REVIEW ARTICLE

Comprehensive Evaluation of Peripheral Immunodynamics in Advanced Non-Small Cell Lung Cancer Associated to Chemotherapy and Immunotherapy

Avaliação Abrangente da Imunodinâmica Periférica no Cancro do Pulmão de Não Pequenas Células Avançado Associado à Quimioterapia e Imunoterapia

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ABSTRACT

Introduction: Immunotherapy, particularly immune checkpoint inhibitors (ICIs), has transformed the treatment landscape for advanced non-small cell lung cancer (NSCLC). Despite significant benefits for some patients, durable responses are limited to 20%–30% of cases, necessitating predictive biomarkers for better therapeutic guidance. While tumor-based markers such as PD-L1 expression and tumor mutational burden offer partial insights, peripheral blood biomarkers have emerged as promising minimally invasive tools for monitoring immune dynamics and predicting outcomes.

Methods: This prospective pilot study analyzed the immune profiles of 51 NSCLC patients across three therapy groups—diagnostic (DX), chemotherapy (CT), and ICI-treated (ICB)—using high-dimensional flow cytometry and advanced computational algorithms.

Results: Compared to healthy controls, NSCLC patients exhibited significant alterations in immune subsets, including elevated regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Therapy-specific changes included increased effector and activated T cells (e.g., HLA-DR$^+$CD38$^+$ Th1 cells) and dendritic cell subtypes, particularly cDC2, in the ICB group. In contrast, chemotherapy was associated with heightened Tc2 and activated CD8 T cells.

Importantly, distinct immune signatures correlated with therapeutic outcomes. Non-progressors (stable disease or partial response) under ICB displayed higher levels of switch-memory B cells and cDC1, while progressors showed increased naïve B cells and cDC2. These findings highlight the immunomodulatory effects of different therapies and the potential of peripheral biomarkers for treatment stratification.

Results: This study underscores the need for further validation of peripheral blood biomarkers and their integration into clinical decision-making to optimize NSCLC immunotherapy outcomes.

Keywords:

Biomarkers, Tumor; Carcinoma, Non-Small-Cell Lung/drug therapy; Carcinoma, Non-Small-Cell Lung/immunology; Immune Checkpoint Inhibitors/pharmacology; Immune Checkpoint Inhibitors/therapeutic use; Immunotherapy.

RESUMO

A imunoterapia, particularmente a utilização dos inibidores de checkpoints imunológicos (ICIs), transformou o panorama do tratamento do cancro do pulmão de não pequenas células (CPNPC) avançado. Apesar dos benefícios significativos para alguns doentes, as respostas duradouras limitam-se a 20–30% dos casos, o que exige biomarcadores preditivos para uma melhor orientação terapêutica. Embora alguns biomarcadores, como a expressão de PD-L1 e a carga mutacional tumoral, ofereçam informações parciais, os biomarcadores no sangue periférico têm emergido como ferramentas promissoras, minimamente invasivas, para monitorizar as dinâmicas imunológicas em tempo real.

Este estudo piloto prospetivo analisou os perfis imunológicos de 51 doentes com CPNPC em três grupos de terapias — diagnóstico (DX), quimioterapia (CT) e tratamento com ICIs (ICB) — utilizando citometria de fluxo de alta dimensão e algoritmos computacionais avançados. Em comparação com os controlos saudáveis, os doentes com CPNPC apresentaram alterações significativas em subgrupos imunológicos, incluindo um aumento de células T reguladoras (Tregs) e células supressoras derivadas da linha mielóide (MDSCs). Alterações específicas de cada terapia incluíram o aumento de células T efetoras e ativadas (por exemplo, células Th1 HLA-DR$^+$CD38$^+$) e subtipos de células dendríticas, particularmente cDC2, no grupo tratado com ICIs. Em contraste, a quimioterapia foi associada a um aumento de células Tc2 e células T CD8 ativadas.

Importa salientar que assinaturas imunológicas distintas foram correlacionadas com a resposta terapêutica. Os não-progressores (doença estável ou resposta parcial) sob ICB apresentaram níveis mais elevados de células B de memória de transição e cDC1, enquanto os progressores exibiram um aumento de células B naïve e cDC2. Estes resultados sugerem efeitos imunomoduladores de diferentes terapias e o potencial de biomarcadores periféricos para a estratificação terapêutica.

Este estudo reforça a necessidade de validação adicional de biomarcadores do sangue periférico e a sua integração na tomada de decisões clínicas para otimizar os resultados da imunoterapia no CPNPC.

Palavras-chave:

Biomarcadores Tumorais; Carcinoma Pulmonar de Células não Pequenas/imunologia; Carcinoma Pulmonar de Células não Pequenas/tratamento farmacológico; Imunoterapia; Inibidores de Checkpoint Imunológico/farmacológico; Inibidores de Checkpoint Imunológico/uso terapêutico

INTRODUCTION

Immunotherapy has revolutionized the treatment paradigm for non-small cell lung cancer (NSCLC), particularly in advanced-stage disease, with immune checkpoint inhibitors (ICIs) such as anti-PD-1, anti-PD-L1, and anti-CTLA-4 monoclonal antibodies now established as standard-of-care options. While ICIs deliver durable clinical benefits for a subset of patients, only 20%–30% demonstrate significant and sustained responses, underscoring the urgent need for predictive biomarkers to guide patient selection and therapy monitoring.[1,2] Although tumor-specific biomarkers such as PD-L1 expression and tumor mutational burden (TMB) have shown some utility, they remain imperfect predictors of therapeutic outcomes.[3,4] Consequently, peripheral blood-based immune biomarkers have gained significant attention for their potential as minimally invasive and dynamic tools for real-time monitoring.

Peripheral blood biomarkers, including systemic inflammatory markers, lymphocyte subsets, and circulating myeloid cells, offer insights into the systemic immune response and its interplay with the tumor microenvironment. The neutrophil-to-lymphocyte ratio (NLR) has been one of the most extensively studied markers. Retrospective analyses have linked pre-treatment NLR to both prognosis and therapeutic response in NSCLC patients treated with ICIs.[5,6] Beyond NLR, eosinophil dynamics during therapy have shown associations with treatment efficacy and immune-related adverse events, reflecting the activation and modulation of systemic immunity.[7,8]

Recent studies have also explored the predictive value of peripheral T-cell populations. Elevated levels of activated or effector memory CD8 T cells before therapy have been associated with favorable outcomes in NSCLC patients.[9,10] Furthermore, circulating tumor-reactive T cells, characterized by T-cell receptor (TCR) clonality and expression of activation markers such as PD-1 and TIM-3, may serve as robust indicators of tumor-specific immune engagement.[11,12] Advances in single-cell technologies and high-dimensional flow cytometry have enhanced the ability to characterize these populations in greater detail, enabling the identification of phenotypic and functional signatures associated with therapeutic success.

Circulating myeloid cells, including myeloid-derived suppressor cells (MDSCs), represent another promising avenue of investigation. Monocytic MDSCs (M-MDSCs) are known to suppress T-cell activation and have been implicated in resistance to immunotherapy. Elevated levels of M-MDSCs in the peripheral blood of NSCLC patients have been correlated with poor prognosis and reduced efficacy of ICIs.[13,14] Monitoring dynamic changes in MDSC populations during therapy may provide early insights into treatment resistance and guide therapeutic adjustments.

Despite these advances, the clinical application of peripheral blood-based biomarkers remains in its infancy. Limitations include variability in study designs, small sample sizes, and lack of standardized methodologies for biomarker quantification and validation. Prospective studies with larger cohorts are critical to establish the predictive and prognostic value of these markers and to develop actionable algorithms for clinical decision-making.

This study aims to systematically evaluate peripheral blood-based immune biomarkers in advanced-stage NSCLC patients undergoing ICI therapy. By leveraging high-dimensional immune profiling techniques and longitudinal sampling, we seek to identify novel predictive markers and further elucidate the immunological dynamics underlying therapeutic responses and resistance in NSCLC.

MATERIAL AND METHODS

Study Design and Inclusion Criteria

This was a single-center, prospective, observational pilot study of 51 patients diagnosed with non-small cell lung cancer (NSCLC) at stages IIB-IV, with an average age of 65 years (range 38-88), with 82% being male. Patients were divided into three groups: Diagnosis (DX), 25 treatment-naïve patients, newly diagnosed with NSCLC; Chemotherapy (QT), 11 patients who had completed chemotherapy and were eligible for immunotherapy; Immune Checkpoint Blockade (ICB), 15 patients actively undergoing immunotherapy. Detailed information for each of the three patient groups is summarized in Table 1.

Patients were recruited between December 2017 and March 2024 from the Oncologic Pulmonology outpatient clinic of the Pulmonology Service at the Unidade Local de Saúde de Coimbra (ULSC). All participants received detailed information about the study, provided informed consent, and were given a copy of the signed consent form.

Samples for the control group were obtained from 39 healthy donors, with a mean age of 66 years (SD ± 17, range 43–87), comprising 71% males.

The study protocol was approved by the ULSC Ethics Committee (CHUC-073-17) and the National Data Protection Commission (17408/2017).

Deep immunophenotyping

For immunophenotyping, peripheral blood samples collected from 51 NSCLC patients and 45 HD controls were analyzed. The 8-color flow cytometer BD FACSCanto II (BD Biosciences, San Jose, CA, USA), was used for collecting peripheral blood samples after they were stained and prepared for flow cytometry analysis according to the previously stated technique (23). Briefly, 100µL of peripheral blood or up to 1x106 PBMC were stained with fluorochrome-conjugated monoclonal antibodies for 15 minutes in the dark at room temperature, then erythrocytes were lysed for 10 minutes in the same conditions, and cells were washed. The panel of antibodies used for extensive immunophenotyping was described in Table 2 and Supplementary File S1, and various lymphocytes subpopulations, monocytes, dendritic cells (DC), and myeloid-derived suppressor cells (MDSC) were examined, along with key receptors associated with maturation, activation, and suppression. The resulting data were analyzed using the software FlowJo v.10.7 (BD Biosciences, Ashland, OR, USA), and the gating strategy was outlined in detail in Supplementary File S2.

Table 1 Characteristics of NSCLC patients included in this study.

CHARACTERISTCS	NSCLC (n=49)	DX (n=23)	CT (n=11)	ICB (n=15)
Age				
mean (range)	65.8 (39-88)	67.4 (39-88)	64.9 (46-78)	64.3 (46-85)
Sex				
male (%)	41 (83.1)	21 (91.3)	7 (63.6)	13 (86.7)
Histology				
Adenocarcinoma	31 (63.3)	13 (56.5)	9 (81.8)	9 (60.0)
Epidermoide	17 (34.7)	9 (39.1)	2 (18.2)	6 (40.0)
Adenosquamous	1 (2.0)	1 (4.3)	-	-
Stage				
IIB	2 (4.1)	1 (4.3)	-	1 (6.7)
IIIB	7 (14.3)	-	4 (36.4)	3 (20.0)
IIIC	4 (8.2)	3 (13.0)	-	1 (6.7)
IV	25 (51.0)	12 (52.2)	7 (63.6)	6 (40.0)
IVA	7 (14.3)	6 (26.1)	-	1 (6.7)
IVB	4 (8.2)	1 (4.3)	-	3 (20.0)
Tumor Proportion Score				
<1%	15 (30.6)	6 (26.1)	5 (45.5)	4 (26.7)
1-50%	3 (6.1)	1 (4.3)	2 (18.2)	-
>50%	28 (57.1)	16 (69.6)	4 (36.4)	8 (53.3)
NA	3 (6.1)	-	-	3 (20.0)
Smoking				
Current	21 (42.9)	10 (43.4)	3 (27.3)	8 (53.3)
Former	18 (36.7)	9 (39.1)	4 (36.4)	5 (33.3)
Never	8 (16.3)	2 (8.7)	4 (36.4)	2 (13.3)
NA	2 (4.1)	2 (8.7)	-	-
ECOG				
PS0	18 (36.7)	10 (43.5)	3 (27.3)	5 (33.3)
PS1	29 (59.2)	12 (52.2)	8 (72.7)	9 (60.0)
PS2	2 (4.1)	1 (4.3)	-	1 (6.7)
EGFR mutation				
Positive	3 (6.1)	1 (4.3)	1 (9.1)	1 (6.7)
Negative	28 (57.1)	12 (52.2)	8 (72.7)	8 (53.3)
NA	18 (36.7)	10 (43.5)	2 (18.2)	6 (40.0)
ALK rearrangement				
Positive	1 (2.0)	-	-	1 (6.7)
Negative	29 (59.2)	13 (56.5)	8 (72.7)	8 (53.3)
NA	19 (38.8)	10 (43.5)	3 (27.3)	6 (40.0)
ROS1 rearrangement				
Positive	-	-	-	-
Negative	29 (59.2)	13 (56.5)	8 (53.3)	8 (53.3)
NA	20 (40.8)	10 (43.5)	7 (27.3)	7 (46.7)
BRAF1 rearrangement				
Positive	-	-	-	-
Negative	26 (53.1)	12 (52.2)	7 (63.6)	7 (46.7)
NA	23 (46.9)	11 (47.8)	4 (36.4)	8 (53.3)
ICB drug				
Pembrolizumab	9 (60.0)	-	-	9 (60.0)
Nivolumab	6 (40.0)	-	-	6 (40.0)

DX – diagnostics; **CT** – chemotherapy; **ICB** – immune checkpoint blockade; **ECOG** - Eastern Cooperative Oncology Group Performance Status score.

Table 2 Panels of fluorochrome-conjugated antibodies used in flow cytometry analysis

Fluorochrome	T cells	T_{reg} cells	Th1/Th2/Th17	B cells	DCs, Mo & NK	MDSC
FITC	PD-1	PD-1	PD-1	PD-1	PD-1	CD45
PE	CCR7	CD25	CXCR3	CD10	CD56	CD33
PerCP-Cy5.5	CD4	CD4	CD4	CD19	CD123	CD3/CD19/CD56
PE-Cy7	CD45RA	CCR4	CCR6	CD27	CC11c	CD15
APC	CD38	CD127	CD38	CD38	CD16	CD11b
APC-H7	CD8	CD45RO	CD8	CD20	CD3/CD19/CD20	CD16
V450	CD3	CD3	CD3	CD3	CD14	CD14
V500	HLA-DR	HLA-DR	HLA-DR	IgD	HLA-DR	HLA-DR

Statistical analysis

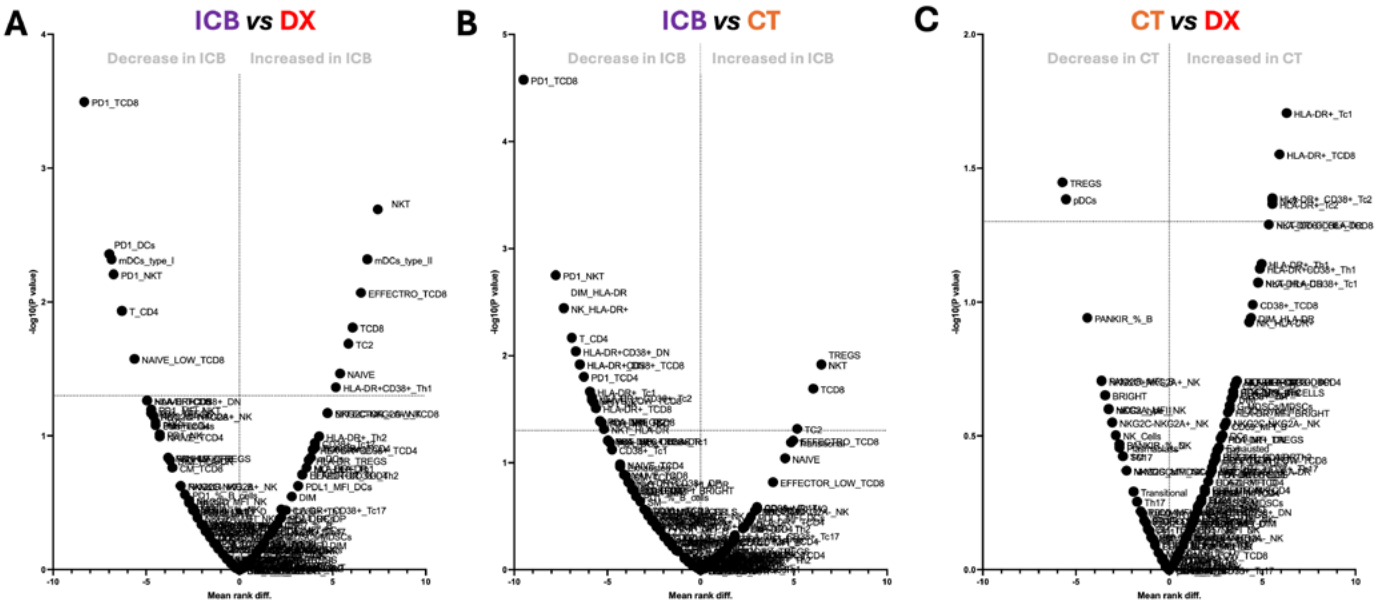
All statistical analyses and graphical representations were performed using GraphPad Prism 10.4.0 for macOS (GraphPad Software, San Diego, CA, USA). Group comparisons were conducted with multiple Mann-Whitney tests, and results were expressed as mean ± standard deviation. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Peripheral blood from NSCLC patients presents distinguished immune subsets of according to therapy

Peripheral blood from NSCLC patients distributed by three groups—DX, CT, and ICB—was analyzed in order to compare frequencies for a comprehensive panel of immune cells, including T, B, and NK cells, monocytes, dendritic cells, and MDSC. For some subsets, activation, maturation, and functional parameters were studied.

To quickly identify changes in large immunophenotyping data sets from the 3 groups of NSCLC patients, scatter-plots (volcano plots) were used to draw significance *versus* fold-change on the y and x axes, respectively (Fig. 1). Comparison of ICB vs. DX patients suggest a significant increase of NKT cells, cDC2, efffector CD8 T cells, total CD8 T cells, Th2, *naïve* B cells, and activated (HLA-DR+CD38+) Th1 cells in ICB group (Fig. 1A). When we compared ICB vs. CT patients indicated significant increases of Tregs, NKT cells, total CD8 T cells, and Th2 cells (Fig. 1B). After chemotherapy, NSCLC patients significantly elevate the frequency of activated (HLA-DR+) total CD8 T cells, Tc1, and Tc2 cells as well as the subset of HLA-DR+CD38+ Tc2 cells (Fig. 1C).



DX – diagnostics; **CT** – chemotherapy; **ICB** – immune checkpoint blockade.

Figure 1 **Volcano plots for comparisons of immunophenotyping parameters for NSCLC patients according to the therapy.** (**A**) ICB vs DX. (**B**) ICB vs CT. (**C**) CT vs DX.

ICB promotes activation and maturation of T cell subsets in NSCLC

Then, the t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm was used to characterize the peripheral T cells of NSCLC patients from the ICB group. By reducing the high-dimensional data obtained from immune phenotyping by multiparametric flow cytometry, t-SNE allowed the visualization of complex T cell phenotypes and states in a low-dimensional space. This approach reveals therapy-specific differences in T cell subsets, such as the enrichment of effector or exhausted T cells in ICB-treated patients (Fig. 2).

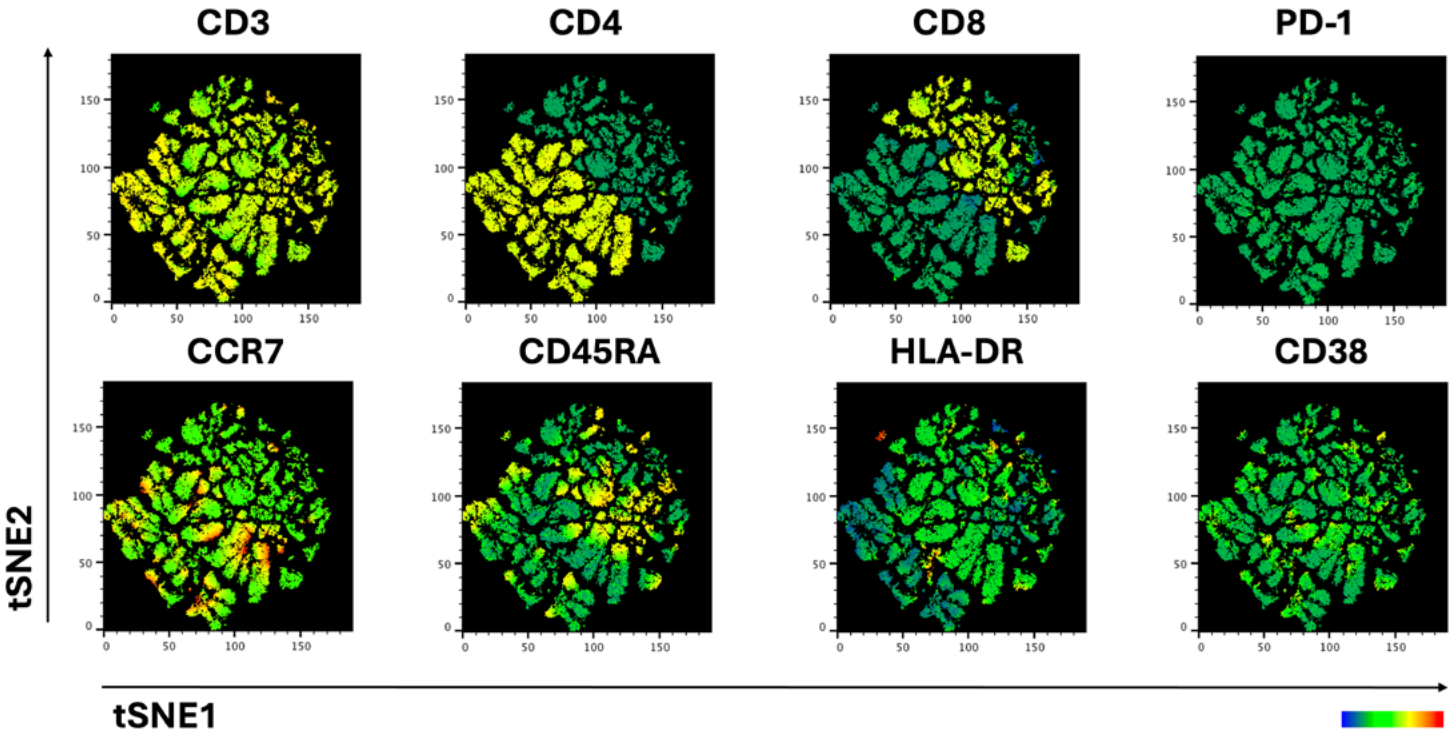
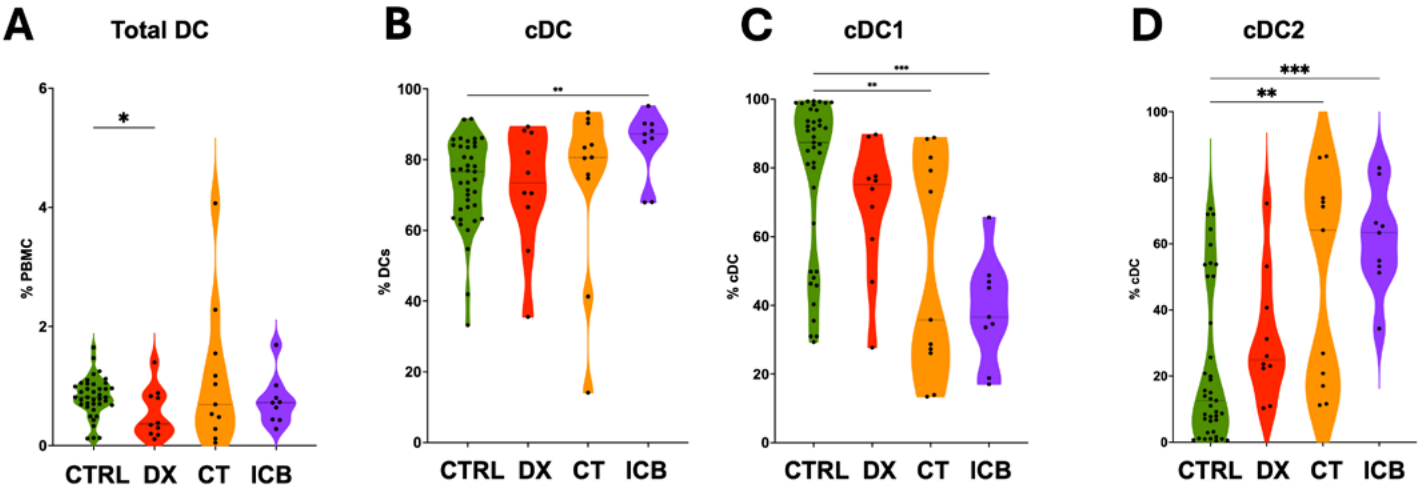


Figure 2 Visualization of high-dimensional data reduction using t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm for T cells obtained from NSCLC patients treated with immune checkpoint blockade.

ICB promotes activation and maturation of T cell subsets in NSCLC

Dendritic cells (DC) are an essential group of myeloid cells that have been identified as one of the most relevant professional antigen-presenting cells. A detailed study of total DC, as well as the major conventional (cDC) and plasmacytoid (pDC) subsets, revealed a statistically significant increase in total cDC, particularly the cDC2 subset (Fig. 3) in the ICB group of NSCLC patients.

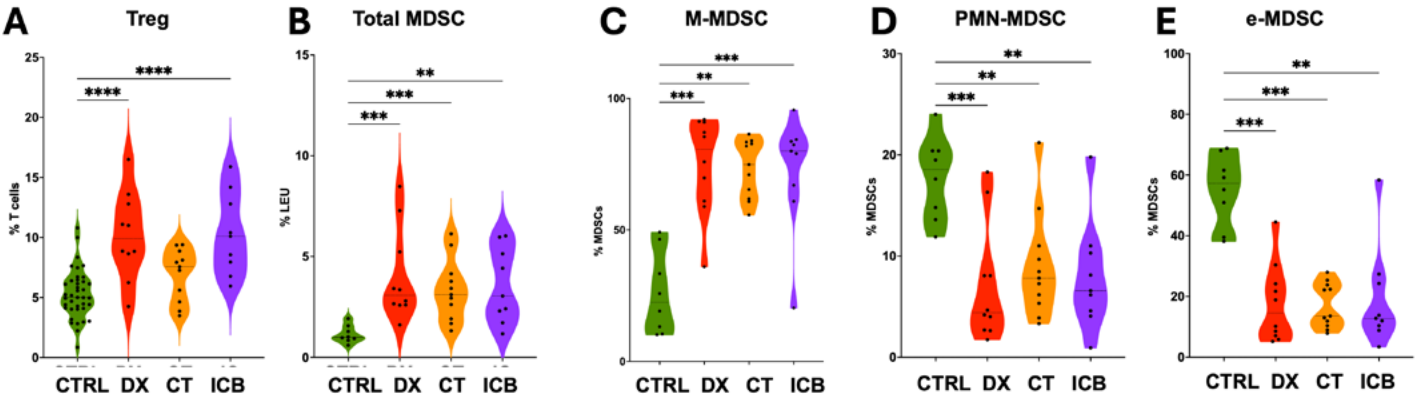


DC – dendritic cells; **cDC** – classical DC; **cDC1** - classical DC type 1; **cDC2** - classical DC type 2; **CTRL** – healthy control group; **DX** – diagnostics group; **CT** – chemotherapy group; **ICB** – immune checkpoint blockade group.
* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Figure 3 **Relative frequency of total dendritic cells of healthy controls and NSCLC patients according to the therapy.** (**A**) Total DCs, (**B**) conventional DCs, (**C**) conventional DCs type 1; (**D**) Conventional DCs type 2.

Tregs and MDSCs are significantly increased in NSCLC and are not affected by therapy

Among the canonical suppressor subsets that can dampen the immune anti-tumor functions, Tregs and MDSC are extensively documented for several tumors. In this study, both suppressor cells were found to be significantly increased in NSCLC patients from the DX and ICB groups ($p < 0.001$) when compared to healthy controls (Fig. 4A). Concerning the MDSCs, total MDSCs also increased in the DX ($p < 0.0001$), CT ($p < 0.0001$), and ICB ($p < 0.001$) groups (Fig. 4B). Analyzing the MDSC subsets, monocytic MDSCs (M-MDSCs) are responsible for those significant differences in DX ($p < 0.001$), CT ($p < 0.01$), and ICB ($p < 0.001$) groups of NSCLC patients (Fig. 4C). Equivalent decreases were observed in the other MDSC subsets—polymorphonuclear MDSCs (PMN-MDSCs) and early MDSCs (e-MDSCs)—for all NSCLC patients (Fig. 4D and Fig. 4E, respectively).



CTRL – healthy control group; **DX** – diagnostics group; **CT** – chemotherapy group; **ICB** – immune checkpoint blockade group.
** - $p<0.01$; *** - $p<0.001$.

Figure 4 **Relative frequency of principal immune suppressor cells in healthy controls and NSCLC patients according to therapy: Treg and MDSC.** (**A**) Treg - regulatory T cells, (**B**) Total MDSC - myeloid-derived suppressor cells. (**C**) M-MDSC – monocytic MDSC; (**D**) PMN-MDSC – polymorphonuclear MDSC; (**E**) e-MDSC – early MDSC

AIn-depth analysis of MDSCs using the s-SNE algorithm demonstrates the predominance of the M-MDSC in the ICB group of NSCLC patients (Fig. 5). M-MDSCs are characterized by the expression of CD45 (leucocyte common antigen), lineage negative (CD3-CD19-CD56-), CD11b, CD14, HLA-DRlow/–, and being negative for CD15 and CD16.

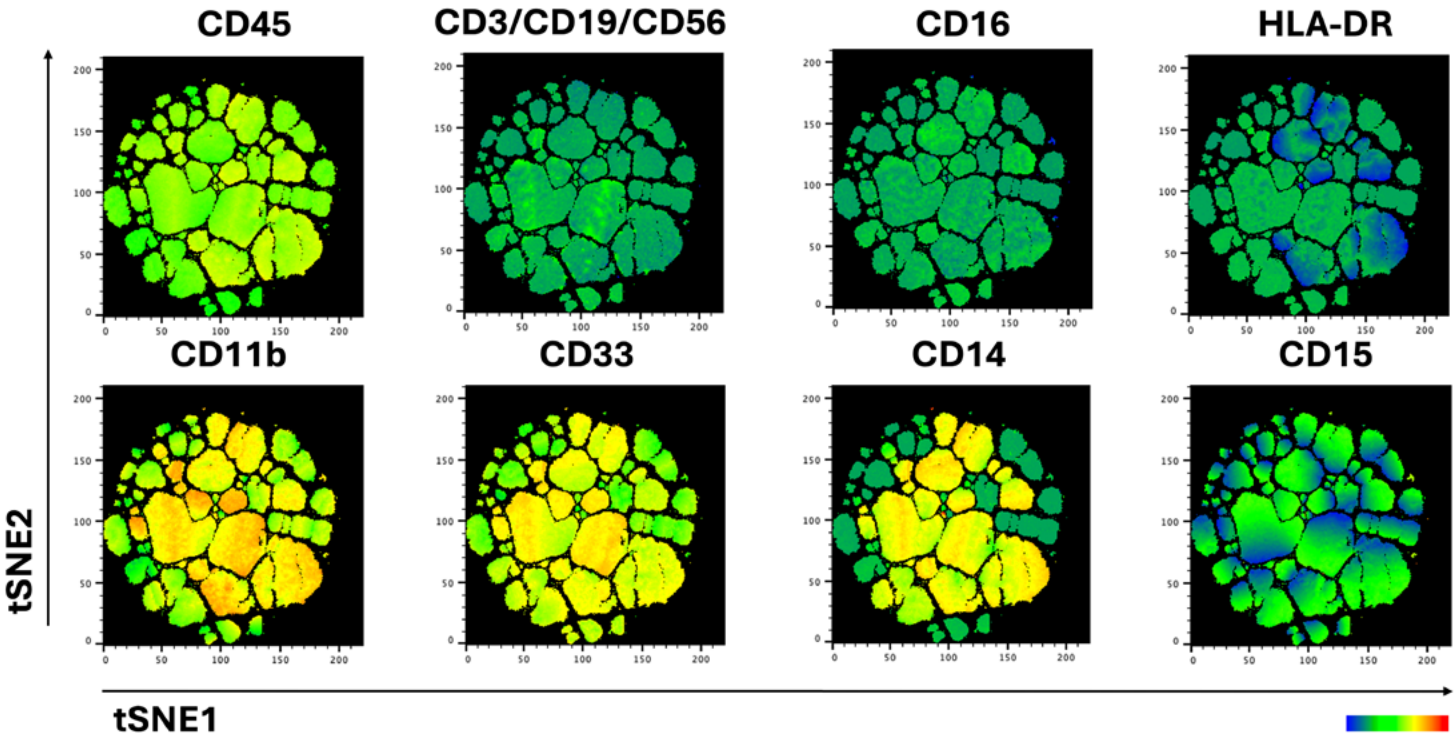
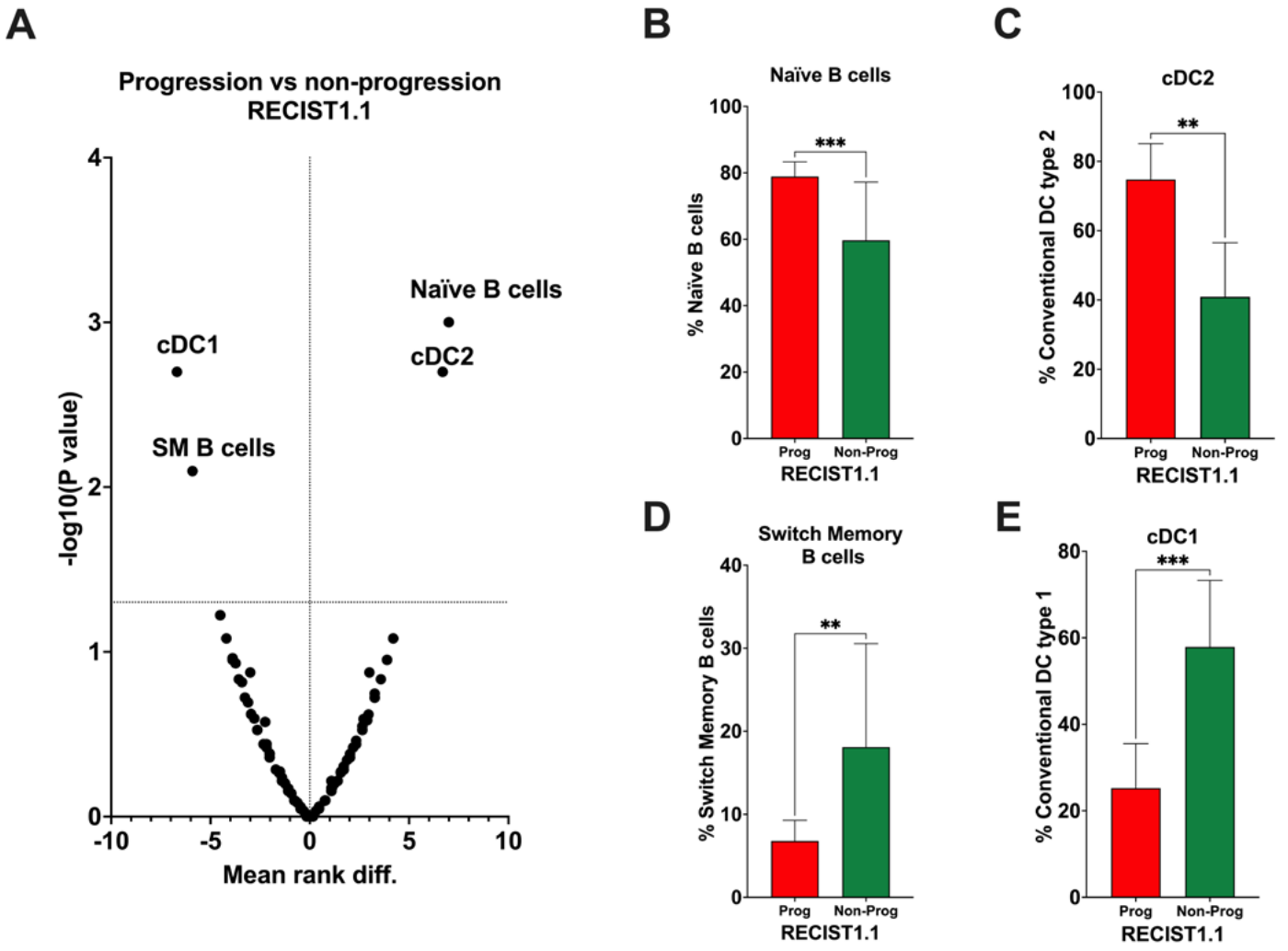


Figure 5 Visualization of high-dimensional data reduction using t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm for myeloid-derived suppressor cells (MDSC) obtained from NSCLC patients treated with immune checkpoint inhibitors.

Peripheral naïve B cells and cDC2 are associated with progression in ICB treated NSCLC patients

The present study identified 5/15 (33%) NSCLC patients that progressed after ICB, according to RECIST 1.1. A volcano plot (Fig. 6A) was used to draw significant changes when comparing progressors (disease progression, DP) with non-progressors (partial response, PR; and stable disease, SD).

Peripheral *naïve* B cells (Fig. 6B) and cDC2 (Fig. 6C) were associated with progression after ICB therapy. On the contrary, cDC1 (Fig. 6D) and switch-memory B cells (Fig. 6E) were significantly increased in the non-progressors.



DX – diagnostics; **CT** – chemotherapy; **ICB** – immune checkpoint blockade.
** - $p<0.01$; *** - $p<0.001$.

Figure 6 **Volcano plots for comparisons of NSCLC progressors and non-progressors (RECIST1.1) after ICB immunotherapy.** (**A**) ICB vs DX. (**B**) ICB vs CT. (**C**) CT vs DX.

DISCUSSION

This study demonstrated the significant impact of the therapy modality used on the peripheral blood immune landscape in patients with non-small cell lung cancer. A comparison of the immune checkpoint blockade (ICB), chemotherapeutic (CT), and diagnostic (DX) groups reveals changes in immune cell composition, activation states, and functional profiles that are distinct to each therapy. The immunodynamic changes linked to various treatment approaches in advanced non-small cell lung cancer are better understood thanks to these findings.

Comparing the ICB group to the DX and CT groups, our data show a considerable enrichment of effector and activated immune cell subsets. In particular, the increased numbers of Th2 cells, effector CD8 T cells, and activated HLA-DR+CD38+ Th1 cells highlight how ICB can strengthen anti-tumor immunity. These results are consistent with previous research demonstrating that immune checkpoint inhibitors (ICIs) revitalize T cells by preventing PD-1/PD-L1-mediated fatigue.[15,16] This is further supported by the visualization of high-dimensional T cell data using the t-SNE

technique, which shows clear effector and fatigued T cell clusters specific to the ICB group. Furthermore, the ICB group's higher levels of NKT cells and cDC2 highlight the therapy's wider effects on both innate and adaptive immunity.

Immunomodulation brought on by chemotherapy was also noticeable, especially in the elevation of Tc2 subsets and activated CD8 T cells. This conclusion is consistent with earlier findings that cytotoxic drugs can cause immunogenic cell death, which in turn activates T lymphocytes that are specific to antigens.[17] Nevertheless, the lack of notable alterations in suppressor cell subsets, such as Tregs and MDSCs, after chemotherapy raises the possibility that this treatment may not be sufficient to reverse immunosuppression on its own. This emphasizes the potential advantages of boosting anti-tumor immunity by combining immunotherapeutic techniques with chemotherapy.[18]

Given their well-established functions in tumor immune evasion, the stability of increased Tregs and MDSCs across all therapy groups is noteworthy.[19] The prevalence of monocytic MDSCs (M-MDSCs) in the ICB group, as demonstrated by the t-SNE analysis, needs additional investigation. Although ICB boosts effector immune responses, the tumor microenvironment may be compensating by expanding suppressor cells like MDSCs to lessen the therapeutic benefit. By focusing on these suppressor cells, ICB may be more effective and patient outcomes may be better.

The varied effect of ICB on dendritic cell subsets demonstrates the complexities of its immunomodulatory properties. The large rise in cDC2 in ICB-treated patients, combined with its link to disease progression, raises critical issues concerning its role in tumor immunity. Conversely, the abundance of cDC1 and switch-memory B cells in non-progressors is consistent with their involvement in fostering effective anti-tumor responses, as previously documented.[20,21] These findings indicate that a better understanding of dendritic cell biology could lead to improved patient classification and combination therapies that boost dendritic cell-mediated anti-tumor immunity.

The link between *naïve* B cells and disease progression underscores the dual character of B cell responses in NSCLC. While B cells have long been associated with humoral immunity, their involvement in immunological control and tumor progression is becoming more widely acknowledged.[22] The correlation between *naïve* B cells and advancement shows that their functional nature, not just their presence, may impact patient outcomes.

CONCLUSION

This study presents a complete assessment of immunodynamic shifts in peripheral blood from NSCLC patients undergoing various therapeutic modalities, revealing therapy-specific alterations that highlight the complexity of immune regulation in advanced disease. Immune checkpoint blockade (ICB) had the greatest immunomodulatory effects, as seen by the concentration of effector CD8 T cells, activated Th1 cells, and NKT cells, as well as an increase in cDC2 subsets. These findings show ICB's potential to boost anti-tumor immunity, but its concurrent relationship with enhanced suppressor populations, such as Tregs and monocytic MDSCs (M-MDSCs), emphasizes the importance of addressing these compensatory mechanisms for optimal therapeutic effects. Chemotherapy significantly activated CD8 T cells and Tc2 subsets, demonstrating its ability to promote immunogenic cell death and stimulation.

Research suggests that *naïve* B cells and cDC2 are linked to disease progression in ICB-treated patients, while cDC1 and switch-memory B cells are linked to better outcomes. These cells can serve as indicators for therapy response and targets for interventions.

Finally, the findings underline the need to incorporate immune profiling into clinical practice for NSCLC, which will allow for more individualized treatment approaches and combination methods. Targeting both effector and suppressor immune dynamics may increase the efficacy of current medicines, paving the door for better outcomes in this complex disease setting.

Contributorship Statement:

PR-S, FB and AF: Research study outline.

PR-S, JSA and LMS: Performed the experiments and acquired the data.

FB and AF: Clinical data and patient management.

PR- S, JSA, LMS and AF: Data analysis.

PR-S and AF: Manuscript writing.

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

Declaração de Contribuição:

PR-S, FB e AF: *Plano do estudo de investigação.*

PR-S, JSA e LMS: *Investigação laboratorial e aquisição de dados.*

FB e AF: *Dados clínicos e gestão de doentes.*

PR- S, JSA, LMS e AF: *Análise de dados.*

PR-S e AF: *Redação do artigo científico.*

Todos os autores leram e concordaram com a versão publicada do artigo científico.

Ethical Disclosures:

Conflicts of Interest: The authors have no conflicts of interest to declare

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Confidentiality of Data: The authors declare that they have followed the protocols of their work center on the publication of patient data.

Protection of Human and Animal Subjects: The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and those of the Code of Ethics of the World Medical Association (Declaration of Helsinki as revised in 2024)

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Confidencialidade dos Dados: *Os autores declaram ter seguido os protocolos da sua instituição acerca da publicação dos dados de doentes.*

Proteção de Pessoas e Animais: *Os autores declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pela Comissão de Ética responsável e de acordo com a Declaração de Helsínquia revista em 2024 e da Associação Médica Mundial.*

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SUPPLEMENTARY FILES

SUPPLEMENTARY FILE S1

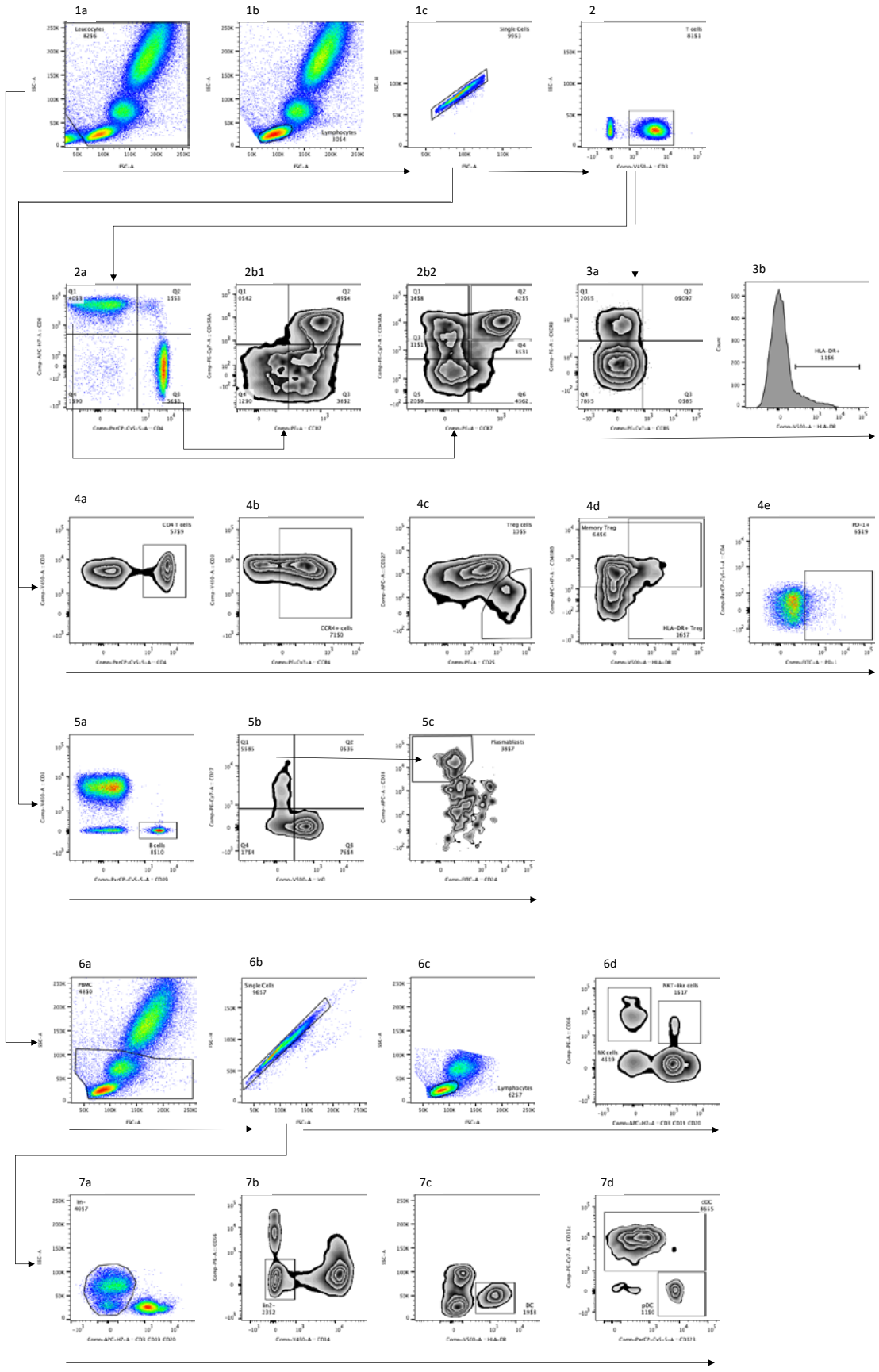
Table 3 Fluorochrome-conjugated monoclonal antibodies used in flow cytometry analysis.

ANTIBODY	CONJUGATE	CLONE	BRAND	CAT#	RRID
CD3	V450	ICHT1	BD Horizon™	561416	AB_10612021
CD3	PerCp-Cy5.5	HIT3a	BioLegend®	300328	AB_1575008
CD4	PerCp-Cy5.5	OKT4	BioLegend®	317428	AB_1186124
CD8	APC-H7	HIT8a	BD Biosciences™	641400	AB_1645736
CD11b	APC	ICRF44	BioLegend®	301310	AB_2564134
CD11c	PE-Cy7	B-ly6	BD Pharmingen™	561356	AB_10611859
CD14	V450	MOP9	BD Horizon™	560349	AB_1645559
CD15	PE-Cy7	HI98	BD Pharmingen™	560827	AB_10563901
CD16	APC-Cy7	3G8	BioLegend®	302018	AB_314218
CD19	APC-H7	SJ2501	BD Pharmingen™	560177	AB_1645470
CD19	PerCp-Cy5.5	HIB19	BioLegend®	302230	AB_2275547
CD20	APC-H7	2H7	BD Pharmingen™	560734	AB_1727449
CD24	FITC	ML5	BD Pharmingen™	555427	AB_395821
CD25	PE	M-A251	BD Pharmingen™	555432	AB_395826
CD27	PE-Cy7	M-T271	BD Pharmingen™	560609	AB_1727456
CD33	PE	WM53	BD Pharmingen™	555450	AB_395843
CD38	APC	HIT2	BD Pharmingen™	555462	AB_398599
CD45	FITC	HI30	BD Pharmingen™	555482	AB_395874
CD45RA	PE-Cy7	5H9	BD Pharmingen™	561216	AB_10611721
CD45RO	APC-H7	UCHL1	BD Pharmingen™	561137	AB_10562194
CD56	PerCp-Cy5.5	HCD56	BD Pharmingen™	560842	AB_2033964
CD56	PE	B159	BioLegend®	318306	AB_604101
CD123	PerCp-Cy5.5	6H6	BioLegend®	306016	AB_2264693
CD127	AF647	HIL-7R-M21	BD Pharmingen™	558598	AB_647113
CD183 (CXCR3)	PE	IC6/CXCR3	BD Pharmingen™	550633	AB_2292853
CD194 (CCR4)	PE-Cy7	IG1	BD Biosciences™	557864	AB_396907
CD196 (CCR6)	PE-Cy7	11A9	BD Pharmingen™	560620	AB_1727440
CD197 (CCR7)	PE	150503	BD Pharmingen™	560765	AB_2033949
CD274 (PD-L1)	FITC	MIH1	BD Pharmingen™	558065	AB_647176
CD279 (PD-1)	FITC	MIH4	BD Pharmingen™	557860	AB_2159176
CD279 (PD-1)	APC	MIH4	BD Pharmingen™	558694	AB_1645458
CD279 (PD-1)	PE	MIH4	BD Pharmingen™	557946	AB_647199
HLA-DR	V500	G46-6	BD Horizon™	561224	AB_10563765
IgD	V500	IAS6-2	BD Horizon™	561490	AB_10679356

FITC - fluorescein isothiocyanate, **PE** - R-phycoerythrin, **PerCP-Cy5.5** - peridinin chlorophyll protein-cyanine 5.5, **PE-Cy7** - R-phycoerythrin – cyanine 7, **APC** – allophycocyanin, **AF647** – Alexa fluor 647, **APC-H7** – allophycocyanin cyanine H7, **V450** - violet 450, **PB** – Pacific blue 3-carboxy-6,8-difluoro-7-hydroxycoumarin, **V500** – violet 500, RRID - research resource identification.

SUPPLEMENTARY FILE S2

A



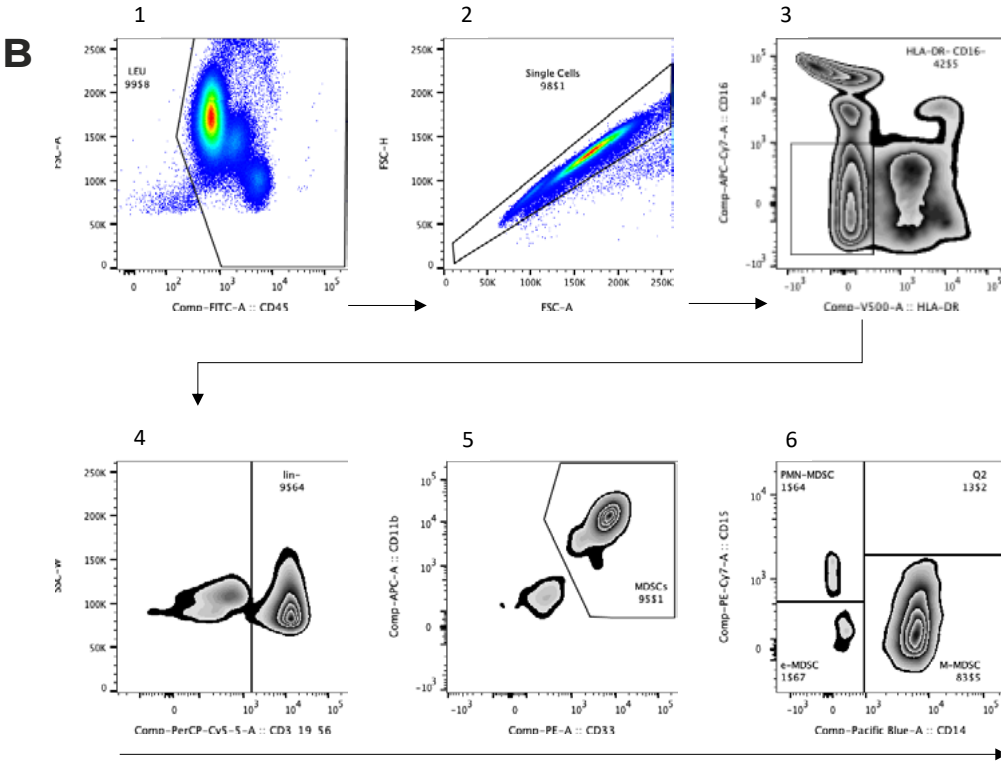


Figure 7 A representative gating strategy was used to analyze the data obtained using multiparametric flow cytometry, identifying the immune populations in peripheral blood samples. A) Gating strategy for T cells, B cells, NK cells, dendritic cells, and monocytes. Lymphocytes were discriminated based on SSC-A and FSC-A and doublets excluded [1a – 1c]. Next, T cells were selected by positivity to CD3 [2]. T cells were plotted on a CD4 versus CD8 plot to identify CD4 T and CD8 T cells, as well as double positive ($CD4^+CD8^+$) and negative ($CD4^-CD8^-$) cells [2a]. Gated on CD4 T [2b1] and CD8 T cells [2b2], naïve cells ($CD45RA^+$ $CCR7^+$), central memory cells ($CD45^-$ $CCR7^+$), effector memory cells ($CD45RA^-$ $CCR7^-$) and effector cells ($CD45RA^+$ $CCR7^-$) were discriminated. Within CD4 T cells, expression of CX3CR1 or CCR6 mAbs were used to differentiate Th1 cells ($CX3CR1^+$ $CCR6^-$), Th17 ($CX3CR1^-$ $CCR6^+$) and Th2 cells ($CX3CR1^-$ $CCR6^-$) [3a]. Activation status of lymphocytes subpopulations were accessed by the presence of the activation marker HLA-DR, representative histogram for HLA-DR expression in Th1 cells [3b]. Treg cells, gated on CD4 T cells were identified by lower or absent expression of CD127 and expression of CD25 and CCR4 [4a – 4c]. The anti-CD45RO mAb were used to discriminate between naïve ($CD45RO^-$) and memory ($CD45RO^+$) Treg cells [4d]. Inhibition status of lymphocytes subpopulations were accessed by the presence of the inhibitory marker PD-1, representative histogram for PD-1 expression in Treg cells [4e]. Gated on lymphocytes, B cells were selected by positivity to CD19 [5a]. B cells were plotted in a CD27 versus IgD plot to identify naïve cells (IgD^+ $CD27^-$), pre-switch memory cells (IgD^+ $CD27^+$), switch memory cells (IgD^- $CD27^+$) and exhausted cells (IgD^- $CD27^-$) [5b]. Gated on naïve B cells, plasmablasts were identified by positivity to CD38 and negativity to CD24 [5c]. Within lymphocytes, NK cells were selected by positivity to CD56 and absence of CD3 and NKT-like cells were identified by the positivity for both [6a – 6d]. Peripheral blood mononuclear cells were selected in a SSC-A and FSC-A plot, then cells positive for CD3, CD19, CD20, CD56 and CD14 mAbs were excluded and the anti-HLA-DR mAb was used as a specific marker to identify the DC [7a – 7c]. CD11c and CD123 identified CD11c+ CD123+/- classical DC (cDC, $CD11c^+$ $CD123^{+/-}$) and plasmacytoid DC (pDC, $CD11c^-$ $CD123^+$) [7d]. B) Gating strategy myeloid-derived suppressor cells (MDSC). First, we selected the positive cells for the CD45 marker and then we proceeded with the exclusion of cells positive for: CD16, HLA-DR, CD3, CD56, and CD19 [1 – 4]. MDSC were then identified by the expression of CD33 and CD11b [5]. Gated on MDSC, three subpopulations were discriminated based on the combination of the anti-CD14 and anti-CD15 mAbs, early-MDSC (e-MDSC: $CD14^-$, $CD15^-$), monocytic-MDSC (M-MDSC: $CD14^+$, $CD15^{-/+}$) and polymorphonuclear-MDSC (PMN-MDSC: $CD14^-$, $CD15^+$) [6]. All samples were analyzed with FlowJo v.10.7 software (BD Biosciences, OH, USA).