Circulating Immune Biomarkers in Peripheral Blood Correlate With Clinical Outcomes in Advanced Breast Cancer

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TITLE: CIRCULATING IMMUNE BIOMARKERS IN PERIPHERAL BLOOD CORRELATE WITH CLINICAL OUTCOMES IN ADVANCED BREAST CANCER.

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Declarations

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Conflicts of interest/Competing interests

Authors declare no potential conflicts of interest with respect to this study

Ethics approval

Protocol was approved by the VMUH's institutional review board (ref. LCM-INM-2015-01/Law 14/2007, of July 3) according to the ethical principles included in Declaration of Helsinki 1964 (2013 update)

Consent to participate

All the patients signed written informed consent to participate in this study.

Consent for publication

All the authors gave their consent to submit this version of the manuscript

Availability of data and material

Data and materials are accessible under formal request

Code availability (software application or custom code)

Not applicable

Authors' contributions

All the authors participated in the following tasks of the work:

1. Substantial contributions to conception and design, acquisition of data, and/or analysis and interpretation of results.
2. Substantial scientific and intellectual contributions to the drafting or rewriting of the initial and/or revised manuscript.
3. Approval of the final accepted version of the manuscript.

ABSTRACT

Identification of the different elements intervening at the immunoediting process, in each body compartment, seems key to explain clinical evolution in several tumor types. In this study, a set of immune biomarkers (myeloid derived suppressor cells, regulatory T cells, and OX40+ and PD-1+ T lymphocytes counts) in peripheral blood of patients diagnosed with advanced breast cancer, were analyzed prior to and along the implementation of first line antineoplastic therapy. Subsequently, a formal comparison between groups with clinical benefit versus progression of disease and with a healthy women cohort was executed.

Results reflected that patients basally showed higher levels of myeloid derived suppressor cells (35.43, IR=180.73 vs 17.53, IR=16.96 cells/ μ l; $p=0.001$) and regulatory T cells (32.05, IR=29.84 vs 22.61, IR=13.57 cells/ μ l; $p=0.001$) in comparison with healthy women. Furthermore, after therapy, an increase in the number of activated T lymphocytes (expressing OX40), and a decrease of immune inhibitory cells (MDSCs, and Tregs), and the number of inhibited (or exhausted) T lymphocytes (expressing PD-1), could be ascertained in patients with clinical benefit ($p\leq 0.001$). The opposite trend was observed in the case of disease progression. These findings suggest that some critical immune elements can be easily detected and measured in peripheral blood, which open a new opportunity for translational research, as they seem to be clearly correlated with clinical evolution, at least in ABC.

Key words: MDSCs, regulatory T cells, OX40, peripheral blood, advanced breast cancer.

INTRODUCTION

Breast cancer (BC) is the leading cause of cancer in women with more than 2 million of new cases worldwide every year. At present, although it is considered that 80-85% of the BC patients can be cured, a sizeable proportion recurs in an advanced stage and eventually die as a consequence of the disease[1].

Immunoediting phenomenon has been postulated as a crucial event in BC initiation and progression, and this condition can be detected and revealed through a wide set of assays, both in the tumor and peripheral blood[2]. Immunoediting hypothesis recognizes the crucial role of the different elements of tumor microenvironment (TME), with respect to development and progression of tumors, including breast cancer[3,4]. Consequently, the balance between immune inhibitory and stimulatory signals is determinant to explain the final effect, anergy or activation of the immune system against cancer cells. Density and function of some cell populations seem extraordinary relevant in this context and, interestingly, they can be detected and measured not just in TME but even in peripheral blood, what renders many advantages over tissue biopsies. At this point, myeloid derived suppressor cells (MDSCs) and different lymphocyte subpopulations, such as regulatory T cells (Tregs) [5], activated CD4+ and CD8+ T lymphocytes (expressing OX-40) or inhibited T lymphocytes (expressing PD-1) [6,7], and other subset of cells can be measured in peripheral blood of patients with advanced breast cancer (ABC) [3,4,8-10].

MDSCs constitute a population of immature myeloid cells with an extensive variety of immunosuppressive properties[11]. Their role in cancer has been recently elucidated, demonstrating in different studies that higher levels of MDSCs represent an adverse prognostic factor[12-14]. The main mechanisms for immunosuppression that MDSCs set up are the following: alteration of L-arginine metabolism through Arginase-1 (Arg-1) production and via iNOS allowing NO generation, promotion of cysteine uptake, stimulation of Tregs and tumor-associated macrophages type-2 (TAM-2) production, and inhibition of CD8+ T lymphocytes and natural killer T (NKT) function[15].

MDSCs can be detected in peripheral blood in cancer patients, especially in advanced stages. However, due to the intrinsic heterogeneity of these cells, analytical characterization has been challenging. At present, MDSCs are generally defined by CD45+CD3-CD19-CD20-CD56-CD16-HLADR-CD33+CD11b+ expression through flow cytometry, being CD14+ and CD15+ subsets assigned as monocytic and granulocytic MDSCs, respectively[16].

In the present work we aimed to analyze circulating MDSC levels from ABC patients in a basal state immediately after diagnosis of metastatic disease and along treatment implementation. Subsequently, a formal comparison between groups with clinical benefit (CB) versus progression of disease (PD) was executed. Other lymphocyte subpopulations were analyzed, including immunosuppressive Tregs, activated (OX-40+) and inhibited (PD-1+) CD4+ and CD8+ T lymphocytes. In addition, a healthy women (HW) cohort was included as control group.

MATERIALS AND METHODS

Patients consecutively diagnosed of ABC and HW were invited to participate in the study at Virgen Macarena University Hospital (VMUH) in Seville (Spain). Protocol was approved by the VMUH's institutional review board (ref. LCM-INM-2015-01/Law 14/2007, of July 3) according to the ethical principles included in Declaration of Helsinki 1964 (2013 update) [17]. Research was partially supported by a grant from the Andalusian Public Foundation Progress and Health (PI-0502-2014 FPS-2014). All the patients gave written informed consent to participate in this study.

Patients received systemic therapies following local protocols based on International Guidelines (NCCN[18] and ABC Guidelines[19]). Clinical response was assigned following Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v 1.1.) [20] considering Clinical Benefit (CB) the sum of Complete Responses (CR), Partial Responses (PR) and Stabilization of Disease (SD). ABC patients were classified in luminal A/B, triple negative and HER2+ subtypes, by immunohistochemistry criteria according to the established St Gallen international guidelines[21].

Flow cytometric analyses in whole blood samples.

Peripheral blood samples were collected from subjects in EDTA-K3 tubes before treatment onset and pre-dose to cycle 3 and cycle 6, to determine MDSCs, Tregs, and OX40+ and PD-1+ T lymphocytes (TL) counts. Cell populations were determined by flow cytometry of whole blood using the BD FACSCanto™ flow cytometry system. M-MDSCs were determined as CD45+CD11b+CD33+HLA-DR-CD14+CD15-, G-MDSC as CD45+CD11b+CD33+HLA-DR-CD14-CD15+, Tregs as CD3+CD4+CD25+CD127-, activated TL were determined as CD3+CD4+OX40+ TL and CD3+CD8+OX-40+ TL, inhibited TL as CD3+CD4+OX-40-PD-1+ and CD3+CD8+OX-40-PD-1+. Leukocyte and lymphocyte counts were obtained from hemotologic count (Sysmex CS-1000).

Monoclonal antibodies.

Antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA, USA) and were used at the manufacturer's recommended concentrations.

MDSCs: PerCP-Cy5.5 Mouse Anti-Human CD 45 (ref no. 564105), APC-Cy7 Rat Anti-CD11b (ref no. 557657), PE Mouse Anti-Human CD 33 (ref no. 555450), PE-Cy7 Mouse Anti-Human HLA-DR (ref no. 560651), FITC Mouse Anti-Human CD 14 (ref no. 555397) and APC Mouse Anti-Human CD 15 (ref no. 551376).

Treg kit (ref no. 560249), including FITC anti-Human CD4, PE-Cy7 anti-Human CD25, and Alexa-Fluor® 647 anti-Human CD127.

Activated and inhibited T Lymphocytes: FITC Mouse Anti-Human OX-40 (CD134) ref no. 555837, PE Mouse Anti-Human CTLA-4 (CD152) (ref no.555853), PerCP-Cy5.5 Mouse Anti-Human CD8 (ref no. 565310), PE-Cy7 Mouse Anti-Human CD4 (ref no. 557852), APC Mouse Anti-Human PD-1 (CD279) (ref no. 558694), APC-H7 Mouse Anti-Human CD3 ref no. 560176).

Data analysis.

Statistical analysis was performed by SPSS 26.0 software package (SPSS Inc., Chicago, IL, USA). Normal distribution of analysed variables was checked by watching histogram, box plot, Q-Q plot- and the outcomes of normality tests of Kolmogorov-Smirnov (samples $\geq$ 50) and Shapiro-Wilk (samples $<$ 50). Due to absence of normality, the lymphocytes distributions before and after systemic therapies were compared using non-parametric Wilcoxon test for related pairs. Lymphocytes distributions from CB vs PD were compared using non-parametric U-Mann-Whitney test for independent samples. Basal lymphocytes distributions from ABC patients versus HW were also compared using non-parametric U-Mann-Whitney test. Statistically significant differences were considered at the 95% level of confidence ($p\leq 0.05$).

RESULTS

From 15 January 2016 to 13 February 2020 a total of 20 HW and 51 ABC patients signed informed consent and were consecutively recruited to participate in the present study. Main clinico-pathological characteristics can be summarized as follows: most of the patients (82%) were included in the luminal A + Luminal B (her2-ve) BC entities. With respect to 1st line treatment, 61 % were treated with cyclin-dependent kinase 4/6 inhibitors plus hormonotherapy, 18% with hormonotherapy, 16% received chemotherapy, 6% were treated with anti-her2 monoclonal antibodies and 2% with chemoimmunotherapy. By 13 February 2020, the following basal measurements from peripheral blood were taken: CD8+ T and CD4+ T lymphocytes with expression of OX-40,

CD8+ T and CD4+ T lymphocytes with expression of PD-1, Tregs and MDSC levels. Furthermore, in ABC patients these levels were determined in cycle 3 (C3) and cycle 6 (C6) to monitor their evolution. In C3, measurements were undertaken in 45 patients since three of them died due to PD; in two patients, response status could not be established by RECIST 1.1 criteria, and finally in three women C3 extraction had not yet been done due to SARS-CoV-2 (COVID-19) pandemic. C6 analysis could be performed in 40 patients since one of them passed away due to clinical PD and in another four patients last extraction had not yet been done either for the same reason as in C3. Objective response by RECIST 1.1 criteria could be established in 48 patients after using computed tomography (CT) in C3 (+/- 30 days) **Figure 1**.

1. Clinical significance in ABC of circulating MDSC

1.1. MDSC levels are increased in ABC patients

Baseline MDSC levels before starting treatment in ABC patients (median cells/ μ l, 35.43; interquartile range (IR)=180.73) were higher than in HW (median, 17.53; IR=16.96) ($p=0.001$).

In addition, basal levels of granulocytic and monocytic MDSCs were also significantly higher in ABC patients compared to HW ($p\leq 0.035$). Medians were 18.46 (IR=30.67) cells/ μ l in ABC vs 10.28 (IR=17.48) cells/ μ l in HW for M-MDSC and 11.38 (IR=57.60) cells/ μ l in ABC vs 4.99 (IR=16.96) cells/ μ l in HW for G-MDSC. **Table 1; Figure 2**.

1.2. Low circulating MDSC levels are associated with clinical benefit

MDSC, M-MDSC and G-MDSC levels decreased along treatment in ABC patients with CB whilst they increased in those with PD, being the difference statistically significant ($p \leq 0.004$) in ABC patients with CB vs PD at C6.

Medians of MDSC were 33.63 (IR=39.34) at baseline vs 10.36 (IR=15.84) cells/ μ l at C6 in ABC patients with CB while they were 37.48 (IR=20.63) at baseline vs 78.54 (IR=128.63) cells/ μ l at C6 in ABC patients with PD. Medians of M-MDSC were 18.46 (IR=33.49) at baseline vs 4.15 (IR=6.48) cells/ μ l at C6 in ABC patients with CB while they were 16.20 (IR=22.97) at baseline vs 42.04 (IR=86.99) cells/ μ l at C6 in ABC patients with PD. Medians of G-MDSC were 12.74 (IR=18.14) at baseline vs 3.36 (IR=7.03) cells/ μ l at C6 in ABC patients with CB while they were 14.20 (IR=28.56) at baseline vs 10.00 (IR=24.23) cells/ μ l at C6 in ABC patients with PD. **Tables 2-3; Figure 3**.

2. OX-40 expression from CD4+ T and CD8+ T lymphocytes in ABC

2.1. Increased OX-40+ T lymphocyte levels are detected in ABC patients

Basal total OX-40+, CD4+ T and CD8+ T lymphocyte levels before starting treatment in ABC patients were higher than in HW. Medians of total OX-40+ T lymphocyte were 9.89 (IR=28.71) cells/ μ l in ABC patients vs 3.01 (IR=3.01) cells/ μ l in HW, being the difference statistically significant ($p=0.001$). **Table 1; Figure 2**.

2.2. Increased OX-40+ T lymphocyte levels in ABC patients are correlated with clinical benefit.

OX-40+, CD4+OX-40+ and CD8+OX-40+ T levels increase along treatment in ABC patients with CB while decrease in those with PD. Values at baseline vs C6 showed statistically significant differences ($p \leq 0.009$) in ABC patients with CB but not in PD.

Medians of OX-40+ T lymphocyte levels in ABC patients with CB were 9.89 (IR=28.64) at baseline vs 37.28 (IR=43.54) cells/ μ l at C6; CD4+OX-40+ T were 8.32 (IR=25.46) at baseline vs 33.22 (IR=40.49) cells/ μ l at C6, and CD8+OX-40+ T were 1.85 (IR=2.37) at baseline vs 2.63 (IR=5.72) cells/ μ l at C6. **Tables 2-3; Figure 3**.

3. PD-1 expression from CD4+ T and CD8+ T lymphocytes in ABC

3.1 PD-1+ T lymphocyte levels decrease in ABC patients

Basal total PD-1+ T and CD4+PD-1+ T lymphocyte levels before starting treatment in ABC patients were slightly lower than in HW, but differences did not reach statistical significance. Medians of total PD-1+ T lymphocytes were 10.77 (IR=8.55) cells/ μ l in ABC patients vs 13.00 (IR=6.19) cells/ μ l in HW, ($p=0.245$). **Table 1; Figure 2**.

3.2 Decreased PD-1+ T lymphocyte levels in ABC patients predict clinical benefit.

PD-1+, CD4+PD-1+ and CD8+PD-1+ T levels diminished along treatment in ABC patients regardless of response, however values at baseline vs C6 showed statistically significant differences ($p \leq 0.001$) only in ABC patients with CB but not in PD. Medians of PD-1+ T lymphocyte levels in ABC patients with CB were 11.16 (IR=8.99) at baseline vs 4.82 (IR=3.74) cells/ μ l at C6; CD4+PD-1+ T were 6.95 (IR=5.08) at baseline vs 2.93 (IR=3.12) cells/ μ l at C6, and CD8+OX-40+ T were 3.62 (IR=2.45) at baseline vs 1.51 (IR=1.05) cells/ μ l at C6. **Tables 2-3; Figure 3**.

4. Levels of regulatory T cells (Tregs) in ABC

4.1. Tregs levels are increased in ABC patients

Basal Tregs levels before starting treatment in ABC patients were higher than in HW with a median of 32.05 (IR=29.84) vs 22.61 (IR=13.57) cells/ μ l, being the difference statistically significant ($p=0.001$). **Table 1; Figure 2.**

4.2. Decreased Tregs levels are observed in ABC patients with clinical benefit

Tregs levels decreased along treatment in ABC patients, being the difference statistically significant ($p \leq 0.001$) in ABC patients with CB.

Medians of Tregs were 31.87 (IR=27.87) at baseline vs 19.01 (IR=16.64) cells/ μ l at C6 in ABC patients with CB while they were 39.30 (IR=52.24) at baseline vs 28.36 (IR=32.03) cells/ μ l at C6 in ABC patients with PD. **Tables 2-3; Figure 3.**

DISCUSSION

In the last few years, growing translational evidence has definitively established immunoediting as a prominent hallmark in BC that should not be overlooked[22-27]. Relationship between the presence of TILs and MDSCs in the tumoral microenvironment (TME) and the response to treatment and survival of patients has been described. Thus, in certain subtypes of breast cancer, i.e. triple-negative and HER2 breast subtypes, a higher presence of TILs in TME implies improved results in pathological complete responses in the neoadjuvant setting and an impact in survival is suggested, although clinical evidence at this point is more debatable[23,28]. A relation between the presence of elevated circulating MDSCs and prognosis have been clearly established, being associated with advanced cancer stage, higher tumor burden, lower progression-free survival (PFS) and overall survival (OS) rates[14, 29-32] as well as with the response to cancer treatment by decreasing the efficacy of chemotherapy, radiotherapy, immunotherapy or other targeted therapies[33-35].

MDSCs represent a heterogeneous population of immature myeloid cells among which we can distinguish two main types based on their phenotype and morphology: the granulocytic subtype (G-MDSC) and the monocytic subtype (M-MDSC). In our research, we have used the following surface markers to identify them: CD45+CD11b+CD33+ HLA-DRlow/- in addition to CD14-CD15+ for G-MDSCs and CD14+CD15- for M-MDSC[16]. Slightly higher levels of M-MDSC over G-MDSCs have been observed in our patients. M-MDSC is a subtype that seems to be related with a greater immunosuppressive role, through NO generation, in comparison with G-MDSC[36].

In physiological conditions, the granulocyte-macrophage colony-stimulating factor (GM-CSF) induces myelopoiesis while the granulocyte colony-stimulating factor (G-CSF) and the macrophage colony-stimulating factor (M-CSF) stimulate the differentiation of granulocytes and macrophages in response to pathogen-derived signals like Toll-like receptors (TLR) ligands, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular pattern (PAMPs) molecules. These events trigger the mobilization of neutrophils and monocytes with the production of inflammatory cytokines and costimulatory molecules. In pathological conditions of chronic inflammation or cancer, the myelopoiesis continues and is characterized, among other events, by the overproduction of MDSCs[37]. Firstly, the expansion phase of immature myeloid cells takes place through the influence of factors produced by the tumor stroma or the bone marrow like GM-CSF, G-CSF, M-CSF, stem cell factor (S-SCF) or the vascular endothelial growth factor (VEGF). Secondly, the suppressing action of MDSC is executed by the production of reactive oxygen species (ROS), cytokines (TGF- β , IL-10, IL-6), the expression of enzymes such as indoleamine 2,3-dioxygenase (IDO) or arginase-1 (ARG1) and the production of prostaglandin E2 (PGE2) [38,39].

At the TME level, tumors that induce expansion of MDSCs in their surrounding stroma contribute to the tumor-induced immune suppression mechanisms by inhibiting the effector function of T cells and natural killer cells through several cellular exhaustion and inactivation mechanisms[40], which are enhanced by the increased production of Tregs. The generation of ROS impair recognition through the class II major histocompatibility complex (MHC II) while nitric oxide (NO) inhibits the IL-2 receptor (IL-2R) that, in turn, affects negatively signaling via T cell receptor (TCR). Furthermore, there is a depletion of L-arginine due to the expression of high levels of ARG1 which leads to a decrease of the average life of mRNA in the CD3 ζ chain of T cells. Aforementioned events, added to decreased secretion of IFN- γ and granzyme B, damage the activity of effector T lymphocytes, and may even lead to their apoptosis[41].

In our research, ABC patients showed increased levels of MDSCs and Tregs in peripheral blood in comparison with healthy women. Therefore, it is plausible to consider that immune profile of the patients is impaired,

facilitating an immune microenvironment that favors the tumor development and progression, as the escape phase of the immunoediting hypothesis dictates. Even more interesting, patients that received cancer treatment and experienced clinical benefit, whether this was a partial or complete response or stabilization of the disease, experienced a drastically drop in MDSCs and Tregs levels, whilst those patients in which disease progressed showed a dramatically increase in MDSCs levels. These findings strengthen the immunoediting postulates in BC, and suggest that this could be a reversible process, as immune profiling of the patients improve when disease back to be under control.

The immunosuppressive role of MDSCs and Tregs and their influence in the anergy of T cells is remarkable in the tumoral microenvironment[42]. The greater expression of immune checkpoint molecules like PD-1/PDL-1 to damage or delay the immune response by inhibiting T cells or the expression of immune system co-stimulators such as OX-40 is remarkable too. These biomarkers seem potential promissory targets for an outstanding performance of immunotherapy and personalized therapy[24]. Furthermore, some new emerging data suggest that the presence of IDO and ARG-1 by MDSCs upregulates the expression of PD-1 over MDSCs, that is, by blocking PDL-1 we would manage to mitigate the activity of MDSCs[43].

In our work, we could demonstrate enhanced levels of CD4+ and CD8+ T lymphocytes with expression of OX-40 in ABC patients compared to healthy women. However, it was in the healthy cohort where we observed a higher percentage of T lymphocytes with expression of the co-repressive molecule PD-1, which reveal the great complexity of the mechanisms of interrelation among the immune system elements beyond those described here: the release of inhibitory cytokines, the expansion of T regulators and tumor-associated macrophages (TAM), the expression of immune checkpoint and the presence of MDSCs working all together to create this immunosuppressive microenvironment[3,15,34-36]. Nonetheless, with respect to CD4+ T and CD8+ T expressing OX-40, we could observe an exceptional rise in these cells in the ABC cohort that reached CB. On the contrary, patients that progressed showed declining and lower levels of OX40 T cells. Furthermore, a significant decrease in CD4+ T and CD8+ T with expression of PD-1 along treatment in ABC patients with CB could be ascertained.

These findings seem quite promising since they highlight the undeniable immunosuppressive role of MDSCs and the co-repressive/co-stimulatory activity of PD-1/OX-40 molecules, respectively. The depletion in the levels of MDSC, Tregs and the increase of OX-40 along antineoplastic treatment, whether this is hormonotherapy alone, chemotherapy, hormonotherapy plus cyclin-dependent kinase inhibitors 4/6, or other combined strategies, could improve immune profile of the patients reverting their immunosuppressive status with respect to cancer cells. These determinations could also become potential biomarkers to monitor in a simple way by means of a simple peripheral blood extraction the response to treatment.

Several combinations of treatments are being carried out in preclinical studies that target MDSC and immune checkpoint, boosting the immune effector activity in cancer. Recently, it has been revealed that the inhibition of PI3K, JAK/STAT and CDK4/6 pathways improve the infiltration of cytotoxic CD8+ T type TILs in mouse models with triple-negative breast cancer. This effect seems tightly related to the decrease of the MDSCs levels, what ultimately facilitate the action of effector T cells. In top of that, this effect may be boosted by blocking some specific immune checkpoint inhibitors[44]. Consequently, targeting MDSCs aimed to deplete them can potentially increase the efficacy of the modern immune checkpoint inhibitors currently in use for several oncological diseases[45].

Results of this study put the focus on potential biomarkers easily detectable in peripheral blood to monitor the response of antineoplastic treatments in ABC. Likewise, it permits foresee new targets for immunomodulatory strategies in breast cancer. There are uncountable therapeutic possibilities at this point, since there are different options to boost the immune response of the host whether employing classic agents (doxorubicin, cyclophosphamide, gemcitabine, and so on) or newer agents such as anti-PD-1 (antibodies that block the inhibiting and proapoptotic effect of their signals in T cells), anti-OX-40 or anti-CD40 (antibodies that stimulate the activating signaling of T lymphocytes) and/or within the context of combinations.

Although data from this work seem clear, additional confirmatory studies with higher sample size and/or with other tumor types are obviously needed. Nonetheless, new avenues of research can be suggested from these findings since a sort of immune liquid biopsy might proportionate extraordinarily valuable and complementary information in BC, not only in the advanced setting but even also during follow-up of BC patients previously treated with curative purposes.

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TABLES

MDSC						
	Total		M-MDSC		G-MDSC	
	Median	IR	Median	IR	Median	IR
ABC	35.43	180.73	18.46	30.67	11.38	57.60
HW	17.53	16.96	10.28	17.48	4.99	16.96
p value	0.001		0.035		0.004	
OX-40+						
	Total		CD4+OX-40		CD8+OX-40	
	Median	IR	Median	IR	Median	IR
ABC	9.89	28.71	7.36	26.33	1.64	2.14
HW	3.01	3.10	2.81	2.83	0.28	0.26
p value	<0.001		<0.001		<0.001	
PD-1+						
	Total		CD4+ PD-1+		CD8+ PD-1+	
	Median	IR	Median	IR	Median	IR
ABC	10.77	8.55	6.95	5.14	3.63	2.57
HW	13.00	6.19	10.04	5.27	3.18	2.47
p value	0.245		0.156		0.591	
Treg						
	Median			IR		
ABC	32.05			29.84		
HW	22.61			13.57		
p value	0.001					

Table 1. Basal medians of MDSC, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ul) in ABC patients and HW. MDSC, myeloid derived suppressor cells; M-MDSC, monocytic MDSCs; G-MDSC, granulocytic MDSCs; Tregs, regulatory T lymphocytes; ABC, advanced breast carcinoma; HW, healthy women; IR, interquartile range.

MDSC						
	Total		M-MDSC		G-MDSC	
	Median	IR	Median	IR	Median	IR
Basal	32.05	29.84	18.46	30.67	11.38	16.77
C3	19.79	16.75	7.09	12.54	4.03	9.64
C6	19.01	21.51	4.89	10.64	4.85	8.31
Basal vs C6 P value	< 0.001		< 0.001		< 0.001	
OX-40+						
	Total		CD4+OX-40		CD8+OX-40	
	Median	IR	Median	IR	Median	IR
Basal	9.89	28.71	7.36	26.33	1.64	2.14
C3	13.75	25.94	12.23	22.72	1.24	2.28
C6	35.10	45.56	30.17	41.34	2.46	4.97
Basal vs C6 P value	0.014		0.007		0.034	
PD-1+						
	Total		CD4+ PD-1+		CD8+ PD-1+	
	Median	IR	Median	IR	Median	IR
Basal	10.77	8.55	6.95	5.14	3.62	2.56
C3	5.02	5.01	3.51	3.69	1.82	1.81
C6	5.17	3.90	2.93	3.33	1.61	1.12
Basal vs C6 P value	< 0.001		< 0.001		< 0.001	
Treg						
	Median			IR		
Basal	32.05			29.84		
C3	19.79			16.75		
C6	19.01			21.51		
Basal vs C6 P value	0.022					

Table 2. Basal, C3 and C6 medians of MDSC, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ul) in ABC patients. MDSC; myeloid derived suppressor cells; M-MDSC; monocytic MDSCs; G-MDSC: granulocytic MDSCs; Tregs, regulatory T lymphocytes; C3, cycle 3; C6, cycle 6; IR, interquartile range.

MDSC												
	Total				M-MDSC				G-MDSC			
	CB		PD		CB		PD		CB		PD	
	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR
Basal	33.63	39.34	37.48	20.63	18.46	33.49	16.20	22.97	12.74	18.14	14.20	28.56
C3	16.54	12.88	25.32	31.26	7.09	12.90	7.50	12.24	3.23	6.70	5.63	20.09
C6	10.36	15.84	78.54	128.63	4.15	6.48	42.04	86.99	3.36	7.03	10.00	24.23
Basal vs C6 P value	<0.001		0.176		<0.001		0.310		<0.001		0.612	
CB vs PD (C6) P value	0.003				0.004				0.002			
OX-40+												
	Total				CD4+OX-40+				CD8+OX-40+			
	CB		PD		CB		PD		CB		PD	
	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR
Basal	9.89	28.64	11.71	36.9	8.32	25.46	6.14	35.77	1.85	2.37	1.40	1.75
C3	15.23	26.78	4.57	12.9	12.93	25.02	4.00	10.97	1.24	2.29	1.20	1.29
C6	37.28	43.54	5.91	57.62	33.22	40.49	2.22	45.44	2.63	5.72	1.75	3.33
Basal vs C6 P value	0.001		0.866		0.001		0.866		0.009		0.917	
CB vs PD (C6) P value	0.199				0.199				0.419			
PD-1+												
	Total				CD4+PD-1+				CD8+PD-1			
	CB		PD		CB		PD		CB		PD	
	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR	Median	IR
Basal	11.16	8.99	11.03	11.18	6.95	5.08	5.95	8.58	3.62	2.45	4.51	4.34
C3	5.02	3.99	4.96	14.81	3.51	3.28	2.16	8.11	1.63	1.62	2.24	6.66
C6	4.82	3.74	7.64	4.55	2.93	3.12	5.98	4.86	1.51	1.05	1.80	3.72
Basal vs C6 P value	<0.001		0.063		<0.001		0.499		<0.001		0.237	
CB vs PD (C6) P value	0.058				0.212				0.240			
Tregs												
	CB				PD							
	Median		IR		Median		IR					
Basal	31.87		27.87		39.30		52.24					
C3	20.79		15.01		17.63		38.37					
C6	19.01		16.64		28.36		32.03					
Basal vs C6 P value	0.001				0.866							
CB vs PD (C6) P value	0.680											

Table 3. Basal, C3 and C6 medians of MDSC, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ul) in ABC patients according to response. MDSC; myeloid derived suppressor cells; M-MDSC; monocytic MDSCs; G-MDSC; granulocytic MDSCs; Tregs, regulatory T lymphocytes; CB, clinical benefit; PD, progression of disease; C3, cycle 3; C6, cycle 6; IR, interquartile range.

FIGURES

Figure 1. Diagram. HW, healthy women; ABC, advanced breast cancer; RECIST v1.1, response evaluation criteria in solid tumors.

Figure 2. Basal medians of MDSC, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ul) in ABC patients and HW. MDSC, myeloid derived suppressor cells; M-MDSC, monocytic MDSCs; G-MDSC, granulocytic MDSCs; ABC, advanced breast carcinoma; HW, healthy women. #, ## and ###, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ compared with ABC patients. Different scales are used.

Figure 3. Basal, C3 and C6 medians of MDSCs, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ul) in ABC patients (A) according to response (B). MDSCs; myeloid derived suppressor cells; M-MDSC; monocytic MDSCs; G-MDSC; granulocytic MDSCs; Tregs, regulatory T lymphocytes; CB, clinical benefit; PD, progression of disease; C3, cycle 3; C6, cycle 6. *, ** and ***, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ compared with basal measurements, respectively. ##, $p \leq 0.01$ compared with CB patients. Different scales are used.

Figures

Figure 1

Diagram. HW, healthy women; ABC, advanced breast cancer; RECIST v1.1, response evaluation criteria in solid tumors.

Figure 2

Basal medians of MDSC, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ μ L) in ABC patients and HW. MDSC, myeloid derived suppressor cells; M-MDSC, monocytic MDSCs; G-MDSC, granulocytic MDSCs; ABC, advanced breast carcinoma; HW, healthy women. #, ## and ###, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ compared with ABC patients. Different scales are used.

Figure 3

Basal, C3 and C6 medians of MDSCs, OX-40+, PD-1+ and regulatory T lymphocytes levels (cells/ μ L) in ABC patients (A) according to response (B). MDSCs; myeloid derived suppressor cells; M-MDSC; monocytic MDSCs; G-MDSC: granulocytic MDSCs; Tregs, regulatory T lymphocytes; CB, clinical benefit; PD, progression of disease; C3, cycle 3; C6, cycle 6. *, ** and ***, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ compared with basal measurements, respectively. ##, $p \leq 0.01$ compared with CB patients. Different scales are used.