ANTICANCER RESEARCH *41*: 661-670 (2021)
doi:10.21873/anticanres.14817

Epithelial-to-mesenchymal Transition Heterogeneity of Circulating Tumor Cells and Their Correlation With MDSCs and Tregs in HER2-negative Metastatic Breast Cancer Patients

MARIA A. PAPADAKI[1*], DESPOINA AGGOURAKI[1*], ELENI-KYRIAKI VETSIKA[1], NIKOLAOS XENIDIS[2,3], GALAKTEA KALLERGI[3,4], ATHANASIOS KOTSAKIS[3,5] and VASSILIS GEORGOULIAS[3,6*]

[1]*Laboratory of Translational Oncology, School of Medicine, University of Crete, Heraklion, Greece;*
[2]*Department of Medical Oncology, Medical School, Democritus University of Thrace, Alexandroupolis, Greece;*
[3]*Hellenic Oncology Research Group (HORG), Athens, Greece;*
[4]*Department of Biology, University of Patras, Patras, Greece;*
[5]*Department of Medical Oncology, University Hospital of Larissa & Laboratory of Oncology, Faculty of Medicine, School of Health Sciences, University of Thessaly, Larissa, Greece;*
[6]*School of Medicine, University of Crete, Heraklion, Greece*

Abstract. *Background: To investigate the correlation between circulating tumor cells (CTCs) bearing cancer stem cell (CSC) and epithelial-to-mesenchymal (EMT) phenotypes and the different immunosuppressive cells in peripheral blood of patients with metastatic breast cancer (mBC). Materials and Methods: Blood was obtained from 38 pre-treated patients with mBC before a new line of treatment. CTC detection and characterization was performed by triple immunofluorescent staining, while Myeloid-derived Suppressor Cells (MDSCs) and T regulatory cells (Tregs) were analyzed by multi-flow cytometry. Results: CTCs were detected in 16 (42.1%) of patients. Based on the co-expression of ALDH1, TWIST and CK, CTCs revealed an important heterogeneity: CTCs with a CSC/partial-EMT, CSC/Epithelial-like, non-CSC/partial-EMT and non-CSC/Epithelial-like phenotype were detected in 7 (18.4%), 7 (18.4%), 1 (1.4%) and 9 (23.7%) of patients, respectively. Immunophenotyping of MDSCs identified 2 monocytic [M-MDSCs; $CD14^+CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ ($CD14^+CD15^+$) and $CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ ($CD14^+CD15^-$)] and one granulocytic [G-MDSCs; $CD14^-CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ ($CD14^-CD15^+$)] subpopulations, expressing inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS), respectively. Patients with detectable CTCs had a higher frequency of Tregs ($CD3^+CD4^+CD25^{high}$; p=0.022) whereas a positive correlation was found between CTC counts and the percentage of Tregs (p=0.005) and $CD14^+CD15^+$ M-MDSCs (p=0.024). Patients with a partial-EMT phenotype had a higher frequency of $CD14^+CD15^+$ M-MDSCs (p=0.023). Patients harboring the non-CSC/epithelial-like CTC subpopulation had an increased frequency of CD14-$CD15^+$ G-MDSCs (p=0.020), along with decreased levels of $CD3^+CD4^+CD25^{high}$ FoxP3+ Tregs (p=0.020). Conclusion: These findings provide evidence that CTCs in $ER^+/HER2^-$ mBC patients may be under the control of the immune system and various immune escape mechanisms might be involved during the different stages of their biological evolution.*

According to the immune-editing theory, most developing cancer cells will be eliminated by the intact immune system. However, progressively, cancer cells may escape and survive from the surveillance effect of immune cells leading to tumor progression; this escape from the immune control is due to several key suppressive mechanisms mediated by both innate and adaptive immunity (1). Nowadays, it is well established that cancer cells activate several types of immunosuppressive cells, including tumor-associated macrophages (TAMs) (2), natural killer T (NKT) cells (3), MDSCs (4), $CD4^+$ and $CD8^+$ cells, Tregs (5) and others. Tregs and MDSCs contribute to the prevalence of immunosuppressive mechanisms (6-8) and their presence has been associated with the patients' clinical outcome (9-11).

*These Authors contributed equally to this study.

Correspondence to: Vassilis Georgoulias, MD, Ph.D., Hellenic Oncology Research Group (HORG), Gregoriou Theologou 5, 11471, Athens, Greece. Tel: +30 2106448450, Fax: +30 2106448450, e-mail: georgulv@otenet.gr

Key Words: CTC, MDSCs, Tregs, immune cells, breast cancer, EMT, cancer stem cells.

MDSCs are a heterogeneous population and, in humans, they have been classified into $CD14^+$ depicting their monocytic origin and $CD15^+$ depicting their granulocytic origin. In the literature, many distinct MDSC populations have been described in BC patients such as $Lin^-HLA\text{-}DR^{-/low}$ (12), $CD15^+$ (13), $HLA\text{-}DR^-Lin^-CD11b^+CD33^+$ (14), $CD14^+HLA\text{-}DR^{-/low}$ (15) and $CD15^+CD11b^+CD33^+HLA\text{-}DR^-$ (16). During the recent years, it has been reported that circulating immunosuppressive cells are increased not only in the tumor microenvironment (TME), but also in the peripheral blood of cancer patients, providing important clinical information (14, 17). We recently described that various $CD4^+$ Treg and MDSC cell subpopulations were increased in the blood of patients with Non-Small Cell Lung Cancer (NSCLC) and that these increased levels were strongly associated with poor clinical outcome (18, 19).

Circulating tumor cells (CTCs), detected in the peripheral blood of cancer patients, constitute an important biomarker with clinical relevance in several tumor types (20, 21). Moreover, there is increasing evidence supporting the hypothesis that the survival of CTCs in the bloodstream is directly linked to their surveillance by circulating immune cells of innate immunity. Indeed, natural killer (NK) cells may target CTCs through direct cell-to-cell contact (22), while the adherence of CTCs on platelets and neutrophils seems to be involved in immune escape mechanisms (23-25). In line with this, CTCs of BC patients might prevent their phagocytosis by macrophages by expressing the CD47 "don't eat me" signal (26-28). Even though the presence of CTCs is correlated with high levels of circulating MDSCs in patients with metastatic breast (29)[15], the role of adaptive immunity in the surveillance of CTCs is less clear. Thus, the expression of the first apoptosis signal (FAS) on peripheral $CD4^+$ T cells of BC patients was shown to be increased among those who had detectable CTCs (30), whereas the expression of programmed cell death ligand-1 (PD-L1) on CTCs of BC patients has been suggested as another mechanism for their immune escape (31).

Epithelial-to-mesenchymal transition (EMT) is a process highly associated with enhanced metastatic potential of tumor cells and it has been recently shown that a partial EMT phenotype, characterized by the concomitant expression of both epithelial and mesenchymal features, confers even more invasive potential to tumor cells (31, 32). In addition, both EMT and stemness properties are characterized by a decreased sensitivity to cytotoxic effector cells (33). Preclinical data support that EMT holds an important role in regulating the immune escape of tumor cells through the induction of TAMs, Tregs and MDSCs (34-36). The current study was designed to investigate the association of the different immune cell populations, both effector and suppressive, with the detection of CTCs bearing EMT or/and stem cell phenotype in patients with $ER^+/HER2^-$ mBC.

Table I. *Patients' demographics.*

Patients (N=38)	N (%)
Age, years	
Median (range)	63 (37-80)
Histology	
Ductal	35 (92.1)
Lobular	3 (7.9)
HR status	
ER(+)/PR(+)	28 (73.7)
ER(+)/PR(–)	10 (26.3)
Disease sites	
Visceral	27 (71.1)
Non-visceral	11 (28.9)
Prior adjuvant treatment	
Yes	26 (68.4)
No	12 (31.6)
Line of treatment	
Second	17 (44.7)
≥Third	21 (55.3)

Materials and Methods

Patients. Thirty-eight female patients with histologically-documented $ER^+/HER2^-$ mBC were enrolled in the study. All patients had received at least one line of systemic treatment for metastatic disease according to the physicians' choice in the context of standard of care (hormone treatment or chemotherapy). In all patients, peripheral blood (25 ml in EDTA) was obtained at the middle of venipuncture, after the first 5 ml of blood were discarded, before the initiation of a new line of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density centrifugation and used for the detection and characterization of CTCs. Peripheral blood was also collected from a normal control (NC) group (n=16 sex and age-matched healthy volunteers; age: 60±2 years). The present study was approved by the Institutional Ethics and Scientific Committees of the University General Hospital of Heraklion, Heraklion, Crete, Greece (#392783); the patients and healthy volunteers provided written informed consent to participate in the study, to the use of samples and to the use of their medical data and publication before sampling, according to the Declaration of Helsinki. The patients' characteristics are shown in Table I.

Cell immunophenotyping by flow cytometry. Fluorescence-active cell sorting (FACS) analysis was performed on freshly isolated PBMCs according to a standard procedure (19). Briefly, PBMCs were stained using a combination of monoclonal antibodies conjugated to fluorochromes for the detection of the following cell populations: (a) MDSCs: anti-CD14/PE-Cy7; anti-CD15/V450; anti-CD11b/PE Dazzle; anti-CD33/Alexa 700; anti-HLA-DR/APC-H7; anti-Lin(CD3/CD19/CD56)/PE and (b) T cells and $CD4^+$ Tregs: anti-CD3/PE-CF594; anti-CD4/V500; anti-CD25/PE-Cy7 and anti-CD8/APC-Cy7 (BD Biosciences, Franklin Lakes, NJ, USA). For intracellular staining, T cells were fixed and permeabilized by BD FoxP3 buffer set (BD Biosciences) according to the manufacturer's instructions and incubated for 1h with anti-FoxP3/FITC. For

intracellular staining, the cells were permeabilized by BD IntraSure kit (BD Biosciences) according to manufacturers' instructions and stained for inducible nitric oxide synthase (iNOS)-PerCP (Santa Cruz, CA, USA). Multicolour analysis was done using a LSRII flow cytometer (BD Biosciences) and analysis of data was performed using FACSDiva Software (BD Biosciences). MDSCs were subclassified into two monocytic populations [$CD14^+CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ and $CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ (referred to as $CD14^+CD15^+$ and $CD14^+CD15^-$, respectively)] and one granulocytic population [G-MDSCs; $CD14^-CD15^+CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ (referred to as $CD14^-CD15^+$ throughout the text) (19). MDSC percentages were calculated within the $CD11b^+CD33^+HLA\text{-}DR^-Lin^-$ cells.

Tregs were defined as $CD3^+CD4^+CD25^{high}$ in $CD3^+$ T cells and $CD3^+CD4^+CD25^{high}FoxP3^+$ within the $CD3^+CD4^+CD25^{high}$ population. For MDSCs analysis all cells, except lymphocytic mononuclear cells, were included. For T cell subset, the acquisition and analysis gates were restricted to the lymphocyte population. Each measurement contained 500,000 events.

Reactive oxygen species (ROS) detection. The intracellular oxidant intensity was determined by using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetateacetylester (DCFDA; Invitrogen, Carlsbad, CA, USA), which is metabolized to fluorescent 2'-7'-dichlorofluorescein (DCF) upon oxidation. Single-cell suspensions from blood were incubated in RPMI1640 medium containing 2.5 μM DCFDA with/or without 30 ng/mL PMA for 30 min at room temperature. Subsequently, cells were washed twice in flow buffer and stained with MDSC mAbs and the mean fluorescence intensity (MFI) of intracellular DFC was determined by flow cytometry.

Detection and phenotypic characterization of CTCs. PBMCs' cytospins (500,000 PBMCs/slide) were prepared and stored at −80˚C until use. Cytospins were used for triple immunofluorescent (IF) staining, using antibodies against putative markers for epithelial cells (Cytokeratin 8, 18 and 19; A45-B/B3; Micromet AG, Munich, Germany), CSCs (ALDH1; Abcam, Cambridge, USA) and EMT (TWIST1; Abcam) as previously reported (37). Briefly, cells were fixed with 3% (v/v) paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100. After an overnight blocking with PBS/1% BSA at 4˚C, the cells were incubated with primary and secondary antibodies. Zenon technology (FITC-conjugated IGg1 anti-mouse antibody; Molecular Probes, Invitrogen), along with Alexa 555- and 633- conjugated secondary antibodies (Molecular Probes, Invitrogen) were used to target primary antibodies and DAPI- -antifade reagent (Molecular Probes, Invitrogen) was then added to each sample for cell nuclear staining. IF analysis was performed using the ARIOL microscopy system (Genetix, Cambridge, UK) and a total of 10^6 PBMCs/patient were analyzed for the detection and characterization of Cytokeratin-positive CTCs. Based on our previous studies, the detection of CTCs was based on the expression of CK; CK^+ CTCs expressing high ALDH1 expression levels were characterized as CSC^+, whereas low or absent ALDH1 expression was defined as a non-CSC phenotype. CK^+ CTCs bearing nuclear TWIST1 localization were characterized as a partial-EMT^+, whereas cytoplasmic or absent TWIST1 was defined as an epithelial-like phenotype (37). The detailed description of the methods used for the characterization of ALDH1 and TWIST1 on CTCs, as well as their sensitivity and specificity have been previously reported (37).

Statistical analysis. Because of the observational nature of this study, it was not possible to make a clear statistical hypothesis to estimate the appropriate number of patients to be enrolled. The Mann Whitney *U*-test was used to compare the incidence of different immune cell subpopulations between different patient groups. Spearman's rho analysis was performed to investigate the correlation between immune cell percentages and different CTC counts. Statistical analysis was performed using IBM SPSS Statistics version 20. *p*-Values were considered statistically significant at the 0.05 level.

Results

Detection and phenotypic characterization of peripheral blood immune cells. Phenotypic analysis of patients' $CD4^+$ and $CD8^+$ T cells demonstrated significantly decreased percentages of $CD4^+$ T cells (56.02%±2.38% *vs*. 77.24%±2.98%, $p<0.0001$), along with slightly elevated $CD8^+$ T cells (23.28%±1.58% *vs*. 17.64%±1.08%, $p=0.06$) compared to NC (Figure 1A). The percentages of both monocytic and granulocytic MDSC subsets were significantly increased in patients compared to NC [M-MDSCs ($CD14^+CD15^+$: 4.66%±1.15% *vs*. 0.79%±0.15%; ($p=0.03$) and $CD14^+CD15^-$; 13.21%±2.11% *vs*. 3.80%±1.08%; ($p=0.02$); G-MDSCs ($CD14^-CD15^+$: 3.96%±0.69% *vs*. 0.88%±0.16%; ($p=0.002$)] (Figure 1B). On the contrary, the percentages of $CD3^+CD4^+CD25^{high}$ Tregs were significantly lower compared to NC (0.42%±0.02% *vs*. 0.58%±0.05%, respectively; $p=0.003$), whereas there was no difference regarding the $CD3^+CD4^+CD25^{high}FoxP3^+$ Tregs between patients and NC (66.66%±4.89% *vs*. 76.74%±6.16%, respectively; $p=0.243$) (Figure 1C).

The functionality of the defined MDSC subpopulations was assessed using the expression of ROS and the production of iNOS. Higher percentages of ROS-expressing cells were detected in G-MDSCs from mBC patients compared to NC (25.35%±3.7% *vs*. 0.45%±0.11%; $p=0.0002$), but the mean fluorescence intensity (MFI) did not differ between the two groups (Figure 2A, B). Conversely, no monocytic MDSCs producing ROS could be detected in both patients and NC.

The immunosuppressive properties of MDSCs were also assessed by iNOS production. A higher frequency of $CD15^+$ M-MDSCs producing iNOS was detected in BC patients compared to NC (2.53%±0.43% *vs*. 0.73%±0.37%; $p=0.028$); moreover, the expression levels of iNOS, as determined by MFI, were higher in both $CD15^+$ and $CD15^-$ M-MDSCs (1219±127.4 *vs*. 133.7±5.35; $p=0.0004$ and 870.8±48.14 *vs*. 116.8±3.75; $p=0.0004$, respectively) in mBC patients compared to NC (Figure 2C, D). The $CD15^+$ M-MDSCs produced increased levels of iNOS both in terms of number of producing cells (2.53%±0.43% *vs*. 0.81%±0.16%; $p=0.003$) as well as in terms of the amount of MFI iNOS expression (1219±127.4 *vs*. 870.8±48.14; $p=0.018$, respectively) compared to $CD15^-$ M-MDSCs.

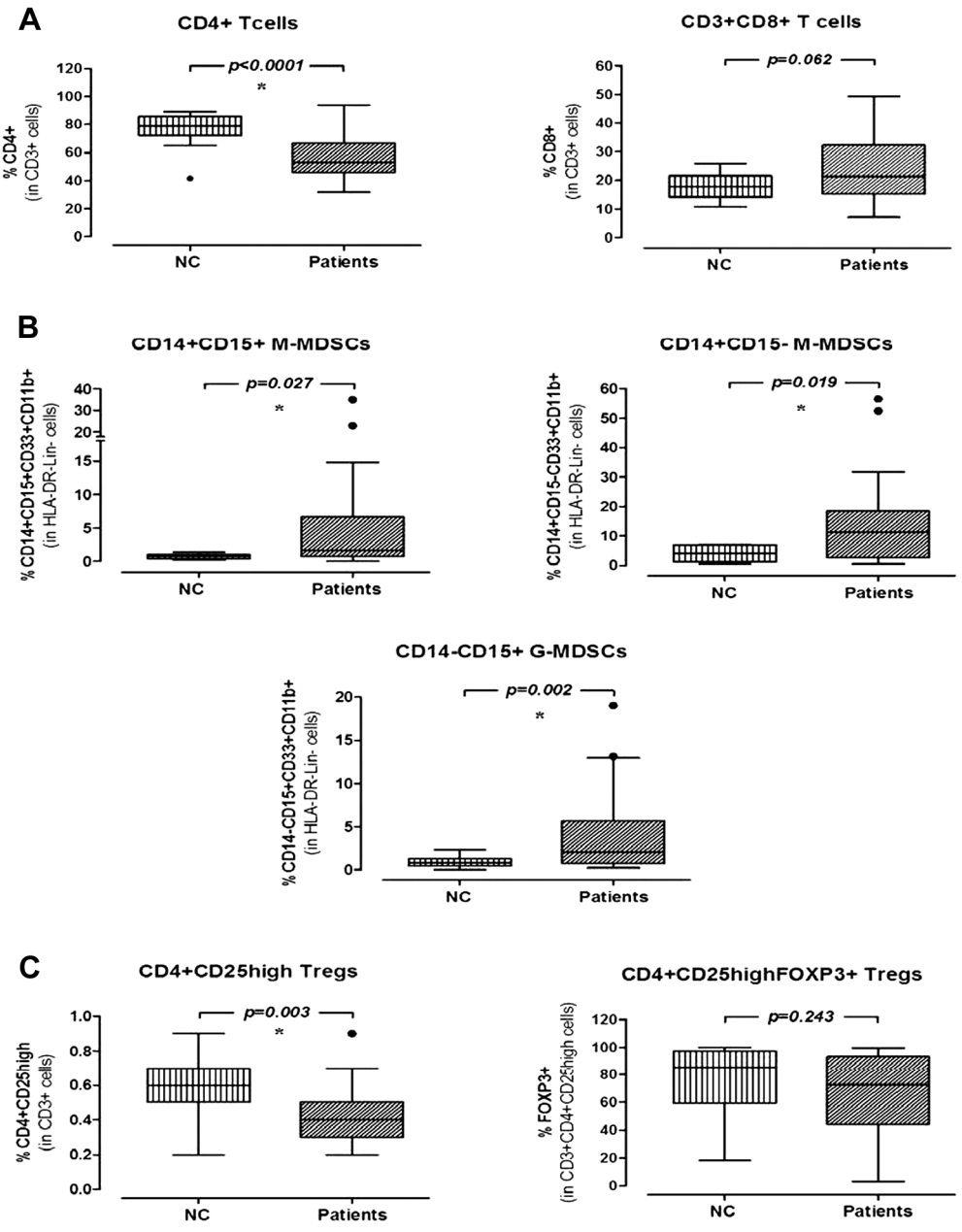

Figure 1. *Incidence of circulating immunosuppressive cells among mBC patients (n=38) and healthy volunteers (normal controls, NC; n=16). (A) Percentage of CD14$^+$CD15$^+$ M-MDSCs and CD14$^+$CD15$^-$ M-MDSCs and CD14$^-$CD15$^+$ G-MDSCs. (B) Percentage of CD3$^+$CD4$^+$CD25^{high} Tregs and CD3$^+$CD4$^+$CD25^{high}FoxP3$^+$ Tregs. The bars denote mean values±SEM and the p-values are determined by the Mann Whitney U-test.*

Detection and phenotypic characterization of CTCs. Cytokeratin-positive CTCs were detected in 16 out of 38 patients (42.1%), with a total number of 70 CTCs [mean CTC number/patient: 1.84 (range=0-14)]. According to the different expression patterns of ALDH1 and TWIST1, four different phenotypic CTC subtypes could be identified, confirming the heterogeneity of these cells in ER$^+$HER2$^-$ mBC patients (Table II). CTCs bearing a CSC$^+$/epithelial-like phenotype were observed in 7 out of 38 (18.4%) patients representing the 47.1% of the total number of detected CTCs whereas, CTCs with a mixed CSC$^+$/partial-EMT$^+$ phenotype were detected in 7 out of 38 (18.4%) patients and represented the 24.3% of the total number of CTCs. Conversely, the non-CSC/partial-EMT$^+$ CTCs subpopulation was rare (1.4% of the total CTCs). Non-CSC/epithelial-like cells were detected in 9 (23.7%) patients representing the 27.1% of the total number of detected CTCs.

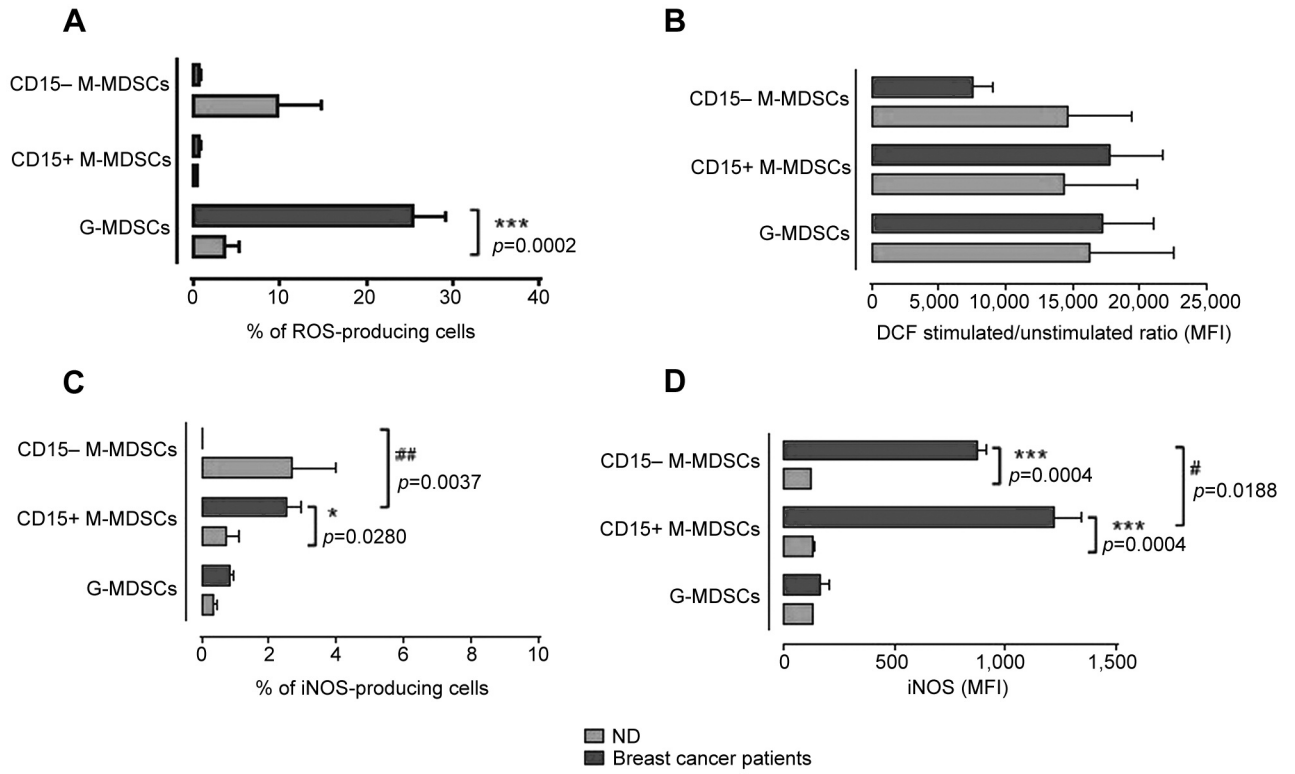

Figure 2. *ROS production and iNOS expression in different MDSCs' subtypes. Percentages of (A) ROS-producing and (C) iNOS-expressing cells from normal donors (n=13) and mBC patients (n=38). Intracellular levels of (B) ROS and (D) iNOS in all tested subpopulations. Intracellular ROS levels are expressed as mean fluorescence intensity (MFI) in subpopulations of MDSCs before and after PMA stimulation. The data are represented as the mean±SEM and the p-values are determined by the Mann-Whitney U-test. ND: Normal donors.*

Table II. *Phenotypic subpopulations of CTCs according to the expression of CSC and EMT phenotypic profiles.*

CTC Phenotypes	Patients No (%)	CTCs No (%)
CSC/partial-EMT (CK+ALDH1^{high}TWIST1^{nuc})	7 (18.4)	17 (24.3)
CSC/epithelial-like (CK+ALDH1^{high}TWIST1$^{cyt/-}$)	7 (18.4)	33 (47.1)
non-CSC/partial-EMT (CK+ALDH1$^{low/-}$TWIST1^{nuc})	1 (0.18)	1 (1.4)
non-CSC/epithelial-like (CK+ALDH1$^{low/-}$TWIST1$^{cyt/-}$)	9 (23.7)	19 (27.1)
Total CK+ cells (n=16 pts)		70 (100)

Cytokeratin-positive CTCs were analyzed using the ARIOL system; high ALDH1 and co-expression of CK with nuclear TWIST1 were defined as CSC and partial-EMT phenotypes, respectively. The first column refers to the number and percentage of patients harvesting each CTC subpopulation. The second column shows the number and percentage of phenotypically different CTCs among the total 70 CTCs detected.

Correlation between CTCs and Tregs. Patients with detectable CTCs had a significantly higher percentage of CD3$^+$CD4$^+$CD25^{high} Tregs compared to patients without CTCs (0.49±0.03 *vs*. 0.36±0.03, respectively; p=0.002) (Figure 3A). Moreover, a positive correlation was observed between the number of CTCs/patient and the percentage of CD3$^+$CD4$^+$CD25^{high} Tregs (p=0.005). Conversely, there was no correlation between the presence of CTCs and the various subpopulations of Tregs identified by the expression of CD127 (IL-7Rα) and CD152 (data not shown). However, the presence of non-CSC/epithelial-like CTCs was associated with decreased levels of CD3$^+$CD4$^+$CD25^{high}FoxP3$^+$

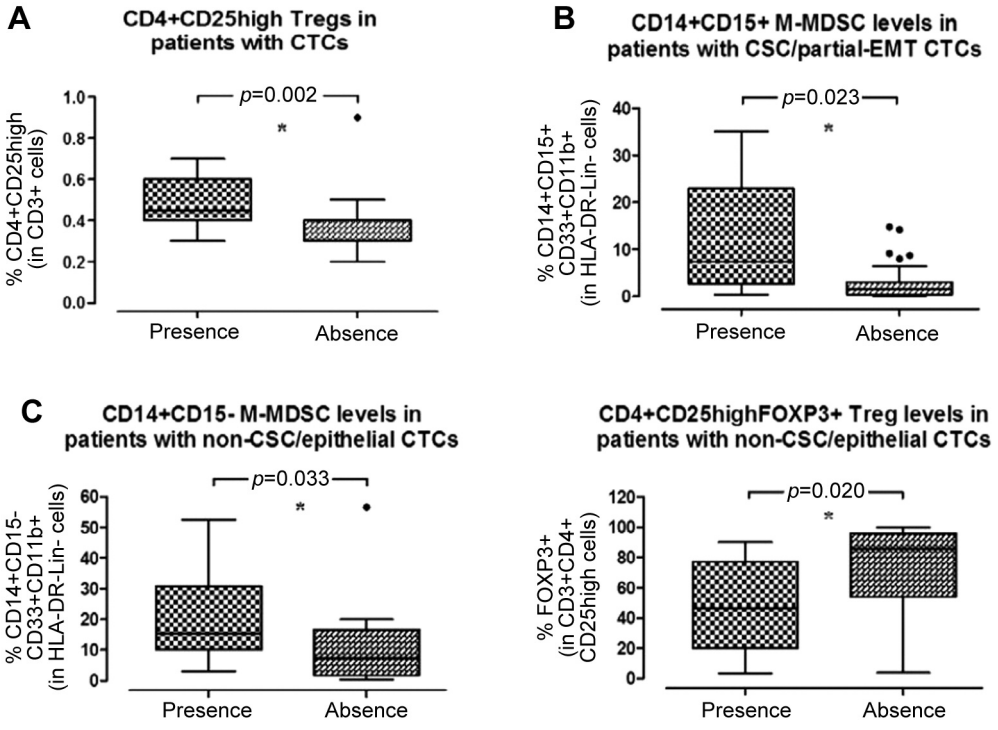

Figure 3. *Correlation between immunosuppressive cells and CTCs with distinct CSC and EMT phenotypes in mBC patients (n=38). Evaluation of the percentage of different immunosuppressive cell populations in the presence and absence of CTCs and within different CTC subpopulations. (A) Percentage of $CD3^+CD4^+CD25^{high}$ Tregs among CTC-positive and CTC-negative patients; (B) Percentage of $CD14^+CD15^+$ M-MDSCs among patients with and without CSC/partial-EMT CTCs; (C) Percentage of $CD14^+CD15^-$ M-MDSCs and $CD3^+CD4^+CD25^{high}$ Tregs among patients with and without detectable non-CSC/epithelial CTCs. p-Values are determined by the Mann-Whitney U-test.*

(46.29%±10.41% *vs*. 72.98%±5.07%; *p*=0.02) (Figure 3C), whereas there was no correlation between the three remaining CTC subsets (CSC^+/epithelial-like, non-CSC/partial-EMT^+ or CSC^+/partial-EMT^+) and the different subpopulations of Tregs, as well as with $CD4^+$ and $CD8^+$ T cells (data not shown).

Correlation between CTCs and MDSCs. Patients with detectable CTCs had a significantly higher percentage of $CD14^+CD15^+$ M-MDSCs (*p*=0.024) whereas there was no correlation with the other subpopulations of MDSCs [$CD14^+CD15^-$ M-MDSCs or $CD14^-CD15^+$ G-MDSCs; (*p*=0.194 and 0.771, respectively)]. Further analysis revealed that the detection of CSC^+/partial-EMT^+ CTCs was associated with a significantly higher percentage of $CD14^+CD15^+$ M-MDSCs compared to patients who did not harbor this CTC subpopulation (11.63%±4.82% *vs*. 3.08%±0.70%; *p*=0.023) (Figure 3B). Conversely, the presence of non-CSC/epithelial-like CTCs was associated with increased $CD14^+CD15^-$ M-MDSC levels (21.53%±5.14% *vs*. 10.62%±2.08%; *p*=0.033). There was no association between the detection of CTCs expressing intermediate phenotypes (CSC^+/epithelial-like and non-CSC/partial-EMT^+) and the percentage of M-MDSCs, as well as of the different CTC subpopulations and the presence of G-MDSCs.

Discussion

The presence of CTCs and their role in the development of distant metastasis in cancer biology are well-established, suggesting that, at least during the metastatic phase of the disease, these cells evade the immune surveillance. Numerous studies have demonstrated the high phenotypic and functional heterogeneity of CTCs; however, it is yet unclear whether the distinct CTC subpopulations have different ways to interact with immune cells. We have previously demonstrated that in mBC patients, single CTCs co-expressing putative stem and partial-EMT phenotypes, as defined by the high ALDH1 expression levels and the nuclear localization of the transcription factor TWIST1, were significantly increased compared to patients with early stage BC (37-39). Similar data were also recently reported by other investigators (38, 39). In addition, our group recently reported that the presence of CSC^+/partial-EMT^+ CTCs in

the peripheral blood of mBC patients represents a strong and independent factor for reduced PFS, whereas in HER2$^-$ disease it is also highly predictive for decreased OS (40).

In the present study we used the same methodology to investigate the association between various subpopulations of CTCs, expressing CSC and partial-EMT phenotypes, and the immune cells in the peripheral blood of patients with mBC. Our findings confirmed the important heterogeneity of CTCs bearing an exclusively epithelial phenotype, as well as CTC subsets with CSC and/or partial EMT phenotype, as previously reported (36). Moreover, the presented data revealed, for the first time, a clear association between the presence of these subpopulations of CTCs and the circulating immune suppressive cells. Indeed, Tregs, both CD3$^+$CD4$^+$CD25^{high} and CD3$^+$CD4$^+$CD25^{high}FoxP3$^+$ subsets, were associated with the detection of CTCs in contrast to Stanzer's *et al*. study which failed to reveal a similar association (41). More importantly, although the presence of non-CSC/epithelial-like CTCs was associated with decreased levels of CD3$^+$CD4$^+$CD25^{high} FoxP3$^+$ cells, there was no correlation between the three remaining CTC subsets (CSC/epithelial-like, non-CSC/partial-EMT or CSC/partial-EMT) and the different subsets of Tregs. However, it is interesting to note that our findings failed to demonstrate an association between CTCs and the levels of CD4$^+$ and CD8$^+$ T cells, which has been previously reported (30, 41).

The present study also demonstrated that two phenotypically distinct monocytic (CD14$^+$CD15$^+$CD11b$^+$CD33$^+$HLA-DR$^-$ Lin$^-$ and CD14$^+$CD15$^-$CD11b$^+$CD33$^+$HLA-DR$^-$Lin$^-$) and one granulocytic population (CD14$^-$CD15$^+$CD11b$^+$CD33$^+$HLA-DR$^-$Lin$^-$) of MDSCs, which have been previously shown to be associated with NSCLC patients' clinical outcome (14), were significantly higher in mBC patients in agreement with previous reports (28, 29). However, to the best of our knowledge, our findings demonstrate, for the first time, that the presence of CTCs is associated with a high percentage of CD14$^+$CD15$^+$ but not with CD14$^+$CD15$^-$ or CD14$^-$CD15$^+$ MDSCs; moreover, it should be noted that CD14$^+$CD15$^+$ MDSCs displayed a stronger immunosuppressive function, as suggested by their higher levels of iNOS production, compared to the CD14$^+$CD15$^-$ MDSCs. Importantly, the presence of CSC$^+$/partial-EMT$^+$ CTCs was associated with a significantly higher percentage of CD14$^+$CD15$^+$ M-MDSCs, whereas the presence of non-CSC/epithelial-like CTCs was associated with increased levels of CD14$^+$CD15$^-$ M-MDSCs. On the other hand, CTCs bearing a non-CSC/epithelial-like phenotype were associated with increased levels of CD14$^+$CD15$^-$ M-MDSCs along with decreased percentages of CD3$^+$CD4$^+$CD25^{high} FoxP3$^+$ Tregs. It is noteworthy that there was no correlation between G-MDSCs, CD8$^+$ or CD4$^+$ T cells and the detection of the above-mentioned CTCs subpopulations. These observations, taken together with the relatively similar data regarding the Tregs, as mentioned above, strongly suggest that different immune cells and effective mechanisms are involved in the clonal selection of various CTC subsets, thus leading to their escape from immunosurveillance.

The data presented in the current study are in line with recent studies which propose different mechanisms of immunological escape of cancer cells. Indeed, tumor cells may evade the host immune system by hiding their tumor-specific antigens (42), through different molecular events, such as down-regulation or loss of MHC class I proteins (43). In addition, it has been shown that CSCs are more resistant to immunological control compared with non-CSCs, and cancer immune surveillance enriches a subpopulation of cancer cells with stem-like properties (44). Furthermore, previous studies have shown that CSCs express low levels of molecules involved in processing and presenting tumor antigens to T cell receptors, which is a crucial stimulatory signal to T-cell response, leading thus, to escape from immune surveillance (45, 46). Interestingly, accumulating evidence indicates that CSCs can also suppress T-cell activation since high PD-L1 expression on CSCs may contribute to CSC immune evasion; however, the regulatory mechanisms contributing to the enriched PD-L1 expression in the CSC populations remain unclear (47-49). Finally, a recent study demonstrated a positive correlation between the levels of MDSCs infiltrating the tumor and CSCs in patients with breast cancer (50). To this direction, both preclinical and clinical findings suggest that different immune cell subpopulations are involved in the surveillance of CSCs.

The current study enrolled previously treated patients with mBC and this could represent a substantial weakness explaining, at least partly, the significant difference of the percentages of the various populations of immune cells compared to NC. It is unknown whether these differences are due to the disease itself or to the various therapies that patients had previously received. The heterogeneity of previous administered treatments limited the possibility to define relatively homogeneous subgroups of patients for further analysis.

The acquisition of CSC properties and EMT are two processes that hold an important role in tumor biology (51). Peripheral blood is considered as a very hostile environment for CTCs, where they are exposed to a high amount of immune cells. Even though previous studies suggest a direct link between tumor cells bearing EMT or CSC features and immune cells (52, 53), the data presented in the current study strongly supports a similar interaction between immune cells and CTCs. Therefore, it is reasonable to hypothesize that the acquisition of distinct EMT and/or CSC features might contribute and facilitate the ability of CTCs to efficiently escape from the immune surveillance. *In vitro* co-cultures and functional assays are required to validate the interactions between immunosuppressive cells and CTCs with CSC and partial-EMT characteristics.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

MP, DA, VG conceived and designed the study; E-KV, NX, GK, AK acquired, analyzed, and interpreted data or made key methodological contributions; MP, DA, VG wrote the paper; VG supervised the study.

Acknowledgements

This work was partially supported by grants from the Cretan Association for Biomedical Research (CABR), and the Hellenic Oncology Research Group (HORG). MAP was a recipient of a CABR fellowship. The authors would like to thank Mrs Vasso Athanasaki, Scientific Secretary of the Hellenic Oncology Research Group, for her attentive editing of this manuscript.

References

1 Dunn GP, Old LJ and Schreiber RD: The immunobiology of cancer immunosurveillance and immunoediting. Immunity *21(2)*: 137-148, 2004. PMID: 15308095. DOI: 10.1016/j.immuni.2004.07.017

2 Solinas G, Germano G, Mantovani A and Allavena P: Tumor-associated macrophages (tam) as major players of the cancer-related inflammation. J Leukoc Biol *86(5)*: 1065-1073, 2009. PMID: 19741157. DOI: 10.1189/jlb.0609385

3 Berzofsky JA and Terabe M: Nkt cells in tumor immunity: Opposing subsets define a new immunoregulatory axis. J Immunol *180(6)*: 3627-3635, 2008. PMID: 18322166. DOI: 10.4049/jimmunol.180.6.3627

4 Gabrilovich DI and Nagaraj S: Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol *9(3)*: 162-174, 2009. PMID: 19197294. DOI: 10.1038/nri2506

5 Curiel TJ: Regulatory t cells and treatment of cancer. Curr Opin Immunol *20(2)*: 241-246, 2008. PMID: 18508251. DOI: 10.1016/j.coi.2008.04.008

6 Umansky V, Blattner C, Gebhardt C and Utikal J: The role of myeloid-derived suppressor cells (mdsc) in cancer progression. Vaccines (Basel) *4(4)*, 2016. PMID: 27827871. DOI: 10.3390/vaccines4040036

7 Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS and Linehan DC: Prevalence of regulatory t cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol *169(5)*: 2756-2761, 2002. PMID: 12193750. DOI: 10.4049/jimmunol.169.5.2756

8 Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M, Egevad L and Pisa P: Cd4+cd25high t cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol *177(10)*: 7398-7405, 2006. PMID: 17082659. DOI: 10.4049/jimmunol.177.10.7398

9 Beyer M and Schultze JL: Regulatory t cells in cancer. Blood *108(3)*: 804-811, 2006. PMID: 16861339. DOI: 10.1182/blood-2006-02-002774

10 Jiang W, Liu K, Guo Q, Cheng J, Shen L, Cao Y, Wu J, Shi J, Cao H, Liu B, Tao K, Wang G and Cai K: Tumor-infiltrating immune cells and prognosis in gastric cancer: A systematic review and meta-analysis. Oncotarget *8(37)*: 62312-62329, 2017. PMID: 28977947. DOI: 10.18632/oncotarget.17602

11 Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, Nair VS, Xu Y, Khuong A, Hoang CD, Diehn M, West RB, Plevritis SK and Alizadeh AA: The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat Med *21(8)*: 938-945, 2015. PMID: 26193342. DOI: 10.1038/nm.3909

12 Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP and Gabrilovich DI: Increased production of immature myeloid cells in cancer patients: A mechanism of immunosuppression in cancer. J Immunol *166(1)*: 678-689, 2001. PMID: 11123353. DOI: 10.4049/jimmunol.166.1.678

13 Schmielau J and Finn OJ: Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. Cancer Res *61(12)*: 4756-4760, 2001. PMID: 11406548

14 Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ and Montero AJ: Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother *58(1)*: 49-59, 2009. PMID: 18446337. DOI: 10.1007/s00262-008-0523-4

15 Bergenfelz C, Larsson AM, von Stedingk K, Gruvberger-Saal S, Aaltonen K, Jansson S, Jernstrom H, Janols H, Wullt M, Bredberg A, Ryden L and Leandersson K: Systemic monocytic-mdscs are generated from monocytes and correlate with disease progression in breast cancer patients. PLoS One *10(5)*: e0127028, 2015. PMID: 25992611. DOI: 10.1371/journal.pone.0127028

16 Mundy-Bosse BL, Thornton LM, Yang HC, Andersen BL and Carson WE: Psychological stress is associated with altered levels of myeloid-derived suppressor cells in breast cancer patients. Cell Immunol *270(1)*: 80-87, 2011. PMID: 21600570. DOI: 10.1016/j.cellimm.2011.04.003

17 Gabitass RF, Annels NE, Stocken DD, Pandha HA and Middleton GW: Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the th2 cytokine interleukin-13. Cancer Immunol Immunother *60(10)*: 1419-1430, 2011. PMID: 21644036. DOI: 10.1007/s00262-011-1028-0

18 Kotsakis A, Koinis F, Katsarou A, Gioulbasani M, Aggouraki D, Kentepozidis N, Georgoulias V and Vetsika EK: Prognostic value of circulating regulatory t cell subsets in untreated non-small cell lung cancer patients. Sci Rep *6(*39247, 2016. PMID: 27976733. DOI: 10.1038/srep39247

19 Vetsika EK, Koinis F, Gioulbasani M, Aggouraki D, Koutoulaki A, Skalidaki E, Mavroudis D, Georgoulias V and Kotsakis A: A circulating subpopulation of monocytic myeloid-derived suppressor cells as an independent prognostic/predictive factor in untreated non-small lung cancer patients. J Immunol Res *2014(*659294, 2014. PMID: 25436215. DOI: 10.1155/2014/659294

20 Ignatiadis M, Xenidis N, Perraki M, Apostolaki S, Politaki E, Kafousi M, Stathopoulos EN, Stathopoulou A, Lianidou E, Chlouverakis G, Sotiriou C, Georgoulias V and Mavroudis D: Different prognostic value of cytokeratin-19 mrna positive circulating tumor cells according to estrogen receptor and her2 status in early-stage breast cancer. J Clin Oncol *25(33)*: 5194-5202, 2007. PMID: 17954712. DOI: 10.1200/JCO.2007.11.7762

21 Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN and Terstappen LW: Circulating tumor cells: A novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol *23(7)*: 1420-1430, 2005. PMID: 15735118. DOI: 10.1200/JCO.2005.08.140

22 Brodbeck T, Nehmann N, Bethge A, Wedemann G and Schumacher U: Perforin-dependent direct cytotoxicity in natural killer cells induces considerable knockdown of spontaneous lung metastases and computer modelling-proven tumor cell dormancy in a ht29 human colon cancer xenograft mouse model. Mol Cancer *13:*244, 2014. PMID: 25373310. DOI: 10.1186/1476-4598-13-244

23 Placke T, Orgel M, Schaller M, Jung G, Rammensee HG, Kopp HG and Salih HR: Platelet-derived mhc class i confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. Cancer Res *72(2)*: 440-448, 2012. PMID: 22127925. DOI: 10.1158/0008-5472.CAN-11-1872

24 Spicer JD, McDonald B, Cools-Lartigue JJ, Chow SC, Giannias B, Kubes P and Ferri LE: Neutrophils promote liver metastasis via mac-1-mediated interactions with circulating tumor cells. Cancer Res *72(16)*: 3919-3927, 2012. PMID: 22751466. DOI: 10.1158/0008-5472.CAN-11-2393

25 Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, Bourdeau F, Kubes P and Ferri L: Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest, 2013. PMID: 23863628. DOI: 10.1172/JCI67484

26 Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, Klein C, Saini M, Bauerle T, Wallwiener M, Holland-Letz T, Hofner T, Sprick M, Scharpff M, Marme F, Sinn HP, Pantel K, Weichert W and Trumpp A: Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. Nat Biotechnol *31(6)*: 539-544, 2013. PMID: 23609047. DOI: 10.1038/nbt.2576

27 Baccelli I, Stenzinger A, Vogel V, Pfitzner BM, Klein C, Wallwiener M, Scharpff M, Saini M, Holland-Letz T, Sinn HP, Schneeweiss A, Denkert C, Weichert W and Trumpp A: Co-expression of met and cd47 is a novel prognosticator for survival of luminal breast cancer patients. Oncotarget *5(18)*: 8147-8160, 2014. PMID: 25230070. DOI: 10.18632/oncotarget.2385

28 Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, Francescato S, Basso G, Zanovello P, Onicescu G, Garrett-Mayer E, Montero AJ, Bronte V and Mandruzzato S: A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood *118(8)*: 2254-2265, 2011. PMID: 21734236. DOI: 10.1182/blood-2010-12-325753

29 Cole S, Montero A, Garret-Mayer E, Onicescu G, Vandenberg T, Hutchens S and Diaz-Montero C: Elevated circulating myeloid derived suppressor cells (mdsc) are associated with inferior overall survival (os) and correlate with circulating tumor cells (ctc) in patients with metastatic breast cancer. Cancer Res *69(24)*: Abstract nr 4135, 2009. DOI: 10.1158/0008-5472.SABCS-09-4135

30 Gruber I, Landenberger N, Staebler A, Hahn M, Wallwiener D and Fehm T: Relationship between circulating tumor cells and peripheral t-cells in patients with primary breast cancer. Anticancer Res *33(5)*: 2233-2238, 2013. PMID: 23645781.

31 Mazel M, Jacot W, Pantel K, Bartkowiak K, Topart D, Cayrefourcq L, Rossille D, Maudelonde T, Fest T and Alix-Panabieres C: Frequent expression of pd-l1 on circulating breast cancer cells. Mol Oncol *9(9)*: 1773-1782, 2015. PMID: 26093818. DOI: 10.1016/j.molonc.2015.05.009

32 Vincent-Salomon A and Thiery JP: Host microenvironment in breast cancer development: Epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res *5(2)*: 101-106, 2003. PMID: 12631389. DOI: 10.1186/bcr578

33 Noman MZ, Messai Y, Muret J, Hasmim M and Chouaib S: Crosstalk between ctc, immune system and hypoxic tumor microenvironment. Cancer Microenviron *7(3)*: 153-160, 2014. PMID: 25337680. DOI: 10.1007/s12307-014-0157-3

34 Su S, Liu Q, Chen J, Chen J, Chen F, He C, Huang D, Wu W, Lin L, Huang W, Zhang J, Cui X, Zheng F, Li H, Yao H, Su F and Song E: A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. Cancer Cell *25(5)*: 605-620, 2014. PMID: 24823638. DOI: 10.1016/j.ccr.2014.03.021

35 Kudo-Saito C, Shirako H, Takeuchi T and Kawakami Y: Cancer metastasis is accelerated through immunosuppression during snail-induced emt of cancer cells. Cancer Cell *15(3)*: 195-206, 2009. PMID: 19249678. DOI: 10.1016/j.ccr.2009.01.023

36 Toh B, Wang X, Keeble J, Sim WJ, Khoo K, Wong WC, Kato M, Prevost-Blondel A, Thiery JP and Abastado JP: Mesenchymal transition and dissemination of cancer cells is driven by myeloid-derived suppressor cells infiltrating the primary tumor. PLoS Biol *9(9)*: e1001162, 2011. PMID: 21980263. DOI: 10.1371/journal.pbio.1001162

37 Papadaki MA, Kallergi G, Zafeiriou Z, Manouras L, Theodoropoulos PA, Mavroudis D, Georgoulias V and Agelaki S: Co-expression of putative stemness and epithelial-to-mesenchymal transition markers on single circulating tumour cells from patients with early and metastatic breast cancer. BMC Cancer *14*: 651, 2014. PMID: 25182808. DOI: 10.1186/1471-2407-14-651

38 Mego M, Karaba M, Minarik G, Benca J, Silvia J, Sedlackova T, Manasova D, Kalavska K, Pindak D, Cristofanilli M, Reuben JM and Mardiak J: Circulating tumor cells with epithelial-to-mesenchymal transition phenotypes associated with inferior outcomes in primary breast cancer. Anticancer Res *39(4)*: 1829-1837, 2019. PMID: 30952723. DOI: 10.21873/anticanres.13290

39 Agnoletto C, Corra F, Minotti L, Baldassari F, Crudele F, Cook WJJ, Di Leva G, d'Adamo AP, Gasparini P and Volinia S: Heterogeneity in circulating tumor cells: The relevance of the stem-cell subset. Cancers (Basel) *11(4)*, 2019. PMID: 30959764. DOI: 10.3390/cancers11040483

40 Papadaki MA, Stoupis G, Theodoropoulos PA, Mavroudis D, Georgoulias V and Agelaki S: Circulating tumor cells with stemness and epithelial-to-mesenchymal transition features are chemoresistant and predictive of poor outcome in metastatic breast cancer. Mol Cancer Ther *18(2)*: 437-447, 2019. PMID: 30401696. DOI: 10.1158/1535-7163.MCT-18-0584

41 Stanzer S, Dandachi N, Balic M, Resel M, Samonigg H and Bauernhofer T: Resistance to apoptosis and expansion of regulatory t cells in relation to the detection of circulating tumor cells in patients with metastatic epithelial cancer. J Clin Immunol *28(2)*: 107-114, 2008. PMID: 17939021. DOI: 10.1007/s10875-007-9139-2

42 Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, Zhang T, Adleff V, Phallen J, Wali N, Hruban C, Guthrie VB, Rodgers K, Naidoo J, Kang H, Sharfman W, Georgiades C, Verde F, Illei P, Li QK, Gabrielson E, Brock MV, Zahnow CA, Baylin SB, Scharpf RB, Brahmer JR, Karchin R, Pardoll DM and Velculescu VE: Evolution of neoantigen landscape during immune checkpoint blockade in non-small cell lung cancer. Cancer Discov *7(3)*: 264-276, 2017. PMID: 28031159. DOI: 10.1158/2159-8290.CD-16-0828

43 Rooney MS, Shukla SA, Wu CJ, Getz G and Hacohen N: Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell *160(1-2)*: 48-61, 2015. PMID: 25594174. DOI: 10.1016/j.cell.2014.12.033

44 Reiman JM, Knutson KL and Radisky DC: Immune promotion of epithelial-mesenchymal transition and generation of breast cancer stem cells. Cancer Res *70(8)*: 3005-3008, 2010. PMID: 20395197. DOI: 10.1158/0008-5472.CAN-09-4041

45 Bruttel VS and Wischhusen J: Cancer stem cell immunology: Key to understanding tumorigenesis and tumor immune escape? Front Immunol *5*: 360, 2014. PMID: 25120546. DOI: 10.3389/fimmu.2014.00360

46 Schatton T, Frank NY and Frank MH: Identification and targeting of cancer stem cells. Bioessays *31(10)*: 1038-1049, 2009. PMID: 19708024. DOI: 10.1002/bies.200900058

47 Schatton T, Schutte U, Frank NY, Zhan Q, Hoerning A, Robles SC, Zhou J, Hodi FS, Spagnoli GC, Murphy GF and Frank MH: Modulation of t-cell activation by malignant melanoma initiating cells. Cancer Res *70(2)*: 697-708, 2010. PMID: 20068175. DOI: 10.1158/0008-5472.CAN-09-1592

48 Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C, Fuhlbrigge RC, Kupper TS, Sayegh MH and Frank MH: Identification of cells initiating human melanomas. Nature *451(7176)*: 345-349, 2008. PMID: 18202660. DOI: 10.1038/nature06489

49 Lee Y, Shin JH, Longmire M, Wang H, Kohrt HE, Chang HY and Sunwoo JB: Cd44+ cells in head and neck squamous cell carcinoma suppress t-cell-mediated immunity by selective constitutive and inducible expression of pd-l1. Clin Cancer Res *22(14)*: 3571-3581, 2016. PMID: 26864211. DOI: 10.1158/1078-0432.CCR-15-2665

50 Peng D, Tanikawa T, Li W, Zhao L, Vatan L, Szeliga W, Wan S, Wei S, Wang Y, Liu Y, Staroslawska E, Szubstarski F, Rolinski J, Grywalska E, Stanislawek A, Polkowski W, Kurylcio A, Kleer C, Chang AE, Wicha M, Sabel M, Zou W and Kryczek I: Myeloid-derived suppressor cells endow stem-like qualities to breast cancer cells through il6/stat3 and no/notch cross-talk signaling. Cancer Res *76(11)*: 3156-3165, 2016. PMID: 27197152. DOI: 10.1158/0008-5472.CAN-15-2528

51 Alix-Panabieres C, Mader S and Pantel K: Epithelial-mesenchymal plasticity in circulating tumor cells. J Mol Med (Berl) *95(2)*: 133-142, 2017. PMID: 28013389. DOI: 10.1007/s00109-016-1500-6

52 Santisteban M, Reiman JM, Asiedu MK, Behrens MD, Nassar A, Kalli KR, Haluska P, Ingle JN, Hartmann LC, Manjili MH, Radisky DC, Ferrone S and Knutson KL: Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. Cancer Res *69(7)*: 2887-2895, 2009. PMID: 19276366. DOI: 10.1158/0008-5472.CAN-08-3343

53 Boyle ST and Kochetkova M: Breast cancer stem cells and the immune system: Promotion, evasion and therapy. J Mammary Gland Biol Neoplasia *19(2)*: 203-211, 2014. PMID: 24997735. DOI: 10.1007/s10911-014-9323-y

Received December 4, 2020
Revised January 3, 2021
Accepted January 14, 2021