Circulating Immune Cell Populations Related to Primary Breast Cancer, Surgical Removal and Radiotherapy Revealed by Flow Cytometry Analysis

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Research article

Keywords: Breast cancer, Radiotherapy, MDSC, CD117, FlowJo, Unsupervised analysis, Biomarker

Posted Date: January 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-146187/v1

Abstract

Background: Advanced breast cancer (BC) impact immune cells in the blood but whether such effects may reflect the presence of early BC and its therapeutic management remains elusive.

Methods: To address this question, we used multiparametric flow cytometry to analyse circulating leukocytes in patients with early BC (n=13) at time of diagnosis, after surgery and after adjuvant radiotherapy, compared to healthy individuals. Data were analysed using a minimally supervised approach based on FlowSOM algorithm and validated manually.

Results: At time of diagnosis, BC patients have an increased frequency of $CD117^+$ Granulocytic-Myeloid Derived Suppressor Cells (G-MDSC), which was significantly reduced after tumor removal. Adjuvant radiotherapy increased the frequency of $CD45RO^+$ memory $CD4^+$ T cells and $CD4^+$ regulatory T cells. FlowSOM algorithm analysis revealed several unanticipated populations, including cells negative for all markers tested, $CD11b^+CD15^{low}$, $CD3^+CD4^-CD8^-$, $CD3^+CD4^+CD8^+$, $CD3^+CD8^+CD127^+CD45RO^+$ cells, associated with BC or radiotherapy.

Conclusions: This study revealed changes in blood leukocytes associated with primary BC, surgical removal and adjuvant radiotherapy. Specifically, it identified increased levels of $CD117^+$ G-MDSC, memory and regulatory $CD4^+$ T cells as potential biomarkers of BC and radiotherapy, respectively. Importantly, the study demonstrates the value of unsupervised analysis of complex flow cytometry data to unravel new cell populations of potential clinical relevance.

Background

Breast cancer (BC) is the most frequent cancer and main cause of cancer-related mortality for women in industrialized countries (1). Three clinically relevant biological BC subtypes (i.e. Oestrogens/Progesterone Receptor positive, Human Epidermal growth factor Receptor 2 (HER2) amplified and triple negative), and multiple molecular subtypes (e.g. Luminal A/B, HER2, basal like, normal like) with distinct features and clinical outcomes, have been defined and characterized (2–5).

Early detection and surgery in combination with adjuvant treatments tailored on biological and molecular subtypes, have improved patients' survival by about 30% in the past three decades (6). Goal of adjuvant therapy, including radiotherapy, is the eradication of tumor cells that disseminated before diagnosis and surgery. Some of these disseminated tumor cells (DTC), however, will escape therapy and later progress to form metastases, which in most patients represents the main causes of cancer-related death. After breast-conserving surgery, radiotherapy reduces the risk of BC recurrence and death. Among women with operable BC, randomized trials have demonstrated equivalent disease-free and overall survival between mastectomy and breast-conserving surgery followed by radiotherapy alone and/or hormonal, anti-HER2, or chemotherapy (7–16).

Mammography is the standard approach for the detection of asymptomatic BC (17). In spite of its benefits in reducing BC specific mortality, mammography has some important limitations (18): low specificity and sensitivity; risk of over-diagnosis; risk of inducing BC due to X-ray exposure, particularly in patients with defective DNA repair genes (19); not recommended before the age of 50 in spite of the fact that 20–25% of all BCs appear before this age. There is therefore an unmet need for complementary or alternative methods for the detection of asymptomatic, early BC (20–22). Circulating tumor cells (CTC), cell free tumor-derived DNA, mRNA and miRNA, proteins, autoantibodies and metabolites are being explored as candidate blood-based biomarkers for BC detection, diagnosis or monitoring, but so far none entered routine clinical practice (23–27). Similarly, there are no effective blood-based biomarkers to actively assess patients' response to treatment and monitoring disease state after therapy. Also the most used in clinical practice biomarker protein such as CA 15 – 3 is not specific and sensitive in early breast cancer diagnosis (28).

Tumors, including BC, mobilize and recruit immuno-inflammatory cells to their microenvironment (29–31). Monocytic and granulocytic cells, mostly immature forms, as well as lymphocytes, contribute to cancer progression by promoting immunosuppression, angiogenesis, cancer cell survival, growth, invasion and metastasis (32,33). We have previously shown that metastatic BC patients have elevated frequencies of $TIE2^+CD11b^+$ and $CD117^+CD11b^+$ leukocytes circulating in the blood, and that circulating $CD11b^+$ cells express higher mRNA levels of the M2 polarization markers CD163, ARG1 and IL-10 (34). Treatment with paclitaxel in combination with bevacizumab decreased the frequency $CD117^+CD11b^+$ leukocytes, IL-10 mRNA levels in $CD11b^+$ cells and IL-10 protein in plasma. We therefore considered that blood circulating leukocytes, or sub-population thereof, may reflect cancer-relevant immuno-inflammatory events that may be further explored as BC-associated biomarkers.

Here, we analysed the phenotype of blood leukocytes of patients with early BC at time of diagnosis, after surgery, and after adjuvant RTX, relative to healthy donors (HD), using flow cytometry and a minimally supervised analytical approach based on FlowSOM algorithm and manual validation. We identified cell populations associated with the presence of a primary BC, tumor removal and adjuvant radiotherapy. Unsupervised clustering analysis approach of the same flow cytometry data allowed discovering unanticipated cell populations associated with BC or adjuvant radiotherapy. These results indicate that phenotypical analysis of peripheral blood leukocytes, with a minimally supervised analytical approach, may be a clinically-relevant strategy for the identification of cellular biomarkers for BC detection and therapy monitoring.

Materials And Methods

Patients and clinical study

The study was approved by the Cantonal ethic commission for human research on Humans of Canton Ticino (CE 2967) and extended to Vaud-Fribourg-Neuchâtel, Switzerland. The study includes 13 female patients (Table 1) who were diagnosed with primary, non-metastatic BC (stage T1-4, N0-N1, M0,). All

patients underwent conservative surgery and received standard fractionated adjuvant radiotherapy (2 Gy per session, total dose : 50 + 10 Gy). Blood samples were collected after confirmed diagnosis/before surgery, after surgery/before radiotherapy, at the end of radiotherapy (week 6), and 6–8 weeks after the end of the radiotherapy (week 12–15). All Patients and HDs gave written informed consent before study entry. Patients were recruited before surgery at Clinica Luganese Moncucco, Lugano, and at Hôpital Neuchâtelois, La Chaux-de-Fonds, once diagnosis was histologically confirmed. Mean age for cancer patients was 60.6 years (all patients were between 43 and 73 years old). HDs were recruited along the study, based on the following criteria: age-matched relative to BC patients, no regular medications in the last 6 months, no previous cancer diagnosis, no chronic diseases and normal blood analyses at time of recruitment.

Blood processing

20 ml of peripheral venous blood was collected using BD Vacutainer® Blood Collection EDTA Tubes (Becton Dickinson, Franklin Lakes, NJ, USA) following manufacturer's instructions and immediately shipped by courier at room temperature to the laboratory. All analyses were performed within 24 hours after blood collection. Antibody staining was performed in whole blood. Plasma and total leukocytes were isolated from the remaining blood using BD Vacutainer® CPT™ Cell Preparation Tube (Becton Dickinson) with Sodium Heparin following manufacturer's instructions. Plasma fraction was frozen at -80 °C and isolated leukocytes were lysed in RA1 lysis buffer (Macherey-Nagel, Düren, Deutschland) and stored at -80 °C.

Flow cytometry

Whole blood staining's were performed within 24 hours after blood collection. Leukocytes were counted using Cell-Dyn Sapphire Hematology System (Abbott Diagnostics, Chicago, IL, USA). For staining, 1 million cells per tube were used. Directly labelled antibodies were added to whole blood and incubated for 20 minutes at 4 °C, followed by 10 minutes red-blood-cells lysis (Bühlmann Laboratories, Schönenbuch, Switzerland) and washing using cold PBS. All anti-human antibodies were used at the concentrations recommended by the manufacturer: anti-CD15-PeCy7 (clone HI98), anti-CD14-Pe (clone MφP9), anti-CD163-FITC (clone GHI/61), anti-CD11b-BV510 (clone ICRF44), anti-CD33-V450 (clone WM53), anti-CD64-APCH7 (clone 10.1), anti-CD117-APC (clone YB5.B8), anti-CD45RA-PeCy7 (clone HI100), anti-CD25-Pe (clone M-A251), anti-CD4-FITC (clone RPA-T4), anti-CD8-V500 (clone SK1), anti-CD45RO-BV421 (clone UCHL1), anti-CD3-APCH7 (clone SK7), and CD127-Alexa Fluor 647 (clone HIL-7R-M21) and 7AAD (all from Becton Dickinson). BD FACSCanto II (Becton Dickinson) instrument was used to analyse samples and FlowJo 10.6.2 (Treestar Inc., Ashland, OR, USA) software and several software plugins (FlowCLEAN, downsample_V3, FlowSOM, tSNE) were used to analyse all data.

Reverse transcription real-time PCR (RT-qPCR)

Total mRNA from total white blood cells was extracted using the NucleoSpin RNA kit from Macherey-Nagel following manufacturer's instructions (Düren, Germany). The purity and quantity of all RNA samples were examined by NanoDrop (Witec AG, Luzern, Switzerland). Total RNA was retro-transcribed

using M-MLV reverse transcriptase kit following manufacturer's instructions (ThermoFisher Scientific, Waltham, Massachusetts, USA) using 500 ng of total RNA. cDNA was subjected to amplification by real time qPCR with the StepOne SYBR System (Life Technologies) using the following primer pairs (Eurofins Genomics, Huntsville, AL, USA) at the indicated hybridization temperatures: GAPDH 58 °C (Fw-TCTTCTTTTGCGTCGCCAGC, Rev-GATTTTGGAGGGATCTCGCTCCT), ARG1 58 °C (Fw-GGAGTCATCTGGGTGGATGC, Rev-CTGGCACATCGGGAATCTTTC), IL-10 58 °C (Fw-CGAGATGCCTTCAGCAGAGT, Rev-AATCGATGACAGCGCCGTAG), CD117 57 °C (Fw-GATTATCCCAAGTCTGAGAATGAA, Rev-CGTCAGAATTGGACACTAGGA), FN1 52 °C (Fw-ACTTCGACAGGACCACTTGA, Rev-TCAAATTGGAGATTCATGGGA). Real-time PCR data were then analysed using the comparative Ct method [55].

Statistical analysis

Acquired data were analysed and graphics were generated using Prism Software (GraphPad, La Jolla, CA, USA). Statistical comparisons between cancer patients and healthy donors were performed by T-test assuming non-homogenous variance. Normality distribution of the samples was checked in case of significance and, if non-Gaussian, a Mann-Whitney replaced the T-test results. Statistical comparisons of all time points to observe the effect of radiotherapy were performed by one-way ANOVA assuming non-homogenous variance using Tukey correction. Normality distribution of the samples was checked in case of significance and, if non-Gaussian, a Kruskal-Wallis assay replaced the ANOVA results. Highest and lowest values from each group were excluded. Results were considered to be significantly from $p < 0.05$. In the figures, the various p values thresholds are presented as follow: ≤0.05=*, ≤ 0.01=**, ≤ 0.001=***, ≤ 0.0001=****.

Results

Increased frequency of $CD117^+$ G-MDSCs in the peripheral blood of patients with newly diagnosed non-metastatic BC

Based on previous observations made in metastatic BC patients (34), we hypothesized that an increased frequency of circulating $CD11b^+$ cells expressing CD117 and/or displaying a M2 activation phenotype, may also occur in patients with early BC. To test this hypothesis, we monitored the frequency of MDSC cells in the blood of non-metastatic BC patients (cT1-4, N0-1, M0) at time of diagnosis using Flow Cytometry. Aged-matched women without BC served as control population (healthy donors – HDs). Monocytic-MDSC (Mo-MDSC) were defined as $CD11b^+CD33^+CD14^{high}CD15^-$ and granulocytic-MDSC (G-MDSC) as $CD11b^+CD33^+CD14^{low}CD15^+$ cells. In both cell populations we monitored the expression of CD117, the receptor for Kit-ligand/stem cell factor widely present in hematopoietic progenitors cells (35,36), and CD163, a M2 polarization marker in monocytes (37). In order to avoid investigator-associated biases and variability in the results inherent to supervised manual analysis of flow cytometry data, we develop a minimally supervised, standardized analytical workflow based on the FlowSOM algorithm (Supplementary Fig. 1), in complement to conventional manual gating and supervised analysis.

Cells clusters revealed by standardized analytical workflow, were considered of interest when their frequency was more than 10% different between HDs and cancer patients. Some of the 14 analysed clusters corresponded to non-standard populations, for example those negative for all tested markers or expressing unanticipated marker combinations (Fig. 1A-C). These populations would have been missed by conventional supervised gating and analysis driven by the marker combination of interest. Interestingly, we observed a significant increase in the frequency of $CD117^+$ cells among the circulating G-MDSC population in cancer patients relative to HDs (Fig. 1D). We observed a similar (but non-significant) trend in the frequency of Mo-MDSC cells, albeit at lower frequency. In addition, the frequencies of some non-classical cell populations, such as those expressing none of the markers of interest (Cluster 13 and 3) or a $CD11b^+CD15^{low}$ cell population (Cluster 22 + 9), were significantly different between BC patients and HDs (Fig. 1E and F). No significant changes were observed for $CD163^+$ cells in both G-MDSC and Mo-MDSC populations (Supplementary Figure S2).

Non-standard CD3 expressing cells are present with increased frequency in the peripheral blood of newly diagnosed BC patients

In parallel we monitored the presence of selected lymphocyte populations in both groups. By conventional supervised analysis we observed no differences in classical $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+CD4^+CD25^+CD127^-$ regulatory T cells (Tregs). Likewise, we observed no changes in the frequency of memory ($CD45RO^+CD45RA^-$) or naïve ($CD45RA^+CD45RO^-$) T cells within the same lymphocyte populations (Supplementary Figure S3).

In contrast, FlowSOM analysis performed on lymphocytes revealed 21 populations that were more then 10% differentially represented between HDs and cancer patients (Fig. 2A-C). A population of cells of the size of lymphocytes, but negative for CD3, CD4 or CD8 expression (Cluster 24 + 29) was significantly less represented in cancer patients relative to HDs. $CD3^+CD8^+$ T cells expressing CD127 and CD45RO markers (Cluster 3 + 7) are present at significantly higher frequency in cancer patients. A cell cluster expressing CD3, but not CD4 or CD8 (Cluster 20) was fond more represented in cancer patients relative to HDs (Fig. 2D-F).

Taken together these results reveal an increased frequency of peripheral blood $CD117^+$ G-MDSC in non-metastatic BC patients at time of diagnosis, as well as significant changes in the frequency of myeloid and lymphocytic cell populations expressing unconventional marker combinations. They also demonstrate that FlowSOM-based analysis can identify cell populations that would have been likely missed by supervised analysis.

No detectable changes in the expression level of transcripts for M2 polarization markers

We previously reported that transcripts of M2-associated genes were expressed at higher levels circulating $CD11b^+$ cells in metastatic BC patients compared to HDs (34). We therefore analysed expression of mRNA for CD117 and the M2 markers IL-10, fibronectin-1 (FN1) and arginase 1 (ARG1) in

total leukocytes from cancer patient and HDs. No differences in expression levels were observed (Supplementary Figure S4).

A proof-of-concept study to monitor the effects of surgical tumor removal and adjuvant radiotherapy on circulating immune cells in BC patients

The differences observed in myelomonocytic and lymphocytic populations in BC patients at time of diagnosis relative to HDs, raised the question whether tumor removal and/or adjuvant therapy may reverse these changes, or induce additional ones. To address this question, we performed a proof-of-concept study, by taking advantage of the fact that the investigated patients were scheduled for breast conservative tumor removal and adjuvant radiotherapy as part of their standard treatment. Adjuvant radiotherapy was selected as therapy of choice as systemic effects on the immune system have been reported (38–40), while on the other side chemotherapy was excluded in order to avoid that myelosuppressive effects induced by chemotherapy could non-specifically impact the results (41). To search for potential changes in cell populations in response to surgery and radiotherapy we analysed G-MDSC and Mo-MDSCs as well as lymphocytes at three time points : after surgery/before radiotherapy start (1_PostOP), at the end of radiotherapy (6 weeks; 2_Post_RTX_6w) and at 6–8 weeks after the end of radiotherapy (12–14 weeks; 3_Post_RTX_12w). Results were compared to values obtained at time of diagnosis (0_PreOP) (Fig. 3).

Tumor removal increased the frequency Mo-MDSCs and G-MDSCs but decreased $CD117^+$ G-MDSCs and radiotherapy induced changes in non-standard myeloid cell populations

Using FlowSOM workflow of analysis we observed distinct expression profiles at the four time-points globally visualized by tSNE. 17 cell clusters were found highly differentially represented in one group compared to the other groups. Surprisingly, when looking at the expression profiles of each cluster of interest, the majority of them was lacking CD33 expression, suggesting that this marker may not be suitable to analyse the monocytes fraction (Fig. 4A-B).

After tumor removal and at the end of radiotherapy the frequency of both Mo-MDSCs and G-MDSCs was significantly increased relative to values at time of diagnosis, and returned to pre-therapy levels 6–8 weeks after the end of radiotherapy (Fig. 4C-D). Strikingly, within the G-MDSCs population, the fractions of CD117 expressing cells significantly decreased after tumor removal and this decrease persisted after the end of radiotherapy (Fig. 4E). Surgery had no impact on the fraction of $CD163^+$ G-MDSCs population (Fig. 4F). Radiotherapy itself had an impact on G-MDSC expressing CD163, but not CD117 (Fig. 4E-F).

The presence of one particular cell cluster expressing only CD11b and CD15 (Pop 1, 6 and 7) clearly decreased after radiotherapy. Another population expressing CD11b but lacking expression of all tested markers significantly increased during and after radiotherapy (Pop 16, 18, 12, 15 and 26) (Fig. 4G-H). The latter observation suggest that some populations defined by non-standard marker combinations may be potentially interesting candidates to investigate further with an extended panel or markers.

Analysis of total blood leukocytes for CD117, IL-10, FN1, and ARG1 mRNA expression by RT-qPCR revealed no observable differences in their expression levels (Supplementary Figure S5).

Adjuvant radiotherapy increases the frequency of $CD4^+$ memory and regulatory T cells, and induces changes in non-standard lymphocytic populations

Likewise, we performed unsupervised analysis of the lymphocyte populations at the three time-points after surgery and radiotherapy. Visualization by tSNE revealed distinctive changes in marker expression profiles. Eleven cells clusters were found highly differentially represented in one group compared to the other ones (Fig. 5A-B). After tumor removal we observed highly variable effects on the frequency of T lymphocyte subpopulations, most of which were inconsistent and statistically non-significant.

Among the stably differentially represented clusters at the various time-points, a $CD3^+$ cell population positive for CD4 and CD8 (Cluster 25), and a $CD3^+CD4^+CD127^+CD45RO^+$ population (Cluster 41) appeared at higher frequency after treatment (Fig. 5C-D). Strikingly, the frequency of this $CD45RO^+RA^-$ memory subset within the $CD3^+CD4^+$ lymphocyte population was significantly and consistently increased at the end of radiotherapy and this increase was still evident 6–8 weeks later. A similar increase was also present among $CD4^+$ regulatory T cells, corresponding to cluster 23 and 30, which also persisted after the end of radiotherapy (Fig. 5E-G).

Discussion

Mammography-based screening significantly reduces BC-related mortality, but intrinsic and practical limitations call for novel screening approaches (17,19,20,42). Blood based biomarkers exploiting cancer-derived CTC, DNA or RNA are being explored, but so far none reached clinical routine practice (22,43). Similarly, there are no valid biomarkers for monitoring patients' response to treatment or detecting relapses before they become symptomatic.

In this study we pursued the use of flow cytometry to analyse the phenotype and frequency of blood leukocytes in patients with non-metastatic BC at time of diagnosis, after surgical tumor removal and after adjuvant radiotherapy. Using a combination of minimally supervised (FlowSOM algorithm), and supervised (manual) analytical approaches, we report that: i) at diagnosis, BC patients have an increased frequency of circulating $CD117^+$ G-MDSC relative to age-matched healthy donors; ii) surgical tumor removal causes transient increase of G-MDSCs and M-MDSCs, and a long-lasting decrease of $CD117^+$ G-MDSC; iii) radiotherapy significantly increases $CD45R0^+$ memory T cells and $CD4^+$ Treg cells; iv) with the FlowSOM algorithm we identified additional unanticipated, non-classical cell populations differentially represented between HD and BC patients and in BC patients in response to therapy.

CD117, the receptor for Kit-ligand/stem cell factor, is widely expressed in hematopoietic progenitor cells in the bone marrow, while virtually no $CD117^+$ leukocytes are present in the circulation under homeostatic conditions (35). We have previously reported a role of $CD117^+$ leukocytes in metastasis in the murine 4T1

metastatic BC model (44) and the presence of $CD117^+CD11b^+$ cells in the blood of mBC patients (34). Here, we observed an increased frequency of $CD117^+$ cells among total $CD11b^+$ G-MDSCs in the peripheral blood of non-metastatic BC patients at time of diagnosis, compared to HD. Interestingly, the frequency of $CD117^+$ G-MDSCs significantly dropped upon tumor removal and remained below pre-treatments levels after radiotherapy. Thus, the increased frequency of $CD117^+$ G-MDSCs may reflect the presence of the primary tumor. No changes were observed in CD117 mRNA expression in total leukocytes. This could be due to that fact that $CD117^+$ cells are lost during leukocyte isolation for RNA extraction (flow cytometry was performed in non-separated total whole blood), or that CD117 mRNA expression has ceased upon cell mobilization (while CD117 protein persisted at the cell surface). The latter possibility is consistent with our previous observation that mobilized $CD117^+$ cells adoptively transferred to a recipient mouse, rapidly became CD117 negative (44). In contrast, the frequency of $CD163^+$ G-MDSCs remained rather constant, with only a transient decrease during radiotherapy. The implication of this decrease is unclear as CD163 expression did not significantly differ between HD and BC patients at time of diagnosis. After surgery and radiotherapy also no change in the mRNA expression of CD117 and ARG1, FN1, IL-10 (i.e., M2 polarization markers) was observed, owing probably to the lack of enrichment of $CD11b^+$ cells for PCR analysis.

In cancer patients at time of diagnosis we observed a higher frequency of atypical T lymphocytes ($CD3^+CD4^-CD8^-$) and of a population of the size of lymphocytes lacking expression of all the tested markers. These observations suggest that some potentially interesting changes may occur in atypical T cells or in non-T cell populations such as B cells or NK cells. Strikingly, after radiotherapy, we observed a steady and significant increase of the fraction of $CD45RO^+$ memory T cells within total $CD4^+$ T cells and within $CD4^+$ Treg. We also observed the increased presence of a T cell population expressing both CD4 and CD8 markers. This suggests that radiotherapy may cause T cell activation leading to the subsequent generation of memory T cell subsets. Indeed, there is evidence that radiotherapy exerts its therapeutic effects, not only in the local treatment field, but also outside the irradiated field and at distant sites (i.e. the so called abscopal effect), at least in part, by eliciting a T cell immune response (38). The recent observation that combination of radiotherapy with immune checkpoint inhibitors in experimental models and cancer patients results in potent synergistic therapeutic effects further support the involvement of T cell-dependent events and the therapeutic effects of radiotherapy (45–48). Through experimental work and mathematical modelling, it has been proposed that anti-tumor T cells may by mobilized by radiotherapy toward peripheral tissues to eliminate DTC (49–51). However, to date there is paucity of human data demonstrating specific changes in circulating T lymphocytes to support such a model. Radiotherapy was reported to cause a global reduction in circulating lymphocyte subsets in patients treated for stage I-II prostate cancer (52), or to induce an increase in $CD4^+$ Treg in the peripheral blood of patients with diverse solid cancers (53). Low-dose radon therapy for chronic inflammatory diseases was shown to induce a long-lasting increase in circulating T cells paralleled with a reduced expression of activation markers (54). Thus, the observed effect of adjuvant radiotherapy on memory $CD4^+$ T cells is

novel and should be further explored in conjunction with patients' outcome, as a possible biomarkers of therapy response or efficacy.

Conclusion

Taken together, this human exploratory study in early, non-metastatic BC revealed changes in blood leukocyte populations associated with the presence of BC, surgical removal and adjuvant radiotherapy. Specifically, we identified $CD117^+$ G-MDSC and $CD45RO^+$ $CD4^+$ memory T cells correlating with the presence of the primary tumor and radiotherapy, respectively. Importantly, the study demonstrates that a minimally supervised, algorithm-based analysis of flow cytometry data is a powerful tool to reproducibly detect phenotypical changes in peripheral blood leukocytes in cancer patients. The approach also identifies non-anticipated population correlated with disease state of therapy. These results should instigate the further investigation of peripheral blood leukocytes as source of reliable candidate biomarkers to detect BC, to monitor response to treatment and possibly disease progression.

List Of Abbreviations

BC Breast Cancer

CTC Circulating Tumor Cells

DTC Disseminated Tumor Cells

G-MDSC Granulocytic-Myeloid Derived Suppressor Cells

HD Healthy Donor

Mo-MDSC Monocytic- Myeloid Derived Suppressor Cells

Treg Regulatory T cell

Declarations

Ethics approval and consent to participate

The study was approved by the Cantonal ethic commission for human research on Humans of Canton Ticino (CE 2967) and extended to Vaud-Fribourg-Neuchâtel, Switzerland. All Patients and HDs gave written informed consent before study entry.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Consent for publication

All authors declare that they consent for the publication of the present manuscript.

Funding

This work was funded by the Swiss National Science Foundation, grant numbers 31003A_159824 and 31003A_179248 and the Medic Foundation to CR.

Authors' contributions

Conceptualization, C.R. and A.F.-P.; Methodology, S.C., M.B. C.R. and A.F.-P; Formal Analysis, S.C.; Investigation, S.C., B.F, L.N. and M.B.; Resources, A.B., A.C., A.CH., A.F.-P., P.T., A.S., L.N. M.B., and C.R.; Writing—original draft preparation, S.C. and C.R..; Writing—review and editing, A.F.-P., C.R., S.C. and P.T.; Supervision, S.C.; Project administration, C.R. and A.F.-P..; Funding acquisition, C.R.

All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors wish to tank Dr. Chiara Secondini for her help in establishing the clinical protocol and patient forms, Nathalie Duffey for her help in processing the samples and Dr. Med. Sebastiano Semini for his help in identifying healthy donors. The authors wish to thank all patients and healthy donors for participating to this study.

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Table

Table 1.

Clinical data of breast cancer patients included in the study.

Patient Number	Age	ER (%)	PR (%)	HER2 (+/-)	Ki67 (%)	Grade	Tumor size	LN mets	Anti-hormonal therapy
1	62	95	70	-	5	1	pT1b	pN1a	-
2	58	95	95	-	10	2	T1b	N0	-
3	50	100	100	+	5	1	pT1	pN0	Tamoxifen
4	73	95	2	-	25	2	pT2	pN1a	-
5	49	90	80	-	10	2	T1b	pN0	Tamoxifen
6	69	100	100	-	15	2	pT1a	pN0	Tamoxifen
7	53	95	60	-	10	2	pT1c	pN0	Letrozole
8	73	100	0	-	20	na	pT1c	pN0	Tamoxifen
9	67	90	80	-	5	1	pT1b	pN0	Tamoxifen
10	66	100	100	-	5	2	pT1c	pN0	Letrozole
11	64	95	80	-	10	1	pT1b	pN0	Letrozole
12	61	80	100	-	10	2	pT1b	pN0	Anastrozole
13	43	95	95	-	10	2	pT1c	pN0	Tamoxifen

Patient’s demographics, tumor subtype, grade, stage (pT and pN) and treatment after conservative surgery. ER (%): oestrogen receptor expression in percent; PR (%): progesterone receptor expression in percent; HER2 (±): overexpression of HER-2; Ki67 (%): fraction of cancer cells positive for Ki67 expression.

Figures

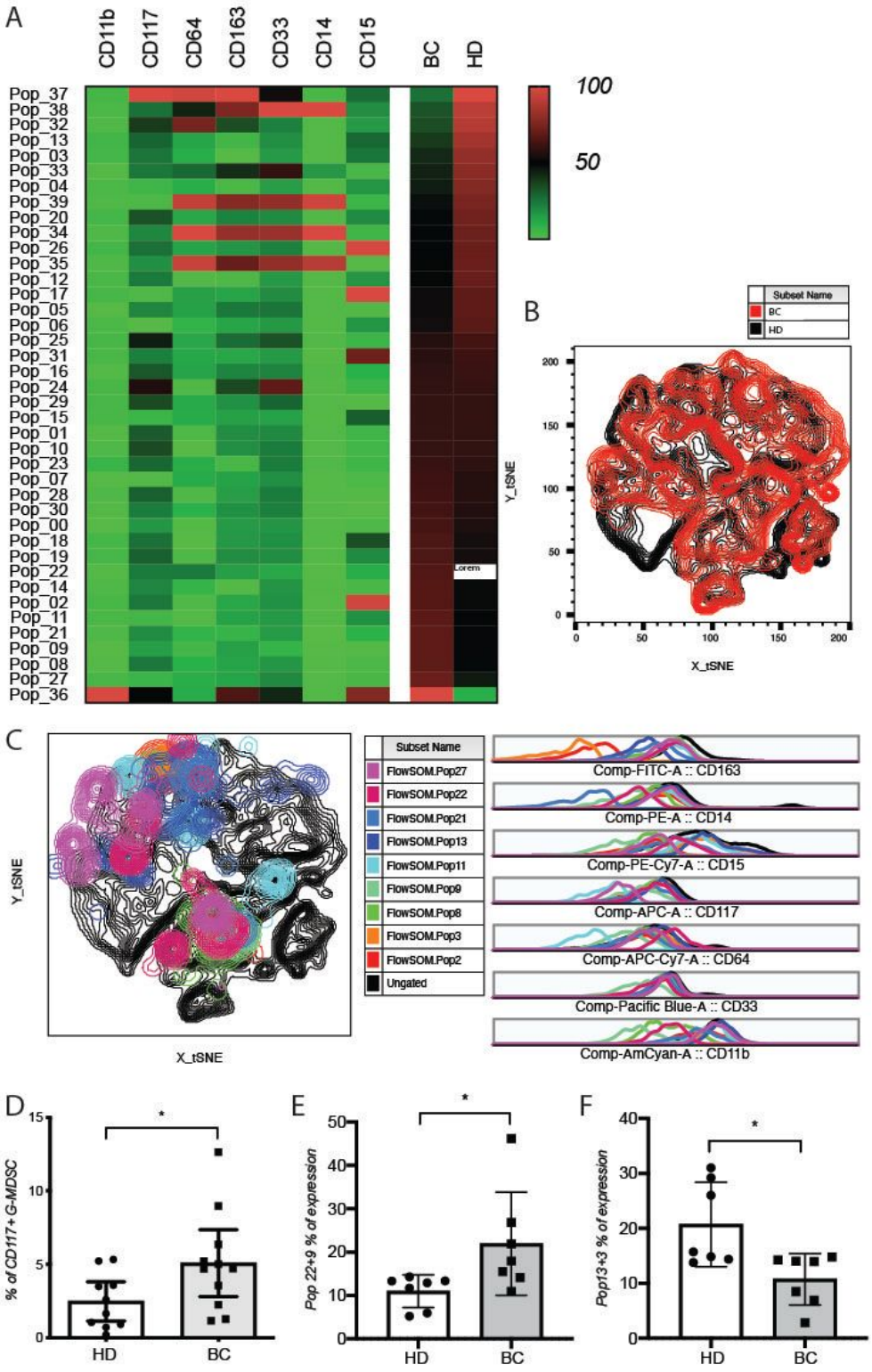

Figure 1

Altered frequency of circulating monocytic populations in cancer patients. (A) Heat map of the FlowSOM clustering between breast cancer patients (BC) and healthy donors (HD). tSNE visualization of (B) the monocytic expression profile and (C) the differentially expressed clusters in the blood of breast cancer patients at time of first diagnosis vs healthy donors. Frequency of (D) CD117+ G-MDSC population, and

the atypical populations (E) 22+9 and (F) 13+3 at the same timing. Cell analysis and quantification were performed by flow cytometry with FlowJo software and results are represented as mean values +/- SD.

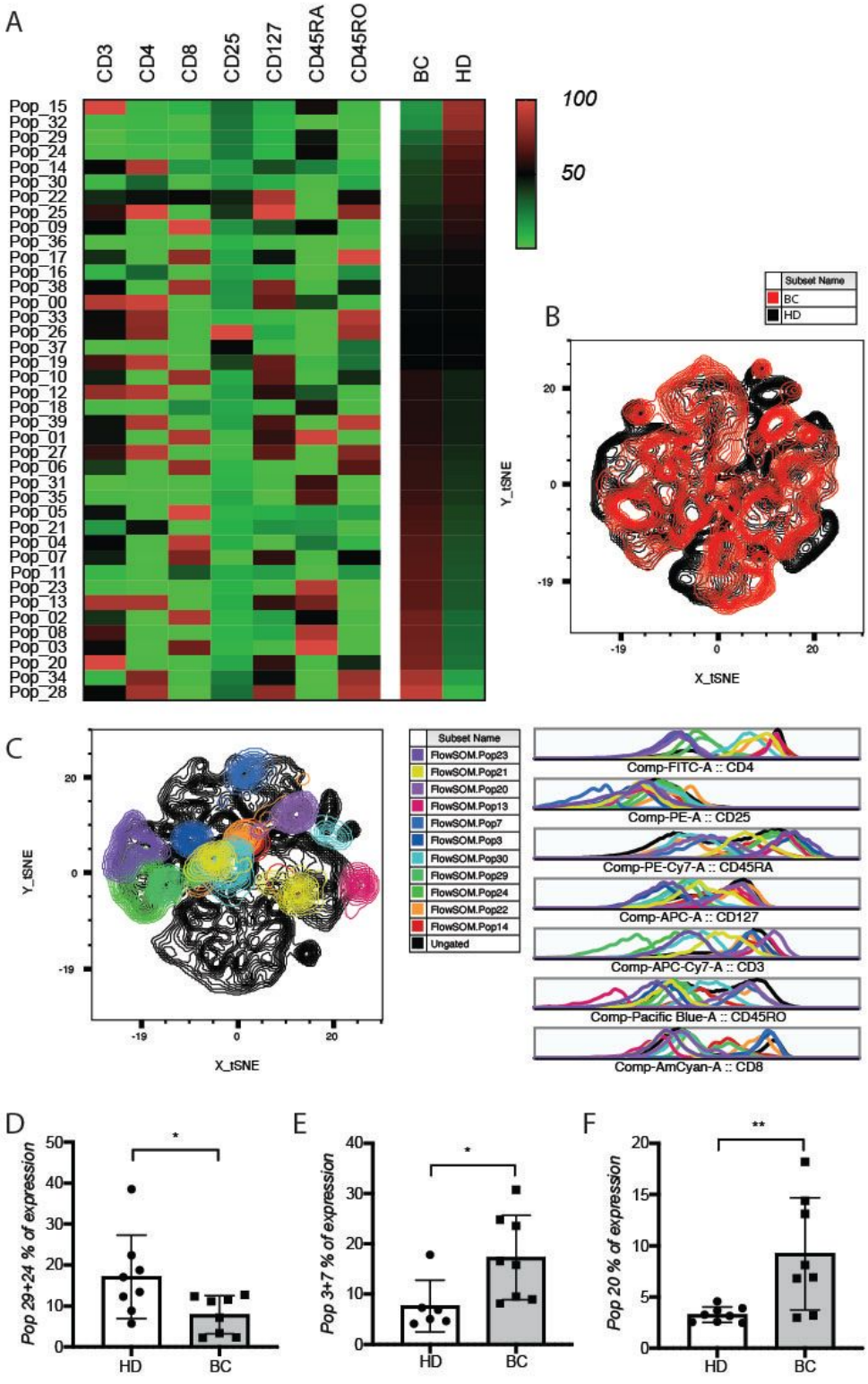

Figure 2

Altered frequency of circulating lymphocyte populations in cancer patients. (A) Heat map of the FlowSOM clustering between breast cancer patients (BC) and healthy donors (HD). tSNE visualization of (B) the lymphocyte expression profile and (C) the differentially expressed clusters in the blood breast

cancer patients at time of first diagnosis of healthy donors. Frequency of the atypical populations (D) 29+24, (E) 3+7 and (F) at the same timing. Cell analysis and quantification were performed by flow cytometry with FlowJo software and results are represented as mean values +/- SD.

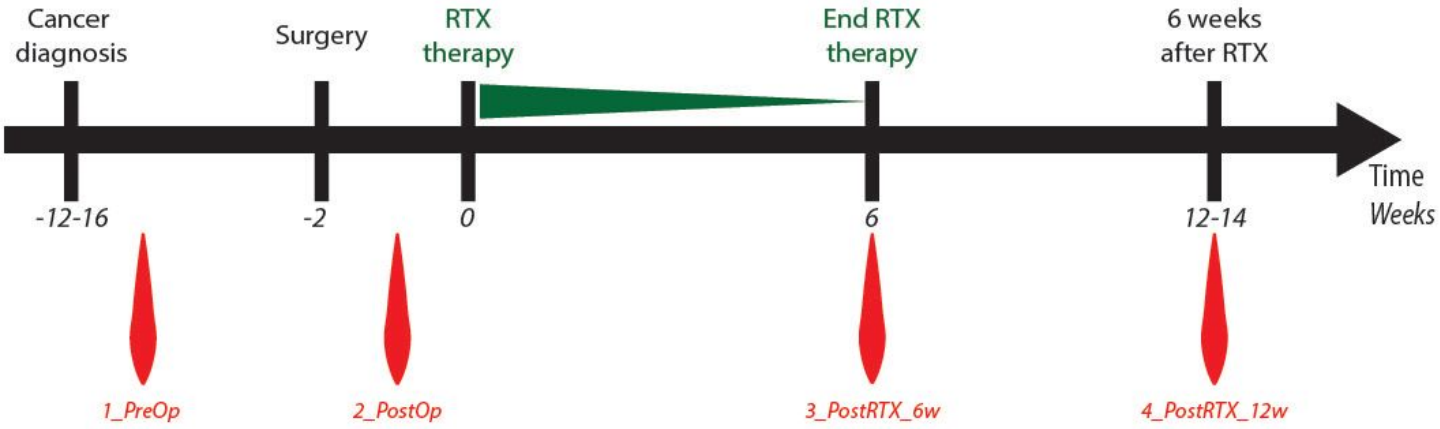

Figure 3

Schematic representation of the radiotherapy study. Patients were enrolled after confirmed histological diagnosis of breast cancer. All patients underwent conservative surgery and received standard fractionated adjuvant radiotherapy (2 Gy per session, total 50+10 Gy). Blood samples were collected after diagnosis/before surgery (Sample 1), after surgery/before radiotherapy (Sample 2), at the end of radiotherapy (Sample 3), and 6-8 weeks after the end of the radiotherapy (Sample 4).

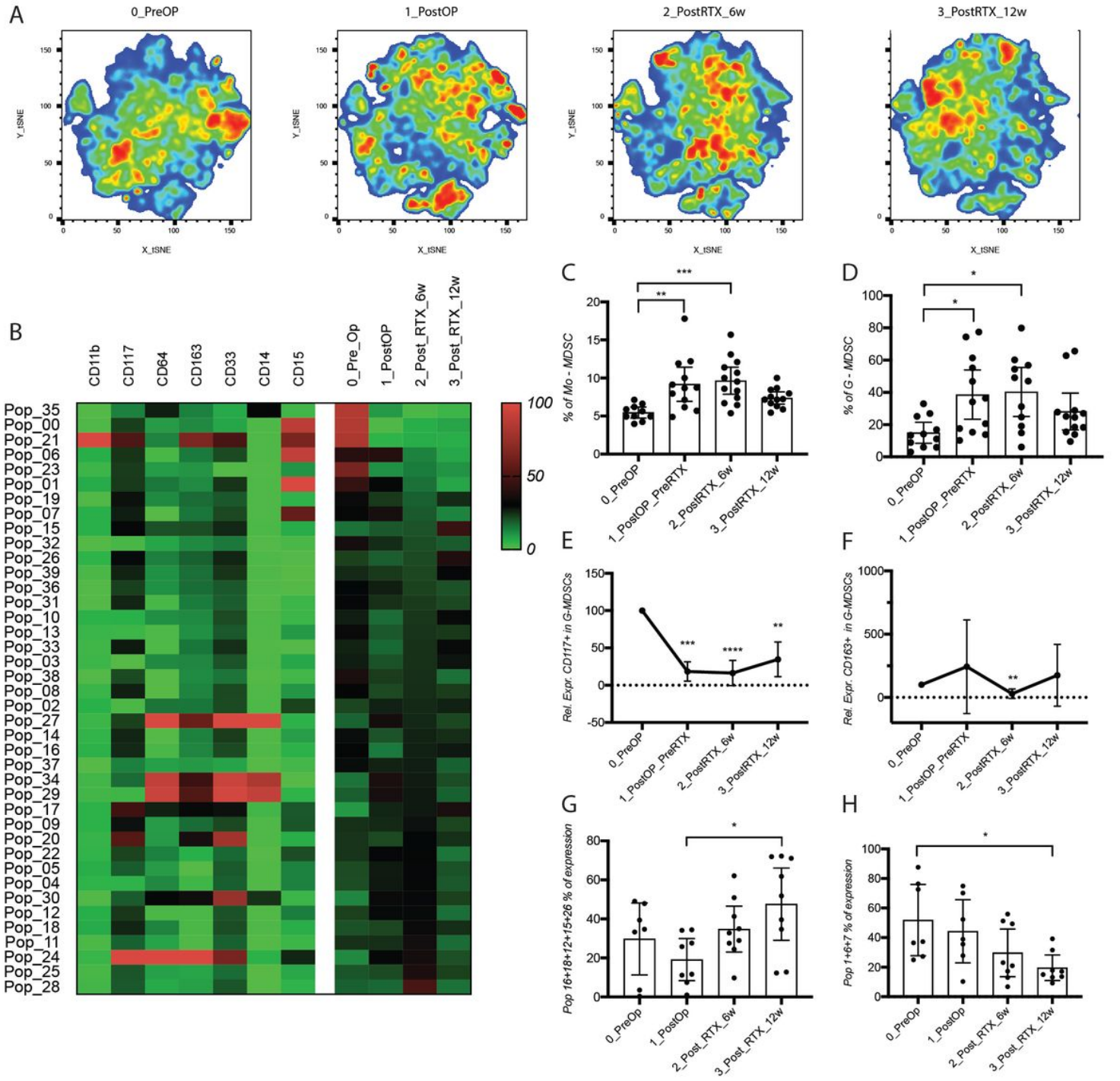

Figure 4

Tumor removal reduces the frequency of circulating CD117+ G-MDSC cells. (A) Comparative visualization of the expression of surface markers in monocytes at different time-points of treatment by tSNE. (B) Heat map of the FlowSOM clustering of breast cancer patients at indicated time-points. Frequency of (C) Mo-MDSC and (D) G-MDSC cell populations in patients at the indicated time points during treatment. Frequency of (E) CD163+ and (F) CD117+ G-MDSCs population at indicated time-points relative to frequency at 0_PreOp time-point. Frequency of the (G) combined 16+18+21+12+15+26 and (H) 1+6+7

atypical cell populations during treatment. Cell analysis and quantification was performed by flow cytometry with FlowJo software and results are represented as mean values +/- SD.

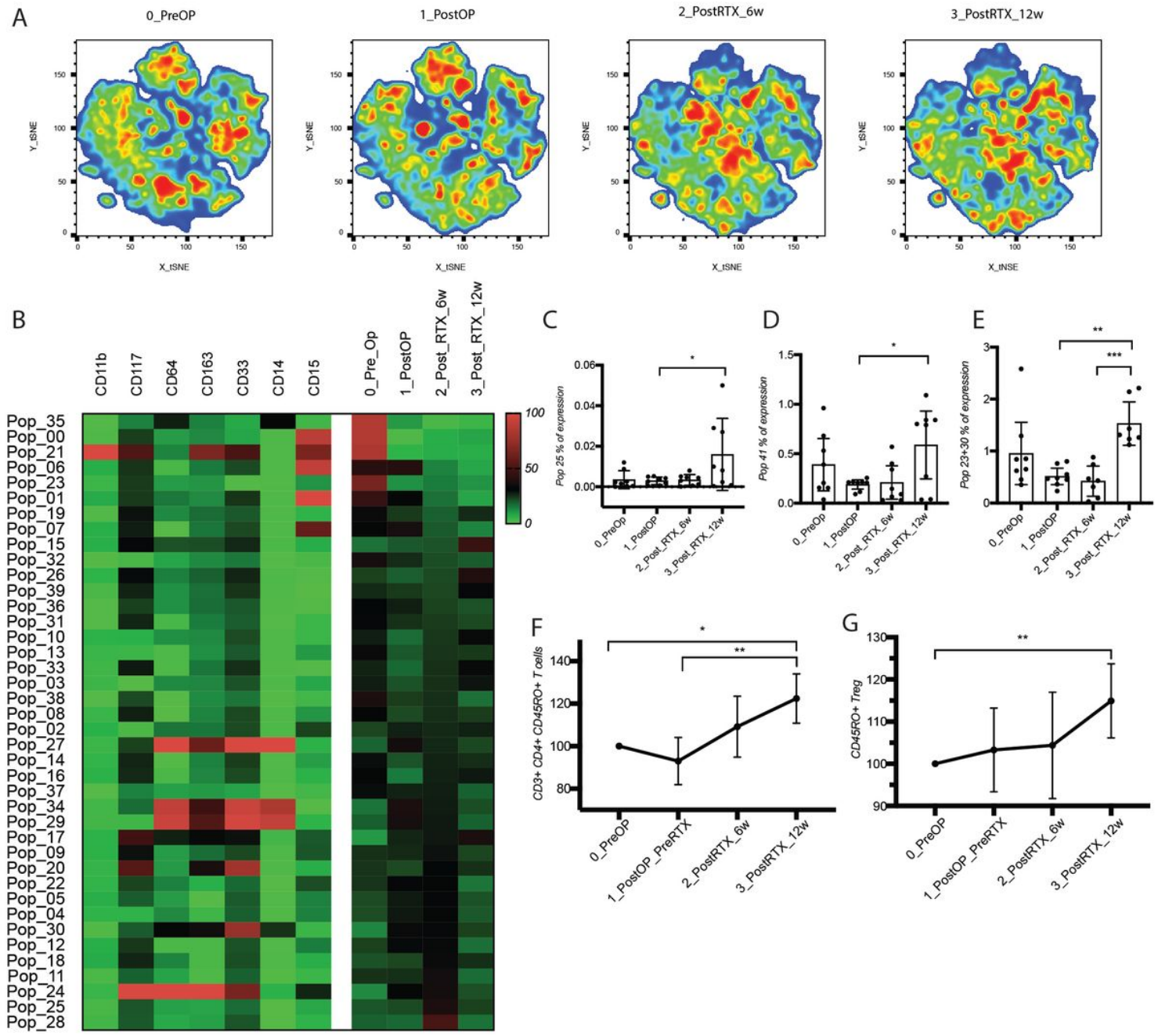

Figure 5

Tumor removal and radiotherapy reduce the fraction of CD117+ cells within the G-MDSC population. (A) Comparative visualization of the expression of surface markers in lymphocytes at different time-points of treatment by tSNE. (B) Heatmap of the FlowSOM clustering of breast cancer patients at indicated time-points during treatment. Frequency of the atypical populations (C) 25, (D) 41 and (E) 23+30 in patients during treatment. Relative quantification to 0_PreOp time-point of (F) CD4+ CD45RO+ lymphocytes and (G) CD45RO+ regulatory T cells during treatment. Cell analysis and quantification was performed by flow cytometry with FlowJo software and results are represented as mean values +/- SD.

Supplementary Files

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