER2/NEU, and family history of disease (Montag and Kumar, 2007).

The widespread occurrence of breast cancer illustrates the complexity of this disease, and that despite ongoing research into treatment optimization, there still remains benefits that could be realized for patients. Currently, conventional treatments for breast carcinomas are chemotherapeutic agents, surgical intervention, and radiotherapy, or some combination thereof. Beyond traditional treatments, biomarkers have emerged as indicators of disease progression, as well as for tailoring patient-specific therapies. These biomarkers include circulating tumor-specific DNA, HER2 receptors on circulating cells, the transcription factor NF- κ B, and body fluid microRNAs (Fehm et al., 2007; Fiegl et al., 2005; Ignatiadis et al., 2011; Nakanishi and Toi, 2005; Patel and Sauter, 2011). Another biomarker of interest is circulating tumor cells (CTCs), which are epithelial tumor markers circulating in the peripheral blood (Lianidou and Markou, 2011; Nadal et al., 2012). CTCs are presently being used in metastatic breast cancer cases to evaluate therapy and examine possible clinicopathological correlations (Alema and Schuur, 2013). Many are currently looking to identify those biomarkers that will most enhance current treatments by supplementation with immunotherapy. The major objective of cancer immunotherapy is to generate an effective antitumor immune response (Igney and Krammer, 2002), which may be measured by NK function. The ultimate goal is to integrate parameters of the host and tumor for optimal disease management and long-lasting immune results (Zitvogel et al., 2008). Analysis of the genetic sequence, molecular structure, and epigenetic observations of tumors in addition to clinical dosing regimens, drug delivery route, and pharmacokinetics will all contribute to overall patient outcome (Bhattacharya and Yusuf, 2012). If achieved, this integration of host and tumor factors would ideally usher in a new era of approaching cancer treatment, but one can also see that there are currently many factors that researchers

are examining. Thus, CTCs emerge as an attractive option as a biomarker, due to easy obtainment and use as a diagnostic tool (Tibbe et al., 2007).

In the present study, we aimed to examine whether a correlation exists between CTCs as markers of both immune function and as a measure of treatment effectiveness in metastatic breast cancer patients. We examined the functional abilities of study participants' NK cells to determine their overall immune function.

Materials and methods

Subjects

The Institutional Review Board at the University of Mississippi Medical Center approved this study. Forty-five patients visiting the Multispecialty Care Cancer Clinic at the Jackson Medical Mall in Jackson, Mississippi were recruited for this study. The attending physician had previously diagnosed each recruited patient with metastatic breast cancer. After obtaining informed consent, the patient's medical record, including age, race, treatment history, diagnoses, staging/pathology, disease progression, and any comorbidities, was documented. Women without documented metastasis or a primary tumor of the breast were not included in this study.

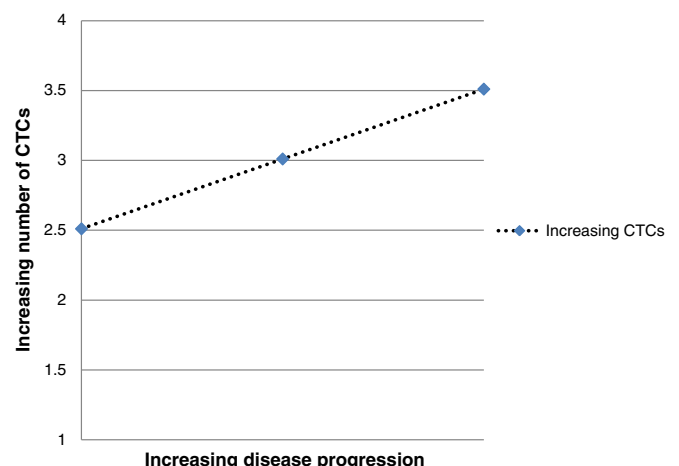
A peripheral blood sample was obtained via venipuncture in a 10.0 mL CellSave tube for CTC analysis and a 6.0 mL sodium-heparin (green top) tube for immune cell analysis.

CTC enumeration

The CellSearch Circulating Epithelial Cell Kit (Veridex, South Raritan, NJ) was used to identify the number of CTCs per 7.5 mL peripheral blood collected in CellSave tubes. Cells that are epithelial cell adhesion molecule (EpCAM)-positive, cytokeratin (CK)-positive, DAPI-positive, and CD45-negative when visualized with fluorescent antibodies are considered a positive CTC. Five or greater CTCs per 7.5 mL whole

Table 2
Progression status and CTCs.

Progression status	CTC group
Progressed	CTC = 0*
n = 14 (31.1%)	n = 29 (64.4%)
Stable	CTC < 5*
n = 31 (68.9%)	n = 6 (13.3%)
CTC > 5*	
n = 10 (22.2%)	

* Significant ($p < .001$) relationship was found between progression status and this group using the Chi-square test and Pearson's r .**Fig. 1.** Correlation between CTCs and disease progression.

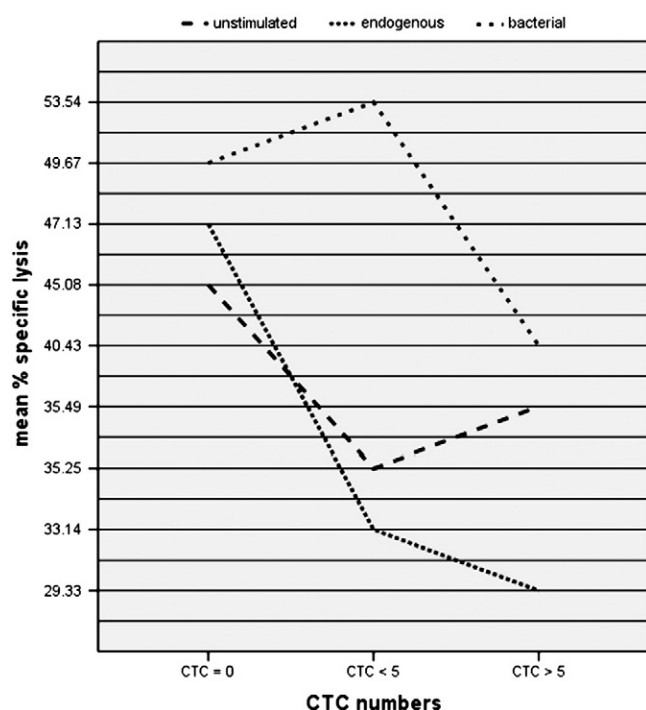


Fig. 2. Mean immune function of CTC groups measured by % specific lysis. The endogenous-stimulated group was found to be statistically significant ($p = .012$) using one-way ANOVA.

blood is considered the threshold at which long term prognosis and survival of breast cancer patients is significantly diminished.

Immune function analysis

Peripheral blood lymphocytes (PBLs) were carefully isolated from the sodium-heparin tube containing peripheral blood using Ficoll-Hypaque centrifugation. The mononuclear cell layer was removed with a sterile transfer pipette and transferred into a sterile tube, which was centrifuged for at least 10 minutes at 3000 RPM at room temperature. The supernatant was discarded and the cell button was resuspended in pre-warmed complete medium (filtered RPMI 1640 supplemented with 10% fetal calf serum (FCS)) (Gibco, Grand Island, NY). Cells were concentrated to be at least 10^5 cells per mL of complete medium.

Isolated PBLs were cultured in RPMI 1640 supplemented with 10% FCS at 37 °C with 5% CO₂. Cells were incubated at a concentration of 3×10^5 cells/well in a 24-well round bottom plate.

To measure the host immune response, one well was left unstimulated, one well was stimulated with an endogenous protein, and one well was stimulated with a bacterial component. Histone proteins (20 µg/mL; Imgenex, San Diego, CA) were used as the endogenous protein, which stimulate TLR2 and TLR4 signaling to cause lasting immune effects. Lipopolysaccharide (10 µg/mL; Invivogen, San Diego, CA) is a component of Gram-negative bacteria that stimulates TLR4. The unstimulated well served as a control compared to the stimulated wells. TLR agonists were chosen because TLRs are expressed on many immune cells and are some of the first responders in the immune response. Further, TLRs have recently been found to have important links to cancer (Gonzalez-Reyes et al., 2010), so elucidation of immune mechanisms was considered pertinent.

To measure immune function, Cr-51 assay was used. The target cell line, K-562 (American Type Culture Collection, Manassas, VA), was grown in suspension in 5% CO₂ at 37 °C. Complete medium for this cell line was made by filtering Iscove's Modified Dulbecco's Medium (IMDM) (ATCC, Manassas, VA) with 10% FCS. Cells were maintained

at a density between 10^5 and 10^6 viable cells/mL complete medium. K562 cells were labeled with Cr-51 (American Radiolabeled Chemicals, Inc., St. Louis, MO) for 90 minutes at 37 °C in 5% CO₂. After the 24 hour culture, PBLs were incubated with target cells at a 20:1 effector to target (E:T) ratio at 37 °C in 5% CO₂ for 4 hours. After incubation, the contents of the wells were microcentrifuged at 1450 RPM for 1 minute. 30 µL of supernatant was collected from each sample and added to 150 µL liquid scintillation cocktail mix (Ultima Gold) (Sigma Aldrich, St. Louis, MO) in a liquid scintillation vial. Samples were counted on a Beckman Coulter (Brea, CA) LS6500 liquid scintillator. Percent specific lysis was calculated as (Experimental release – Spontaneous release) / (Maximum release – Spontaneous release) × (100). Spontaneous release was obtained by labeling target cells with medium alone. Maximum release was determined by incubating target cells with the detergent Triton X-100 (Sigma Aldrich, St. Louis, MO) at a concentration of 10%.

Statistical analysis

Chi-squared analysis was used to evaluate differences in experimental values from observed (population) values. These were further used to confirm findings using Pearson's correlation coefficient r , with a value of -1 representing a completely negative correlation between variables and 1 representing a wholly positive correlation. Independent t -tests were used for evaluating significant differences between disease progression and the unstimulated, the endogenous TLR agonist-stimulated, and the bacterial TLR agonist-stimulated percent specific lysis by the patient's NK cells. One-way ANOVA was used to evaluate differences among and between patients grouped by CTC numbers and their NK response. Post hoc analysis using Tukey's test was performed. For all analyses, $p < .05$ was considered statistically significant. All analyses were performed using IBM (Armonk, NY) SPSS Statistics 19 software.

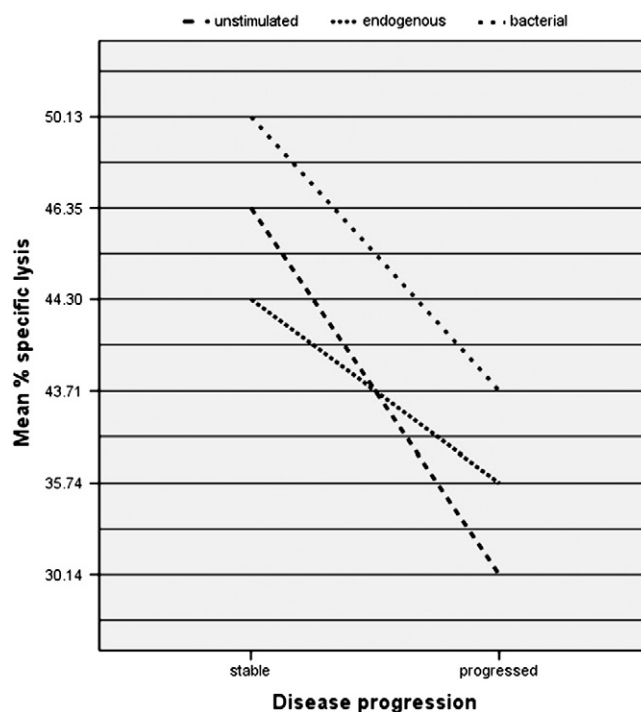


Fig. 3. Immune function of metastatic breast cancer patients measured by % specific lysis and divided into disease progression states. The unstimulated and endogenous-stimulated groups % specific lysis were significantly decreased in progressed patients ($p < .05$, $p = .054$, respectively). The bacterial-stimulated group did not show statistically significant differences ($p = .13$).

Results

Study population

After patients were consented, patient records were used to obtain their hormone receptor (HR) status, HER2 status, any past surgical or radiotherapies, any previous combination of chemotherapeutic agents, and progression status. Disease progression and HR status were found to be related ($p < .05$), as well as HER2 status ($p = .01$). Disease

progression and past chemotherapies were found to be marginally significant ($p = .07$). These results are summarized in Table 1.

CTC numbers and disease progression

The determination of CTCs was next evaluated in relation to disease progression to determine any association between them (Table 2). All three CTC groupings were correlated with progression status ($p < .01$). Table 1 further indicates any significant relationships between CTC

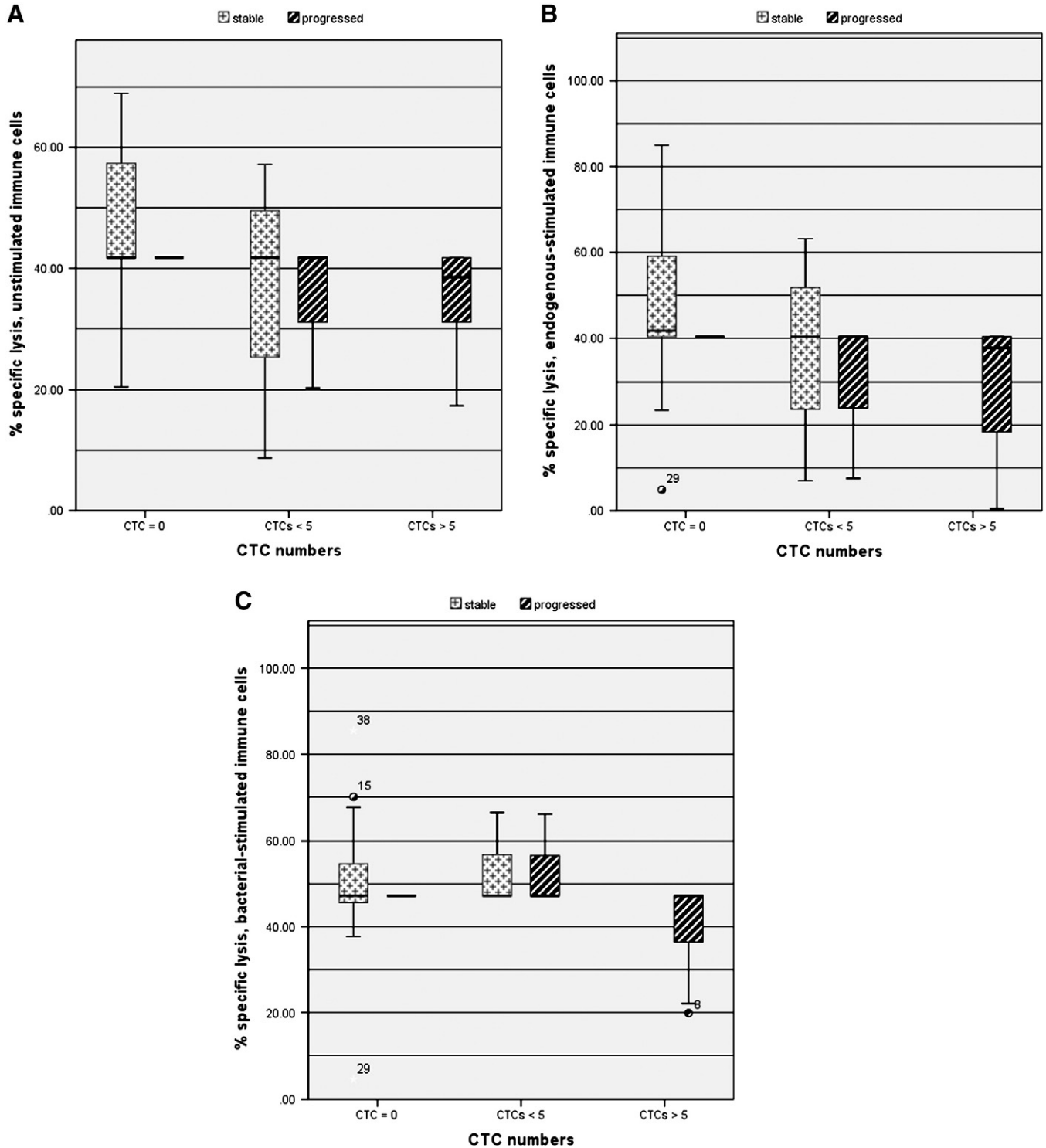


Fig. 4. (A, B, and C) The unstimulated, endogenous-stimulated, and bacterial-stimulated, respectively, responses of patients divided into CTC numbers and then progression status. Tukey's post-hoc analysis showed a significant decrease in the last group's (CTC > 5) response to the endogenous-stimulated ligand compared to the group with 0 CTCs ($p = .02$).

groupings and other patient characteristics. The only significant relationship was found to be CTCs and HER2 ($p = .03$), with HR status being marginally significant ($p = .07$). Fig. 1 shows the correlation between CTCs and disease progression. This line of regression was calculated using Pearson's r and the standard deviations of the means of each respective group.

CTC numbers and NK cell function as measured by radioimmunoassay

As a measure of ability of immune response to potential host or foreign pathogens, their NK percent specific lysis was calculated in response to stimulation with above-mentioned agonists of TLR ligands. There was found to be a significant difference between the mean % specific lysis of the stable versus progressed group in the unstimulated group ($p < .05$) and the endogenous group ($p < .01$) but not the bacterial group ($p > .05$) when measured by an independent t -test. One-way ANOVA analysis showed significant differences between the endogenous-stimulated CTC groups ($p = .012$), with Tukey's post hoc analysis showing that the group with 0 CTCs had a significantly increased response compared to those with > 5 CTCs ($p = .02$). The unstimulated and bacterial-stimulated groups showed marginally significant results ($p = .08$, both). The following figures depict these results: Fig. 2 shows the mean response to each stimulus by CTC grouping, Fig. 3 shows the mean response to each stimulus by progression status, Figs. 4A, B, and C show the % specific lysis and are grouped into both CTC and progression status.

Discussion

The use of CTCs as an independent marker of progression and response to treatment has emerged as a potential tool in cancer patients (Lianidou and Markou, 2011). The low invasiveness and quick interpretation of results allows for direct impact to patient health and future treatment (Swaby and Cristofanilli, 2011). Our findings support the use of CTCs, especially using the threshold number of 5 CTCs or more per 7.5 mL blood, to monitor progression of the disease, in tandem with other conventional tests.

We found that progression status did correlate with CTC positivity, notably more than 5 CTCs per 7.5 mL blood, although previous studies have reported that as little as one CTC causes damaging effects (Bidard et al., 2010). The majority of patients had 0 CTCs ($n = 29$), and the majority of patients also had stable disease ($n = 31$). Those patients showed that progressive disease universally presented with CTCs in their peripheral blood (Allard et al., 2004). This indicates that some process of metastasis likely does cause residual disease or tumor cells to circulate in some bodily process that we are still illuminating (DeNardo et al., 2008). A further direction of research would be to isolate CTCs and characterize them in terms of other markers they may express. Finally, culturing these cells and using real-time PCR to evaluate any gene regulation would provide insight into the complexities of metastasis. For example, one might expect the upregulation of matrix metalloproteinases to digest the extracellular matrix and allow easier migration of tumor cells into the tissues (de Visser and Coussens, 2005; Gonzalez et al., 2007). It is likely that CTCs have an important role in the spread of tumors and those elucidations of this role will only further our understanding of immunity and tumorigenesis.

Furthermore, we found that patients with 5 or more CTCs had reduced immune function in comparison to those with CTCs less than 5 or equal to 0. This is especially pertinent because these patients still have metastatic cancer, and therefore represent a very ill population. Thus, the decreased functional immunity is a sign of worsening health conditions and need for aggressive treatment options. We observed significantly decreased % specific lysis results compared to their counterparts with 4 or less CTCs in the endogenous-stimulated group, and marginally significant results in the other two groups.

Finally, CTCs have important, and almost immediate, significance in that the presence of CTCs results in poorer prognoses, which is evident even after just a few months. This should indicate that aggressive treatment and possible change in chemotherapeutic agent should be considered. The majority of people, even with cancer, do not have CTCs, but of those that do, it appears that it represents a larger dire clinical situation. Thus, it seems that CTCs are a good complement to current tests of cancer patients' health as a gauge of overall metastatic ability (Zhang et al., 2012).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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