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The role of CD20$^+$ T cells: Insights in human peripheral blood

Aryane Cruz Oliveira Pinho[1,2,3] | Pedro Barbosa[1,2,4] | Maria João Pereira[5] | Artur Paiva[2,6,7,8,9] | Eugenia Carvalho[1,2,4,10] | Paula Laranjeira[1,2,6,7,8]

[1]CNC-UC- Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

[2]CIBB -Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

[3]Department of Life Sciences, Faculty of Science and Technology, University of Coimbra, Coimbra, Portugal

[4]Institute for Interdisciplinary Research, University of Coimbra, Coimbra, Portugal

[5]Department of Medical Sciences, Clinical Diabetology and Metabolism, Uppsala University, Uppsala, Sweden

[6]Flow Cytometry Unit, Clinical Pathology Department, Hospitais da Universidade de Coimbra, Unidade Local de Saúde de Coimbra, Coimbra, Portugal

[7]Coimbra Institute for Clinical and Biomedical Research (iCBR), Group of Environmental Genetics of Onco-biology (CIMAGO), Faculty of Medicine (FMUC), University of Coimbra, Coimbra, Portugal

[8]Clinical Academic Center of Coimbra (CACC), Coimbra, Portugal

[9]Instituto Politécnico de Coimbra, ESTESC-Coimbra Health School, Ciências Biomédicas Laboratoriais, Coimbra, Portugal

[10]APDP-Portuguese Diabetes Association, Lisbon, Portugal

Correspondence
Eugenia Carvalho, Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra 3004-504, Portugal.
Email: ecarvalh@cnc.uc.pt

Paula Laranjeira, Unidade Funcional de Citometria de Fluxo, Centro Hospitalar e Universitário de Coimbra, Av. Bissaya Barreto, Bloco Hospitalar de Celas, n.° 205, Coimbra 3000-076, Portugal.
Email: 1979paula@gmail.com

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Abstract

CD20$^+$ T cells constitute a small subset of T cells. These are found among CD4$^+$, CD8$^+$, CD4$^+$CD8$^+$, CD4$^-$CD8$^-$ T, and TCRγδ$^+$ T cells, and have been poorly characterized. The aim of this study was to characterize peripheral blood (PB) CD20$^+$ T cells and compare them to their PB CD20$^-$ T cell counterparts. PB from 17 healthy individuals was collected. The distribution of CD20$^+$ T cells among maturation-associated T cells compartments (naïve, central memory, transitional memory, effector memory, and effector T cells), their polarization, activation status, and expression of immune-regulatory proteins were evaluated by flow cytometry. Their function was also assessed, by measuring IFN-γ, TNF-α, and IL-17 production. Compared with CD20$^-$ T cells, CD20$^+$ T cells represent a higher proportion of transitional memory cells. Furthermore, CD20$^+$ T cells display a proinflammatory phenotype, characterized by the expansion of Th1, Th1/17, and Tc1 cell subsets , associated to a high expression of activation (CD25) and exhaustion (PD-1) markers. In addition, the simultaneous production of the proinflammatory cytokines IFN-γ, TNF-α, and IL-17 was also detected in CD4$^+$CD20$^+$ T cells. Our results show that CD20$^+$ T cells are phenotypically and functionally different from CD20$^-$ T cells, suggesting that these cells are a distinct subset of T cells.

KEYWORDS
activation and exhaustion status, CD20$^+$ T cells, cytokines, flow cytometry, functional compartments, polarization

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1 | INTRODUCTION

The cluster of differentiation 20 (CD20) is a cell membrane protein found predominantly in B cells. It participates in B cell proliferation and cell cycle progression (Cragg et al., 2005). However, CD20 is also expressed, at a low level, on a small subset of T cells, called $CD20^+$ T cells (Hultin et al., 1993; Quintanilla-Martinez et al., 1994).

The origin of $CD20^+$ T cells is controversial, as recently reviewed (Pinho et al., 2024). The hypothesis that T cells may acquire CD20 by trogocytosis from B cells is the most common (de Bruyn et al., 2015; Ochs et al., 2022). However, $CD20^+$ T cells have been found in the thymus of very young children (<93 days old) (Schuh et al., 2016; Wilk et al., 2009). Moreover, the detection of CD3- and CD20-encoding mRNA, by reverse transcription polymerase chain reaction, has been repeatedly demonstrated in this cell population (Ochs et al., 2022; Schuh et al., 2016; Wilk et al., 2009). It has been suggested that CD20 may be a marker of T cell activation (Li et al., 2020; Murayama et al., 1996), and the unique characteristics of $CD20^+$ T cells reinforce the hypothesis that they should be considered an independent T cell subpopulation (Palanichamy et al., 2014; Wilk et al., 2009).

Our group and others (Eggleton et al., 2011; Hultin et al., 1993; Vlaming et al., 2021) have demonstrated that $CD20^+$ T cells can be identified within $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (double positive), and $CD4^-CD8^-$ (double negative) T cell populations, as well as in $TCR\gamma\delta^+$ T cells in peripheral blood from healthy individuals. The immunobiology of $CD20^+$ T cells has not been well established even under healthy conditions in humans. In order to fill this knowledge gap, the present study characterizes peripheral blood (PB) $CD20^+$ T cells from healthy individuals and compares them to their PB $CD20^-$ T cell counterparts. This study describes their cytokine production profile (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and interleukin [IL]-17 expression), and their distribution among maturation-associated T cells compartments (naïve, central memory [CM], transitional memory [TM], effector memory [EM], and terminally differentiated/effector [TE] T cells). In addition, their polarization, activation status (measured by the early-activation marker CD25), and the expression of immune-regulatory molecules (programmed cell death protein [PD]-1 and T cell immunoglobulin and mucin domain 3 [TIM-3]), have also been evaluated.

2 | MATERIALS AND METHODS

2.1 | Participants

Seventeen PB samples from healthy individuals (disease-free individuals, i.e., individuals without clinically reported diseases, without current or past medical history of cancer or autoimmunity, without current inflammatory diseases, and not taking medication) were collected. Thirteen women and four men, ranging from 25 to 60 years, with a mean age of 41 ± 10.8 years, were recruited at the Flow Cytometry Unit of the Centro Hospitalar e Universitário de Coimbra (CHUC). This study was conducted in accordance with the Declaration of Helsinki (1975) guidelines, last revised in 2013, and was approved by CHUC's Ethics Committee (Ref.: CHUC-202-20). All individuals gave their written informed consent prior to entering the study. Study-specific exclusion criteria consisted of subjects taking anti-inflammatory drugs and pregnancy.

2.2 | Flow cytometry staining protocols

The immunophenotypic study of $CD20^+$ T cells was carried out using a cell surface marker staining protocol for tubes 1 and 2 (Table 1) and an intracellular cytokine staining protocol for tube 3 (Table 1).

For the study of maturation-associated T cells compartments (naïve, CM, TM, EM, and TE T cells, $n = 7$), the monoclonal antibody (mAb) combination described in tube 1 (Table 1) was used. Briefly, for cell surface marker staining, 100 µL of EDTA-collected PB was stained with the mAbs detailed in tube 1 (Table 1), and incubated for 10 min in the dark, at room temperature. After a lyse-and-then-wash method, the sample was acquired in the FACSCanto II (BD Biosciences, San Jose, CA, USA) flow cytometer equipped with FACSDiva software (version 6.1.2, BD), as previously described (Laranjeira et al., 2018; Laranjeira et al., 2023; Oliveira et al., 2019; Silva et al., 2022).

To evaluate the T cell polarization, activation status, and expression of the immunosuppressive molecules PD-1 and TIM-3 ($n = 12$), the mAb combination described in tube 2 (Table 1) was used. First, EDTA-collected PB (100 µL) was incubated with 50 µL of Brilliant stain buffer (Becton Dickinson Biosciences [BD], San Jose, CA, USA) and the mAbs combination for 30 min, at room temperature, in the dark. After this incubation, a lyse-and-then-wash method was applied as described by Barbosa et al. (2023). Sample acquisition was performed in a FACSLyric flow cytometer (BD) using FACSuite acquisition software (v1.5.0.925, BD).

To assess the production of cytokines, heparin-collected PB ($n = 10$) was incubated with phorbol myristate acetate (PMA, 50 ng/mL; Sigma–Aldrich, St. Louis, MO, USA) and ionomycin (1 µg/mL; Boehringer Mannheim, Germany), along with brefeldin A (10 µg/mL, Sigma–Aldrich), which stopped the synthesized proteins in the Golgi complex and prevented them from reaching the extracellular medium, for 4 h, at 37°C, in a sterile and humidified atmosphere containing 5% CO_2. After stimulation, cells were stained with the mAbs for cell surface markers: CD3, CD4, CD8, and CD20, as described in tube 3 (Table 1). After 10 min incubation in the dark, at room temperature, the samples were washed with Dulbecco's phosphate-buffered saline (PBS, Corning, Manassas, VA, USA). For intracellular staining, the Fix&Perm Cell Permeabilization Kit (Invitrogen, Hamburg, Germany) was used, in accordance with the instructions of the manufacturer, in parallel with the mAbs for the intracellular antigens: TNF-α, IFN-γ, and IL-17. Samples were incubated for a 15 min incubation period, washed twice with PBS, and immediately acquired in a FACSCanto II (BD) flow cytometer, equipped with FACSDiva software (version 6.1.2, BD).

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TABLE 1 Panel of monoclonal antibodies used for T cell immunophenotyping, indicating the clone and commercial source.

Fluorochromes											
Tube	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-C750	V450	V500-C	BV605	BV711
1	CD3	CD8	CD27	CD45RO	CD45RA		CD4	CD20			
	UCHT1	SK1	L128	UCHL-1	HI100		SK3	L27			
	Beckman Coulter	BD Bioscience	BD Bioscience	BD Bioscience	BD Pharmingen		BD Pharmingen	BD Bioscience			
2	TIM-3	CD25	CD4		CD3	CD20	CD8		CCR6	PD-1	CCR5
	7D3	M-A251	SK3		UCHT1	2H7	SK1		11A9	EH12.1	2D7/CCR5
	BD Horizon	BD Pharmingen	BD Pharmingen		BD Pharmingen	BD Horizon	BD Pharmingen		BD Horizon	BD Horizon	BD Horizon
3	cyIFN-γ	cyIL-17	CD4	cyTNF-α	CD3		CD8	CD20			
	4S.B3	SCPL1362	SK3	MAb11	UCHT1		SK1	L27			
	BD Pharmingen	BD Pharmingen	BD Pharmingen	BD Pharmingen	BD Pharmingen		BD Pharmingen	BD Bioscience			

Abbreviations: APC, allophycocyanin; BV, brilliant violet; CD, cluster of differentiation; cy, cytoplasmatic staining; CCR, C-C motif chemokine receptor; FITC, fluorescein isothiocyanate; IFN-γ, interferon γ; IL-17, interleukin 17; PD-1, programmed cell death protein 1; PE, phycoerythrin; PE-Cy7, phycoerythrin-cyanine 7; PerCP-Cy5.5, peridinin chlorophyll protein-cyanine 5.5; TIM-3, T-cell immunoglobulin and mucin containing protein 3; TNF-α, tumor necrosis factor α.

Source: BD Biosciences (San Jose, CA, USA); BD Horizon (Franklin Lakes, NJ, USA); BD Pharmingen (San Diego, CA, USA); Beckman Coulter (Miami, FL, USA).

2.3 | Flow cytometry analysis

Data were analyzed using Infinicyt software (version 2.0.5; Cytognos SL, Salamanca, Spain). The gating strategy used to identify $CD20^+$ T cells, T cell maturational/differentiation status, polarization, activation status, and the expression of the immunosuppressive molecules is depicted in Figure 1. After the exclusion of doublets and cell debris (based on FSC-A and FSC-H properties), T cells were identified by CD3 expression (Figure 1a), and $CD20^+$ T cells as T cells with $CD20^{dim}$ expression (Figure 1b). After identifying $CD20^+$ T and $CD20^-$ T cells within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cell compartments (Figure 1c,d), we proceed to their distribution among T cell functional compartments to evaluate maturational/differentiation status, based on the expression of CD27 and CD45RA (Figure 1e,f). Naïve T cells were defined as $CD27^+CD45RA^+$, CM as $CD27^+CD45RA^-$, EM corresponded to $CD27^-CD45RA^-$ events, and TE to $CD27^-CD45RA^+$. CM and EM cells were further characterized according to CD45RO expression (Supplementary Figure 1), wherein $CD45RO^+$ cells corresponded to TM T cells. T cells were also divided according to the expression of C-C motif chemokine receptor (CCR)5 and CCR6, as follows: $CCR5^+CCR6^-$ (T1-like), $CCR5^-CCR6^+$ (T17-like), $CCR5^+CCR6^+$ (T1/17-like), and $CCR5^-CCR6^-CD20^+$ T cells (Figure 1g,h). In each one of these four latter T cell subsets, the percentage of activated T cells (analyzed by CD25 expression, Figure 1i,j) and the expression of the immunosuppressive PD-1 (Figure 1k,l) and TIM-3 (Figure 1m,n) were measured. Finally, the percentage of TNF-α, IFN-γ, and IL-17-producing T cells was also evaluated within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cell compartments (Figure 1o–r).

2.4 | Statistical analysis

All results are expressed as mean ± standard deviation. Normal distribution was assessed by the Shapiro–Wilk test. Differences between $CD20^+$ T cells and $CD20^-$ T cells were evaluated using the Wilcoxon matched-pairs signed-rank test. Statistically significant differences were considered when the p value was lower than 0.05. Statistical analyses were performed using the Prism Software (GraphPad, version 9.5.1).

3 | RESULTS AND DISCUSSION

3.1 | Frequency of the $CD20^+$ T cell population within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells

A small percentage of T cells in PB from healthy individuals is dimly CD20 positive (mean ± standard deviation: 1.24% ± 0.98), representing 4.44% ± 3.81 of $CD20^+$ T cells within total T cells in PB. The detailed information on the absolute number of $CD20^+$ T cells in the PB, and their percentage in leukocytes and within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cell subsets, for each participant of our cohort, can be

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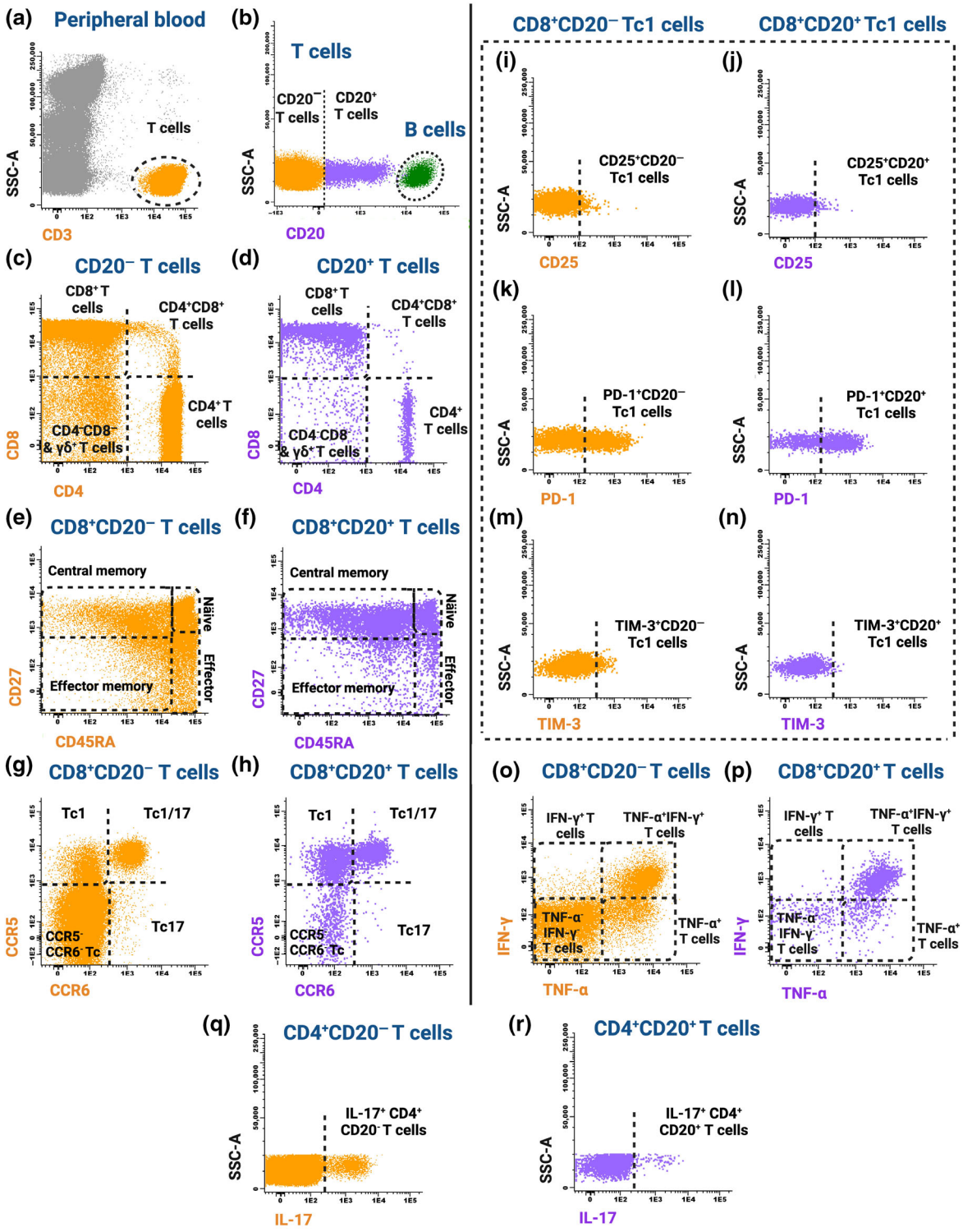

FIGURE 1 Dotplot histograms illustrate the gating strategy to identify $CD20^+$ T cells as well as their different subsets. (a) T cells were identified by the expression of CD3 and (b) $CD20^+$ T cells were identified by the expression of CD20. B cells are also represented. (c) $CD20^-$ T and (d) $CD20^+$ T cells were identified within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cell compartments. (e–p) The following schemes are specifically representing the $CD8^+$ T cells subsets, but the same gating strategy was applied to the $CD4^+$ and $CD4^+CD8^+$ T cells. (e) Identification of $CD8^+CD20^-$ T and (f) $CD8^+CD20^+$ T cells maturational/differentiation status, based on the expression of CD27 and CD45RA. (g, h) Identification of Tc1, Tc17, Tc1/17, and $CCR5^-CCR6^-$ Tc cells, based on CCR5 and CCR6 expression. (i, j) Identification of Tc1 cells expressing the early activation marker CD25. Strategy for the identification of Tc1 cells expressing the (k, l) immunosuppressive molecules PD-1 and (m, n) TIM-3. (o, p) Identification of TNF-α and IFN-γ-producing $CD8^+$ T cells. (q, r) Identification of IL-17-producing $CD4^+$ T cells. [Color figure can be viewed at wileyonlinelibrary.com]

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TABLE 2 Absolute number and percentage of $CD20^+$ T cells in peripheral blood and within $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cell subsets.

			Absolute number (cells/μL)			% in leukocytes					
Participants	Age	Sex	$CD4^+CD20^+$ T cells	$CD8^+CD20^+$ T cells	$CD4^+CD8^+CD20^+$ T cells	$CD4^+CD20^+$ T cells	$CD8^+CD20^+$ T cells	$CD4^+CD8^+CD20^+$ T cells	% $CD4^+CD20^+$ T cells (within $CD4^+$ T cells)	% $CD8^+CD20^+$ T cells (within $CD8^+$ T cells)	% $CD4^+CD8^+CD20^+$ T cells (within $CD4^+CD8^+$ T cells)
1	44	F	10.35	18.45	0.32	0.23	0.41	0.0072	1.88	6.52	6.51
2	41	F	0.64	32	0.43	0.01	0.5	0.01	0.17	6.9	1.09
3	60	M	72.9	135	105.30	0.81	1.5	1.17	7.39	11.13	19.37
4	35	M	15.75	44.1	0.45	0.35	0.98	0.01	2.33	10.7	6.49
5	49	F	7.81	75.26	1.42	0.11	1.06	0.02	0.95	11.71	4.99
6	49	F	8.1	57.15	0.45	0.18	1.27	0.01	0.77	13.3	1.24
7	58	F	15.95	75.35	2.75	0.29	1.37	0.05	1.45	12.51	5.45
8	27	M	5.28	20.64	0.48	0.11	0.43	0.01	1.12	3.25	4.38
9	40	M	5.58	29.14	0.05	0.09	0.47	0.0008	1.48	7.68	0.66
10	25	F	48.38	63.14	3.85	0.59	0.77	0.047	3.1	5.49	3.79
11	35	F	59.15	108.55	0.65	0.91	1.67	0.01	4.8	11.56	5.88
12	59	F	53.96	48.64	33.44	0.71	0.64	0.44	7.63	15.23	8.93
13	29	F	NA	54.52	2.44	NA	0.94	0.042	NA	9.68	27.16
14	33	F	19.76	38	0.08	0.26	0.5	0.001	1.38	7.6	6.77
15	37	F	20.77	34.17	1.34	0.31	0.51	0.02	1.1	4.84	14.06
16	43	F	15.66	23.78	0.52	0.27	0.41	0.009	1.53	6.54	9.27
17	37	F	19.53	20.16	3.21	0.31	0.32	0.0051	1.27	4.97	4.29
Mean	41	13 F	23.7	51.6	9.24	0.34	0.80	0.10	2.39	8.80	7.66
SD	10.8	4 M	22.1	32.3	25.9	0.26	0.43	0.29	2.26	3.44	6.85

Abbreviations: F, female; M, male; NA, not analyzed; SD, standard deviation.

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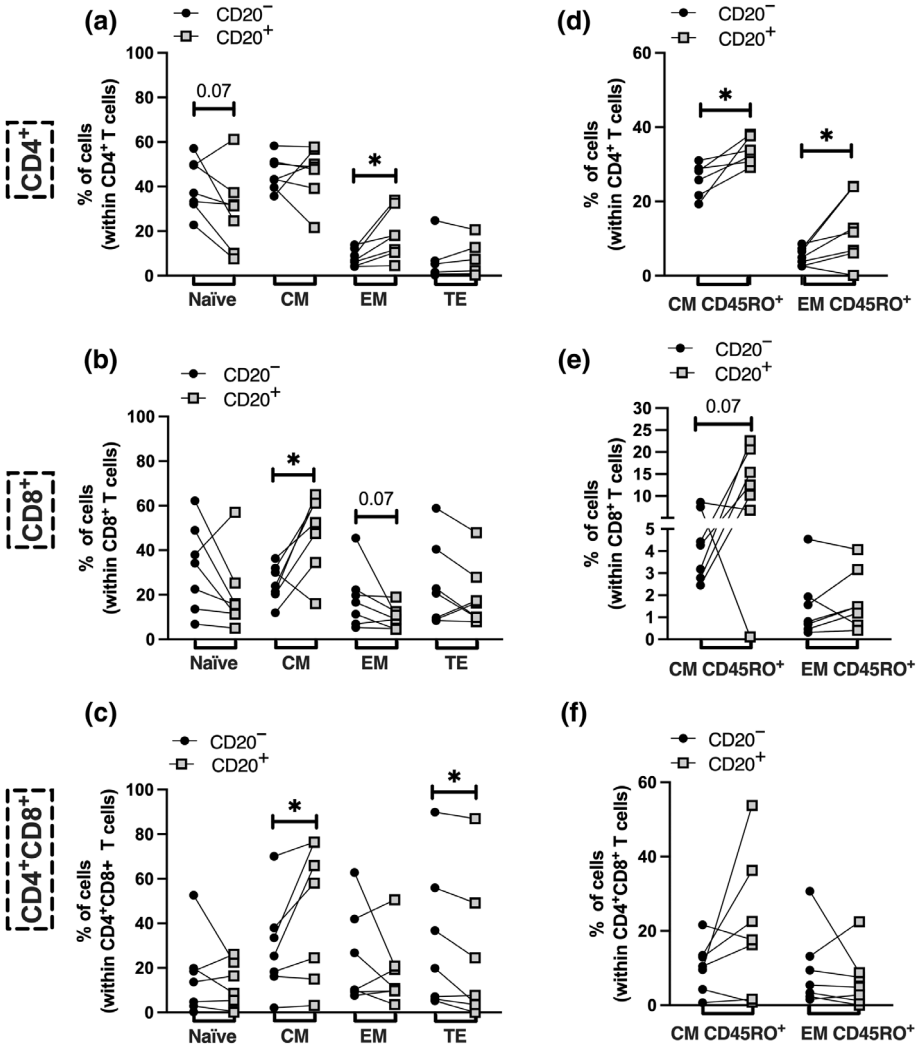

FIGURE 2 Distribution of CD20$^+$ T and CD20$^-$T cells based in the maturational/differentiation status. The maturational/differentiation status were identified by CD27, CD45RA, and CD45RO expression, in (a) CD4$^+$ T, (b) CD8$^+$ T, and (c) CD4$^+$CD8$^+$ T cells ($n = 7$). (d–f) The percentage of CD45RO$^+$ T cells (TM compartment) was further measured in the CM and EM compartments. Wilcoxon matched pairs signed rank test was applied. * $p < 0.05$. CM, central memory; EM, effector memory; TE, terminal effector; TM, transitional memory.

found on Table 2. In agreement with previous studies (Hultin et al., 1993; Quintanilla-Martinez et al., 1994; Schuh et al., 2016), CD8$^+$ T cells in PB from healthy individuals are particularly enriched of CD20$^+$ T cells.

3.2 | T cell maturational/differentiation status: CD20$^+$ T cells versus CD20$^-$ T cells

After identifying CD20$^+$ T and CD20$^-$ T cells within the CD4$^+$, CD8$^+$, and CD4$^+$CD8$^+$ T cell compartments, each one of the six subsets identified was distributed among the T cell maturational/differentiation status, based on the expression of CD27, CD45RA, and CD45RO.

Within the CD4$^+$ T cell compartment, CD20$^+$ T cells displayed a higher percentage within the EM compartment ($p < 0.05$), compared with CD20$^-$ T cells (Figure 2a), at the expense of naïve T cells. In turn, in the CD8$^+$ T cell compartment, CD8$^+$CD20$^+$ T cells presented a marked higher percentage in the CM compartment ($p < 0.05$), and a tendency for a lower percentage in the EM compartment ($p = 0.07$), compared with CD8$^+$CD20$^-$ T cells (Figure 2b). Finally, a higher percentage of CD4$^+$CD8$^+$CD20$^+$ T cells was found in the CM functional compartment. This was accompanied by a lower percentage of CD4$^+$CD8$^+$CD20$^+$ T cells in the TE compartment ($p < 0.05$ vs. CD4$^+$CD8$^+$CD20$^-$ T cells, Figure 2c).

Furthermore, both CD4$^+$CD20$^+$ T and CD8$^+$CD20$^+$ T cells displayed a higher percentage of CD45RO$^+$ T cells in the CM and EM compartments. This corresponds to an expansion of the TM compartment ($p < 0.05$ for CD4$^+$CD20$^+$ T cell vs. CD4$^+$CD20$^-$ T cells, Figure 2d,e). TM T cells are defined as cells that have an intermediate differentiation between CM and EM T cells (Botafogo et al., 2020; Hunt et al., 2023). In addition, T cells found in the TM compartment present a high proliferative potential and effector function (Hunt et al., 2023). Our results agree with previous studies (Hultin et al., 1993; Schuh et al., 2016; Vlaming et al., 2021) showing that PB CD20$^+$ T present a higher percentage of memory T cells, compared with CD20$^-$ T cells. EM CD20$^+$ T expressing CD45RO have the capacity to migrate to inflammatory sites (Hultin et al., 1993; Schuh et al., 2016).

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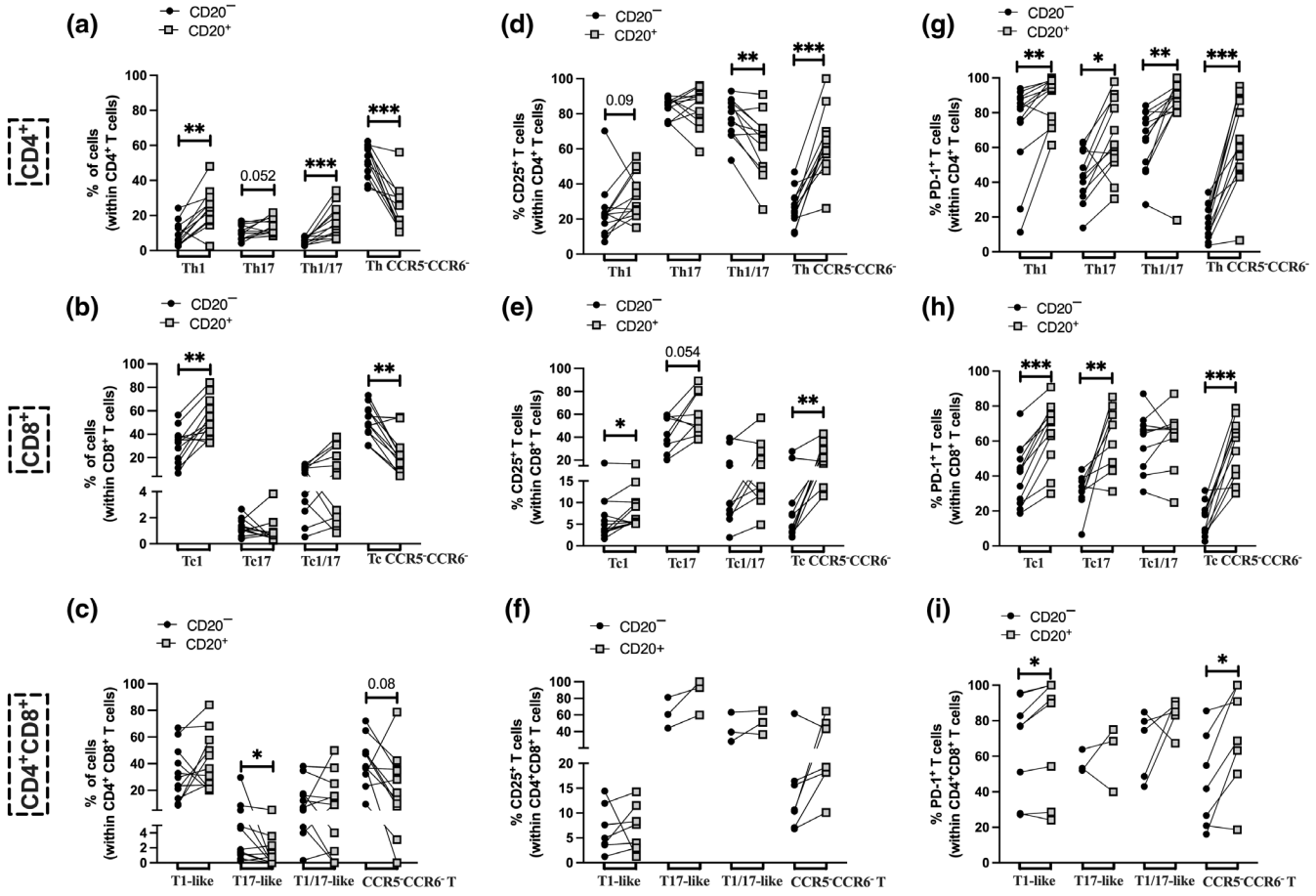

FIGURE 3 Sense of polarization of CD20$^+$ T cells versus CD20$^-$ T cells. Percentage of polarized CD20$^+$ T and CD20$^-$ T cells, from the peripheral blood of healthy individuals ($n = 12$), identified within (a) CD4$^+$, (b) CD8$^+$, and (c) CD4$^+$CD8$^+$ T cells, and (d–f) their activation (CD25$^+$) and/or (g–i) exhaustion (PD-1$^+$) status. Wilcoxon matched pairs signed rank test was applied. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. In some samples, the activation and exhaustion status of CD20$^+$ T cells were no analyzed due to low numbers of events. Th, T helper cells; Tc, T cytotoxic cells.

3.3 | Polarization of CD20$^+$ T cells versus CD20$^-$ T cells

The expression of the C-C motif chemokine receptor (CCR)5 and CCR6 enable blood T cells to navigate to inflammatory sites (Brodie et al., 2013; von Essen et al., 2019). Interestingly, the percentage of CD4$^+$CD20$^+$ and CD8$^+$CD20$^+$ T cells presenting a Th1 and Tc1 phenotype, respectively (i.e., T cells expressing CCR5), and the percentage of CD4$^+$CD20$^+$ T cells presenting a Th1/17 phenotype (expressing CCR5 and CCR6 simultaneously), was significantly higher than CD20$^-$ T cells ($p < 0.05$, Figure 3a,b), in PB from healthy individuals. On the other hand, CD4$^+$CD8$^+$CD20$^+$ T cells exhibited a lower percentage of T17-like cells ($p < 0.05$ vs. CD4$^+$CD8$^+$CD20$^-$ T cells, Figure 2c). Importantly, CD20$^+$ T cells displayed a lower percentage of cells in the CCR5$^-$CCR6$^-$ T compartment compared with CD20$^-$ T cells ($p < 0.05$ for CD4$^+$ and CD8$^+$ T cell compartment, Figure 3a,b). The latter T cell subset corresponds to T cells that are neither T1- nor T17-polarized, mainly comprising T0- and T2-like T cells.

Then, we investigated the CD20$^+$ T cell activation status, based on the expression of the early activation marker CD25 (Laranjeira et al., 2023), and the immunosuppressive molecules TIM-3 (Huang et al., 2022; Sun et al., 2020) and PD-1 (Huang et al., 2022; Lewis et al., 2022; Mudd et al., 2021), also considered exhaustion (Cai et al., 2016; Porsche et al., 2021) or activation (Gros et al., 2014; Inozume et al., 2010; Simon et al., 2016) markers. CD20$^+$ T cells presented a higher percentage of cells expressing the early activation marker CD25 compared with CD20$^-$ T cells (Figure 3d–f), except for Th1/17 cells (Figure 2a). In contrast, Vlaming et al. (2021) reported that there was no difference between CD20$^+$ T cells and CD20$^-$ T cells expressing CD25.

Moreover, a higher percentage of PD-1$^+$ T cells was found among CD20$^+$ T cells (Figure 3g–i), suggesting that PD-1 may play an important role in CD20$^+$ T cell inhibition. This finding agrees with a study revealing that the tumor microenvironment suppressed CD8$^+$CD20$^+$ T cell subset through the PD ligand 1 (PD-L1)-PD-1 pathway (Mudd et al., 2021), resulting in an impaired host anticancer immune response and tumor progression. Furthermore, the CD20 expression on tumor infiltrating-T cells has provided a positive prognostic marker in ovarian cancer (Milne et al., 2009). Accordingly, our data showed that CD20$^+$ T cells display a proinflammatory and

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activated phenotype, which may favor the immune response against tumor cells.

Furthermore, lower expression of TIM-3 was found among $CD20^+$ T cells (2.3% ± 2.13% and 4.3% ± 4.14%, for $CD4^+CD20^+$ T and $CD8^+CD20^+$ T cells, respectively) compared with $CD20^-$ T cells (3.5% ± 1.66% and 9.0% ± 5.70%, for $CD4^+CD20^-$ T and $CD8^+CD20^-$ T cells, respectively; $p < 0.05$, data not showed). The TIM-3/galectin-9 pathway can induce T cell exhaustion and tolerance (Tang et al., 2019); in that regard, the upregulated/ downregulated TIM-3 expression on T cells has been found in obesity and obesity-related type 2 diabetes, respectively (Sun et al., 2020).

3.4 | Cytokines production $CD20^+$ T cells versus $CD20^-$ T cells

To further elucidate the functional properties of $CD20^+$ T cells, flow cytometry was also used to measure the production of cytokines by T cells, upon stimulation with PMA plus ionomycin. We found the highest production of TNF-α, IFN-γ, and IL-17 by $CD20^+$ T cells (Figure 4), according to previous reports in healthy individuals (Schuh et al., 2016) and those with autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis (Eggleton et al., 2011; Ochs et al., 2022; Schuh et al., 2016; von Essen et al., 2019; Wilk et al., 2009). Interestingly, besides the highest percentage of $CD20^+$ T cells producing each one of these proinflammatory cytokines, we also found that the percentage of T cells with the ability to produce simultaneously TNF-α and IFN-γ, or even TNF-α, IFN-γ, and IL-17, was also the highest among the $CD20^+$ T cell compartment (1.05% ± 0.67% of TNF-α+IFN-γ+IL-17+CD4+CD20+ T cells within $CD4^+$ T cells), as depicted in Figure 4a.

Our study is limited by the number of participants. Nevertheless, there is consistency with previous studies and new findings were found including the characterization of the $CD4^+CD8^+CD20^+$ T cell subset, TM phenotype of $CD20^+$ T cell subsets, and activation and exhaustion status within T1-, T17-, T1/17-like, and $CCR5^-CCR6^-$ PB $CD20^+$ T cell subsets. To the best of our knowledge, this is the first time that $CD20^+$ T cells producing IFN-γ, TNF-α, and IL-17 simultaneously were identified. Future studies aiming the description of $CD20^+$ T cells phenotype and function in inflammatory conditions are

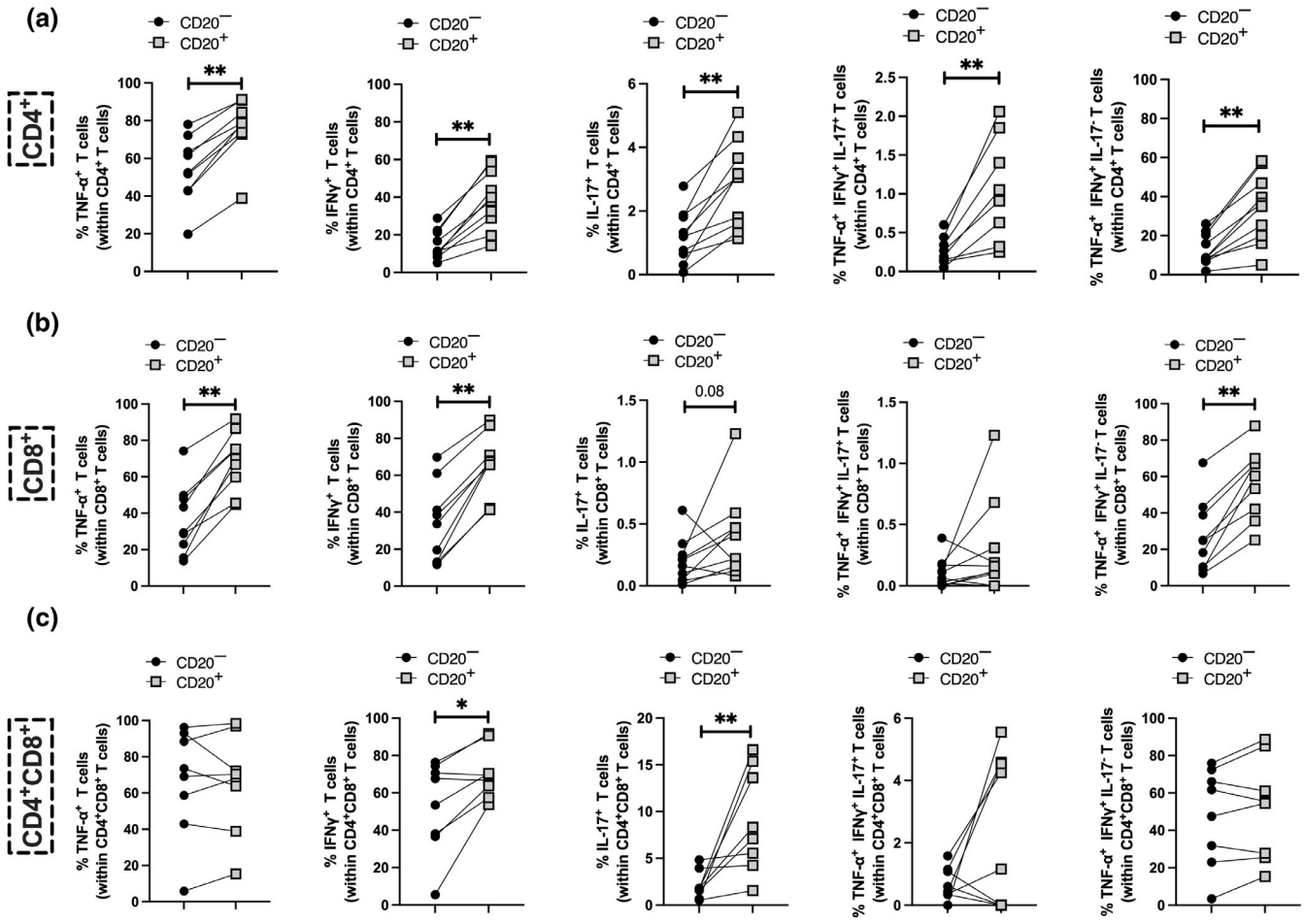

FIGURE 4 Cytokine production profile of $CD20^+$ T versus $CD20^-$ T cells. Percentage of $CD20^+$ T and $CD20^-$ T cells, in peripheral blood of healthy individuals ($n = 10$), identified within (a) $CD4^+$, (b) $CD8^+$, and (c) $CD4^+CD8^+$ T cell compartments, producing TNF-α, IFN-γ, and IL-17. Wilcoxon matched pairs signed rank test was applied. * $p < 0.05$; ** $p < 0.01$. In some samples the cytokine expression of $CD20^+$ T cells were no analyzed due to low numbers of events. IFN-γ, interferon γ; IL-17, interleukin 17; TNF-α, tumor necrosis factor α.

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well under way, as well as to clarify if these phenotypes change in regard to age and sex.

4 | CONCLUSION

We have demonstrated that CD20$^+$ T cells are associated with a central, transitional, and EM phenotype, with enhanced activation, and high susceptibility to be immunoregulated by the PD-1/PD-L1 axis. These cells are particularly prone to differentiate into Th1, Th1/17, and Tc1 cells and produce high levels of proinflammatory cytokines, with a remarkable percentage of Th and Tc cells producing TNF-α and IFN-γ simultaneously, or even TNF-α, IFN-γ, and IL-17. Their propensity to assume a proinflammatory phenotype but possible to be immunoregulated may impact the course of several human diseases, where the CD20$^+$ T cell number has been demonstrated to be altered. This study contributes to a better understanding of the sparsely characterized CD20$^+$ T cell subset in humans.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ORCID

Aryane Cruz Oliveira Pinho https://orcid.org/0000-0003-2235-2237
Pedro Barbosa https://orcid.org/0000-0002-0048-2615
Maria João Pereira https://orcid.org/0000-0001-5498-3899
Artur Paiva https://orcid.org/0000-0002-6562-5859
Eugenia Carvalho https://orcid.org/0000-0001-6264-3632
Paula Laranjeira https://orcid.org/0000-0002-1136-6118

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