Immune-phenotyping of peripheral blood mononuclear cells from patients with early triple-negative breast cancer: Identification of a systemic immunosuppressive CD3+ CD4+ CD39+ Treg subset.

Bernardo Leon Rapoport [1,2], Nicole Chicken [2], Pieter Meyer [2], Ronald Anderson [2], Carol Benn [3], Liezl Heyman [1], TeresaSmit [1], Catherine Worsley [2]

[1] Department of Immunology, Faculty of Health Science, University of Pretoria, South Africa

[2] The Medical Oncology Centre of Rosebank, Johannesburg, South Africa.

[3] The Netcare Breast Centre of Excellence, Netcare Milpark Hospital, Johannesburg, South Africa

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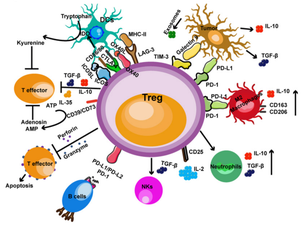
Background

- Immune cells and malignant cells interact in the tumor microenvironment, and this process influences treatment response and survival.
- TNBC is associated with an immunosuppressive microenvironment.
- The immuno-suppressive cells involved include Tregs, MDSC, and M2 macrophages.
- Tumor-infiltrating Tregs interact with both tumor and stromal cells as well as extracellular matrix components in the TME.
- Understanding the mechanism of Tregs in protecting TNBC from antitumor immune responses in the TME will pave the way for developing novel, immune-based therapeutics.
- The role of systemic, circulating immune cells, particularly those with immune suppressive activity is not well-characterized.

Figure 1. Immune suppressive functions of Tregs in the TME.



Targeting regulatory T cells for immunotherapy in melanoma Lili Huang et al, Molecular Biomedicine, Apr 19, 2021 (permission obtained)

Aim

- In this prospective, exploratory study we aim to delineate the clinical relevance of a broad panel of circulating immune cells in patients with newly-diagnosed localized TNBC before the start of neo-adjuvant treatment.
- The current study was undertaken to assess the incidence of immune cells systemically in patients with early triple-negative breast cancer (TNBC).

Methods

- 20 localized or loco-regional TNBC patients and 10 controls.
- The flow cytometry results for only 19 TNBC patients were evaluable.
- Multi-parameter flow cytometry was used to examine the percentages and phenotypes of immune cell subsets in the peripheral blood of the TNBC patients. These included:
  - cytotoxic T cells
  - natural killer (NK) cells
  - monocyte subsets
  - regulatory T cells (Tregs)
  - B Cells
- Multicolor flow cytometry was carried out using DURAClone IM phenotyping (basic and Treg tubes containing the following fluorochrome-labeled monoclonal antibodies) with cellular expression detected using a CytoFLEX Flow Cytometer (Beckman Coulter, California, USA).
- Basic Tube: CD16, CD56, CD19, CD14, CD4, CD8, CD3, and CD45.
- Treg Tube: CD3, CD4, CD25, CD39, CD45, CD45RA, FoxP3 and Helios.
- Peripheral blood samples of the patients were freshly collected and evaluated. Three flow cytometry tubes were used for control, basic- and Treg cell analysis.
- All flow cytometric data were analyzed using Kaluza Analysis Flow Cytometry software (v.2.2; Beckman Coulter).

Statistical Analysis:

- The normality of the obtained data was determined by Shapiro–Wilk tests and histogram analysis.
- Data in this study were expressed as medians and interquartile ranges.
- The Mann-Whitney U-test was used to compare non-parametric data where appropriate.
- In all analyses, $p < 0.05$ was considered statistically significant.
- The area under the ROC curve (AUC) was used as a measure of discriminatory ability for the biomarkers. The Youden index, a summary measure of the ROC curve, was used as an agnostic method for choosing an optimal cut-off value on the biomarker value to illustrate potential clinical usefulness.
- Data analyzed with FlowJo™ v 10.10.0 software (BD Life Sciences, USA).
- NCSS 2021 software for Windows (USA) was used for statistical analyses

Results

- Significant increases in the percentages of immunosuppressive CD3+ CD4+ CD39+ Tregs cells in patients with early TNBC.
- A significant decrease of CD19+ B cells was observed.
- Tumor size, nodal status, age, and ki-67 did not correlate with the percentages of any circulating immune cell.

Table 1. Patient Characteristics.

Age	
Median Age	49
Range	29-74
BMI	
Median 28	Range: 18-36
Menopausal Status	
Peri-menopausal	1 (5%)
Pre-menopausal	9 (45%)
Post-menopausal	10 (50%)
Ki-67 Grouped	
< 14%	0 (0%)
14% - 40%	3 (15%)
> 40%	17 (85%)
Tumor Size	
< 2cm	5 (25%)
≥ 2cm	15 (75%)
Nodal Involvement	
No	8 (40%)
Yes	12 (60%)
Stage	
I	3 (15%)
II	13 (65%)
III	4 (20%)

Figure 2. Immunophenotypic classification of a healthy participant

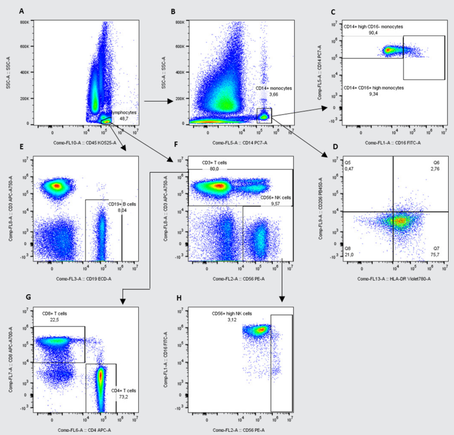


Figure 3. Characterization of Tregs in a healthy participant

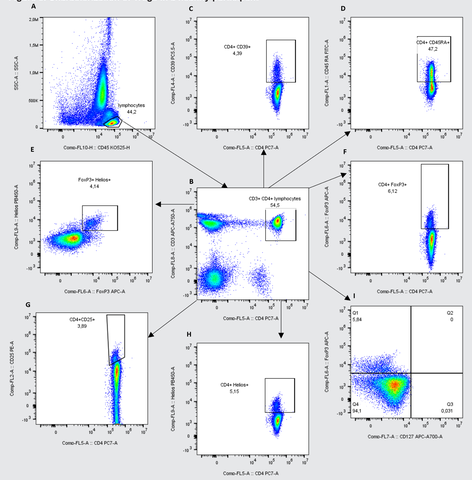


Figure 4. Characterization of immune cells in TNBC patients.

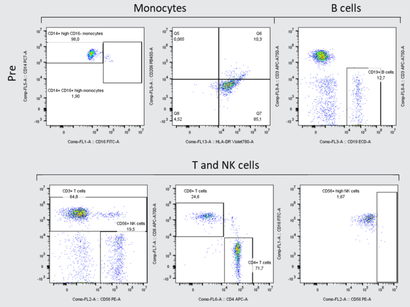


Figure 5. Characterization of Tregs in TNBC patients.

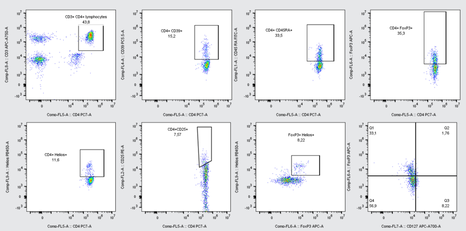


Table 2. Median levels of immune cells in TNBC patients and controls.

	BASIC TUBE	ROC (auc)	Group	Median	95% CI	p-value
	CD45+ leukocytes | Freq. of Parent	0,7235	Breast Cancer	98,5	97,1-99,3	0,039
			Control	96,9	82,6-98,8	
Monocytes	CD45+ leukocytes/**CD14+ monocytes | Freq. of Parent**	0,5889	Breast Cancer	5,25	4,44-5,95	0,464
			Control	6,53	3,92-7,67	
	CD45+ leukocytes/**CD14+ monocytes/CD14high CD16+ monocytes | Freq. of Parent**	0,4588	Breast Cancer	39,75	12,5-49,4	0,906
			Control	34,2	28,1-60,6	
	CD45+ leukocytes/CD14+ monocytes/CD14high CD16+ monocytes | Median (CD206)	0,4889	Breast Cancer	3500	2339-5533	0,923
			Control	3733	2503-4455	
	CD45+ leukocytes/CD14+ monocytes/CD14high CD16+ monocytes | Median **(HLA-DR)**	0,3917	Breast Cancer	39193	20476-143329	0,35
			Control	24616,5	17935-47754	
B Cells	CD45+ leukocytes/lymphocytes | Freq. of Parent	0,7944	Breast Cancer	26,55	17,6-31,7	0,01
			Control	42,6	20,7-45,7	
	CD45+ leukocytes/lymphocytes/CD19+ B cells | Freq. of Parent	0,7444	Breast Cancer	3,93	2,15-7,13	0,035
			Control	6,58	-9,21	
	CD45+ leukocytes/lymphocytes/CD19+ B cells | Median (CD206)	0,3278	Breast Cancer	-1508	-(1636)-5305	0,02
			Control	-1636	-(1636)-12,7	
Lymphocytes	CD45+ leukocytes/lymphocytes/**CD3+ T cells | Freq. of Parent**	0,7972	Breast Cancer	55,8	52,8-65,6	0,01
			Control	67,3	65,6-71,2	
	CD45+ leukocytes/lymphocytes/**CD56+ NK cells | Freq. of Parent**	0,5667	Breast Cancer	9,92	8,61-17,3	0,588
			Control	11,5	9,56-16,6	
	CD45+ leukocytes/lymphocytes/**CD3+ T cells/ CD3+ CD4+ T cells | Freq. of Parent**	0,3528	Breast Cancer	51,95	49,3-59,9	0,204
			Control	49,65	39-61,7	
	CD45+ leukocytes/lymphocytes/**CD3+ T cells/ CD3+ CD8+ T cells | Freq. of Parent**	0,5917	Breast Cancer	34,65	25,2-38,8	0,429
			Control	36,5	21-43,6	
	CD45+ leukocytes/lymphocytes/**CD56+ NK cells/ CD56+ high NK cells | Freq. of Parent**	0,5167	Breast Cancer	5,28	3,27-7,38	0,906
			Control	5,56	4,73-6,19	

Table 3. Median levels of Tregs in TNBC patients and controls.

	TREGS	ROC (auc)	Group	Median	95% CI	p-value
	CD45+ lymphocytes | Freq. of Parent	0,2056	Breast Cancer	25,8	11,8-35	0,011
			Control	40,95	32-51,8	
	CD45+ lymphocytes/**CD3+ CD4+** T cells | Freq. of Parent	0,5111	Breast Cancer	34,2	19,4-37,6	0,944
			Control	28,95	24,6-46,4	
CD25+	CD45+ lymphocytes/CD3+ CD4+ T cells/ **CD3+CD4+CD25+ | Freq. of Parent**	0,3889	Breast Cancer	4,63	3,96-6,55	0,356
			Control	5,9	4,06-6,71	
	CD45+ lymphocytes/CD3+ CD4+ T cells/ CD3+CD4+CD25+ | Median **(FoxP3)**	0,5412	Breast Cancer	94,65	91,1-95,8	1,0000
			Control	94,3	92,9-95,6	
	CD45+ lymphocytes/CD3+ CD4+ T cells/ CD3+CD4+CD25+ | Median **(CD127)**	0,6588	Breast Cancer	373	-(333)-815	0,175
			Control	-243	-(572)-964	
CD25-	CD45+ lymphocytes/**CD3+ CD4+ T cells/ CD3+CD4+CD25- | Freq. of Parent**	0,5000	Breast Cancer	2271	1543-2681	0,047
			Control	2943,5	2283-4823	
	CD45+ lymphocytes/CD3+ CD4+ T cells/ CD3+CD4+CD25- | Median **(FoxP3)**	0,2676	Breast Cancer	8352	7534-11473	0,749
			Control	8866	6439-10323	
	CD45+ lymphocytes/CD3+ CD4+ T cells/ CD3+CD4+CD25- | Median **(CD127 APC)**	0,7118	Breast Cancer	2778	1564-7488	0,074
			Control	1671	566-2937	
CD4+ FoxP3+	CD45+ lymphocytes/**CD3+ CD4+ T cells/CD4+ FoxP3+** | Freq. of Parent	0,6833	Breast Cancer	8,81	6,21-15,5	0,121
			Control	6,62	4,42-9,67	
CD4+ Helios+	CD45+ lymphocytes/**CD3+ CD4+ T cells/CD4+ Helios+** | Freq. of Parent	0,6167	Breast Cancer	10,4	4,85-12,3	0,314
			Control	7,9	4,41-11	
CD4+ CD39+	CD45+ lymphocytes/**CD3+ CD4+ T cells/CD4+ CD39+** | Freq. of Parent	0,7778	Breast Cancer	9,44	6,28-15,5	0,016
			Control	5,3	2,8-8,35	
CD4+ CD45RA+	CD45+ lymphocytes/**CD3+ CD4+ T cells/CD4+ CD45RA+** | Freq. of Parent	0,7778	Breast Cancer	7,8	4,15-13,4	0,016
			Control	20,3	9,48-41,8	
FoxP3+ Helios+	CD45+ lymphocytes/**CD3+ CD4+ T cells/ FoxP3+Helios+** | Freq. of Parent	0,5389	Breast Cancer	7,15	3,41-9,06	0,76
			Control	6,6	3,56-9,53	

Figure 6. Comparison of plasma levels of Tregs between TNBC patients and healthy controls

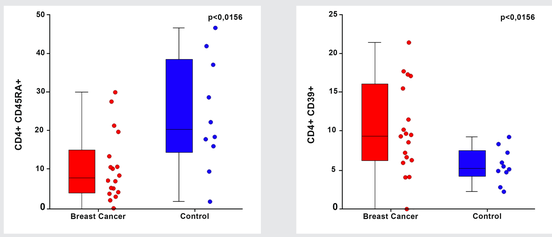
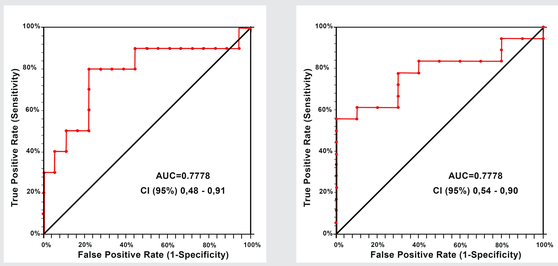


Figure 7. ROC curve of CD45RA+ and CD39+



Conclusions

- TNBC is associated with severe immunosuppression.
- In this context, percentages of circulating CD19+ B cells were significantly lower, while CD3+ CD4+ CD39+ Tregs were significantly higher in early TNBC patients.
- The peripheral blood immunome of TNBC patients identified a subset of immunosuppressive Tregs.

Future Directions

- A study is ongoing to identify the prognostic and predictive biomarkers (potential targets) for pCR of a subset of Tregs in early TNBC patients undergoing neoadjuvant chemotherapy.
- Follow-up encompasses flow cytometry analyses after 3 neo-adjuvant treatments.
- Tregs will be correlated with circulating MDSC's and M2 macrophages.

References

- **Rama RM, Padmaraju V, Rahul KV, Marni R, Neha M, Ganji PN. Regulatory T cells: Their role in triple-negative breast cancer progression and metastasis. Cancer. 2022; 15;128(6):1171-1183. DOI: 10.1002/cncr.34084**
- **Huang P, Zhou X, Zheng M, Yu Y, Jin G, Zhang S. Regulatory T cells are associated with the tumor immune microenvironment and immunotherapy response in triple-negative breast cancer. Front Immunol. 2023 Sep 12;14:1263537. doi: 10.3389/fimmu.2023.1263537. eCollection 2023.PMID: 37767092**

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