CD8\(^+\) Treg cells play a role in the obesity-associated insulin resistance

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ABSTRACT

Obesity-related chronic low-grade inflammation may trigger insulin resistance and type 2 diabetes (T2D) development. Cells with regulatory phenotype have been shown to be reduced during obesity, especially CD4\(^+\) Treg cells. However, little is known about the CD8\(^+\) Treg cells. Therefore, we aim to characterize the CD8\(^+\) Treg cells in human peripheral blood and adipose tissue, specifically, to address the effect of obesity and insulin resistance in this regulatory immune cell population. A group of 42 participants with obesity (OB group) were recruited. Fourteen of them were evaluated pre- and post-bariatric surgery. A group of age- and sex-matched healthy volunteers (n = 12) was also recruited (nOB group). CD8\(^+\) Treg cell quantification and phenotype were evaluated by flow cytometry, in peripheral blood (PB), subcutaneous (SAT), and visceral adipose tissues (VAT). The OB group displayed a higher percentage of CD8\(^+\) Treg cells in PB, compared to the nOB. In addition, they were preferentially polarized into Tc1- and Tc1/17-like CD8\(^+\) Treg cells, compared to nOB. Moreover, SAT displayed the highest content of CD8\(^+\) Treg cells infiltrated, compared to PB or VAT, while CD8\(^+\) Treg cells infiltrating VAT displayed a higher percentage of cells with Tc1-like phenotype. Participants with pre-diabetes displayed a reduced percentage of TIM-3\(^+\)CD8\(^+\) Treg cells in circulation, and PD-1\(^+\)CD8\(^+\) Treg cells infiltrated in the VAT. An increase in the percentage of circulating Tc1-like CD8\(^+\) Treg cells expressing PD-1 was observed post-surgery.

In conclusion, obesity induces significant alterations in CD8\(^+\) Treg cells, affecting their percentage and phenotype, as well as the expression of important immune regulatory molecules.

1. Introduction

Obesity-related chronic low-grade inflammation is a potential trigger for comorbidities, such as insulin resistance (IR) and type 2 diabetes (T2D) [1]. Weight gain caused by excessive food intake, physical inactivity, and poor diets, leads to adipose tissue (AT) expansion due to adipocyte growth. The hypertrophic growth of adipocytes induces metabolic stress and metabolic dysfunction, leading to alterations in...
their secretome [1]. This has been observed by the increased secretion of inflammatory molecules, including tumor necrosis factor (TNF-α), interleukin (IL)-6 [1], as well as the regulated upon activation, normal T cell expressed and secreted (RANTES or CCL5) [2]. These alterations instigate the infiltration of immune cells into the AT microenvironment [1,2].

The infiltration of monocytes/macrophages polarizing towards M1, together with CD4+ T cells and CD8+ T cells displaying a pro-inflammatory profile, has been observed in obesity [3,4]. This pro-inflammatory profile observed in the AT microenvironment contributes to the development, but also enhances insulin action impairment, and systemic inflammation [3,4]. In fact, a shift in the immune profile during obesity towards a pro-inflammatory profile has been described, displaying a reduction of anti-inflammatory cell populations, including M2-polarized macrophages, Th2 cells and, especially, the regulatory T (Treg) cells [3,4]. CD4+ Treg cells are one of the most well-described regulatory immune cell populations. This is in part owing to their capacity to suppress local and systemic inflammation and to keep whole-body homeostasis [5]. Different authors indicate a reduction of this cell population during obesity [4,5].

More recently, the CD8+ Tregs have drawn the attention of researchers despite their reduced number [5,6]. This is in part, due to their highly suppressive and regulatory function [5,7]. Additionally, CD8+ Tregs share a similar transcriptome with CD4+ Treg cells but are different in the expression patterns of some immune regulatory molecules, which may indicate distinct functional pathways between subpopulations [5,7]. Alterations in CD8+ Treg cells have been described in different diseases, such as allergic asthma [8], multiple sclerosis [9], type 1 diabetes [10] and cancer [11]. Studies reporting human CD8+ Treg cells in obesity are limited. An increase in the number of CD8+ Tregs has been described in cord blood units from mothers displaying type 1 diabetes [10] and cancer [11]. Furthermore, Ruiz and collaborators observed a decrease in the percentage of CD8+ Tregs in obesity compared to those with normal-weight mothers [12].

The main aim of this study was to extensively characterize CD8+ Treg cells from people with obesity and insulin resistance, not only in peripheral blood (PB) but, more importantly, those infiltrated in the subcutaneous and visceral adipose tissue (VAT). Moreover, CD8+ Treg cells have been described in cord blood units from mothers displaying type 1 diabetes [10] and cancer [11]. Studies reporting human CD8+ Treg cells in obesity are limited. An increase in the number of CD8+ Tregs has been described in cord blood units from mothers displaying type 1 diabetes [10] and cancer [11]. Furthermore, Ruiz and collaborators observed a decrease in the percentage of CD8+ Tregs in obesity compared to those with normal-weight mothers [12].

The main aim of this study was to extensively characterize CD8+ Treg cells from people with obesity and insulin resistance, not only in peripheral blood (PB) but, more importantly, those infiltrated in the subcutaneous and visceral adipose tissue (VAT). Moreover, CD8+ Treg cells from the three tissues were further characterized according to their polarization patterns, chemokine receptor expression, distribution by the distinct functional compartments, and the expression of immunosuppressive molecules.

2. Material and methods

2.1. Subjects

Peripheral blood (PB), SAT and VAT were collected from a group of 42 adults with obesity (OB), at the Bariatric Surgery Unit, Department of General Surgery from Centro Hospitalar e Universitário de Coimbra (CHUC), Portugal. A group of 36 individuals (24 women and 12 men; age: 45 ± 11.4 years) was studied immediately before undergoing bariatric surgery (T1), while 6 individuals (4 women and 2 men; age: 42 ± 9.5 years) were only studied 9 to 18 months (11 ± 2.9 months) after bariatric surgery (T2). From T1 participants, a total of 8 individuals were followed up and studied at both T1 and T2. All subjects participated in the study after giving their written informed consent. The study was approved by the Ethics Committee of Centro Hospitalar e Universitário de Coimbra (CHUC; reference: CHUC-115-20 & CHUC-202-20). The study was carried out following the Declaration of Helsinki (1964) guidelines last revised in 2013. As inclusion criteria for the present study, the clinical indication for bariatric surgery was used. Briefly, individuals aged between 18 and 75 years old with BMI ≥ 35 kg/m², or BMI ≥ 35 kg/m² associated with the clinical diagnosis of T2D, hypertension, sleep apnea, or dyslipidemia were included. The exclusion criteria consisted of the presence of another known chronic illness and disorders that could affect the study outcomes, including inflammatory, neurodegenerative, and autoimmune diseases, as well as the use of immunosuppressive drugs. Pregnant women and individuals under 18 years old were not included in the study. This study consisted of a comprehensive 3-year observational study of the immune-metabolic parameters from people with obesity. Specifically, circulating immune cells and those infiltrated in subcutaneous and visceral adipose tissue were evaluated. The primary endpoint of this study was to quantify CD8+ Treg cells and assess their phenotype, as well as their distribution among functional/maturational compartments, both in the peripheral blood (PB) and in the adipose tissue immune infiltrate. Due to lack of adipose tissue samples from healthy individuals, we compared the

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**Fig. 1.** Study design. We characterized the phenotype and the functional compartment of CD8+ Tregs from peripheral blood, and those infiltrating adipose tissue, subcutaneous and visceral, by flow cytometry. Furthermore, the phenotype of these cells was assessed among different groups of study participants distinguished by HOMA-IR, with different glucose-impairment status.
percentage of CD8+ Tregs infiltrating the adipose tissue deposits, and their phenotypic features, with those from PB CD8+ Tregs from the same individuals. We also carried out comparisons between obesity groups. Obesity and obesity-related insulin resistance are known to be linked to chronic low-grade inflammation, which subsequently impacts cell metabolism. T cells play a pivotal role as orchestrators of the inflammatory response. Consequently, obesity-related alterations on T cell subsets, particularly on CD8+ Treg cells, may constitute as novel biomarkers for metabolic diseases. These specialized cells could become useful tools to stratify individuals with obesity into distinct immune profile groups, which may be associated with different outcomes in obesity-related comorbidities (Fig. 1). Approximately 12 mL of fasting PB was collected in EDTA tubes prior to surgery while about 12 mL of fasting adipose tissue was collected during the surgery. In addition, an age- and sex-matched group of voluntary participants (n = 12; 8 women and 4 men; age: 43 ± 11.9) without obesity (NOB) or any known health condition was also recruited to participate, following the same guidelines and procedures.

2.2. Anthropometric and biochemical characterization of participants

Body weight (BW, kg) was measured using a scale (seca 515 mBCA, seca, Hamburg, Germany) which was used to calculate the BMI (kg/m²), according to the formula: [BMI = BW (kg) / Height²(m²)]. Waist circumference (WC), hips circumference (HC), and neck circumference (NC) were measured using a flexible measuring tape (cm). The systolic and diastolic blood pressure (mmHg) were measured in the left arm, using a digital sphygmomanometer (Philips IntelliVue MP20, Philips, Boeblingen, Germany), after 5 min of resting. The measurements were performed during the same timeframe for all participants.

The biochemical parameters were analyzed in peripheral blood pre-surgery after 8–12 h of fasting. Glucose (mg/dL), insulin (μU/mL), C-peptide (µg/mL), HbA1c (%), C-reactive protein (CRP) (µg/mL), and the lipid profile – high-density lipoprotein cholesterol (HDL, mg/dL), triglycerides (TG, mg/dL) and total cholesterol (mg/dL) – were measured according to the standard methods and best practices at the Department of Clinical Pathology at CHUC. Low-density lipoprotein cholesterol (LDL, mg/dL) was calculated according to the Friedewald formula – [LDL = TC – (HDL + TG / 5)] [14]. The fasting glucose and insulin levels were further used to calculate the homeostatic model assessment insulin resistance index (HOMA-IR), as previously described [15] – [HOMA-IR = (Glucose (mg/dL) x Insulin (µU/mL)) / 405].

The leptin and adiponectin plasma levels were measured by immunoassay, according to the manufacturer’s instructions (ref: ELH-Leptin and ref.: ELH-Adiponectin, respectively, RayBio Tech Life Inc., GA, USA).

2.3. Isolation of the adipose tissue stromal vascular fraction

Abdominal subcutaneous and visceral adipose tissue from the great omentum was collected into a flask with saline solution, during the bariatric surgery, from each participant with obesity. The samples were immediately minced into small pieces and digested using 1 mg/mL of collagenase A (Clostridium histolyticum, ref.: 11088793001, Roche, Mannheim, Germany) in Krebs-Ringer HEPES medium [pH 7.4] (KRH) enriched with 4 % BSA (ref.: A4503 Sigma), 6 mM of glucose (ref.: G6152, Sigma) and 150 mM adenosine (ref.: P4532, Sigma, MO, USA), for 1 h at 37 °C and 105 rpm in a water bath, in order for the release of the stromal vascular fraction (SVF). The digested tissue was filtered using a 250 µm nylon mesh to retain the undigested extracellular matrix. The floating mature adipocytes were removed and washed using enriched KRH, to acquire the highest number of cells from the SVF. Further, the SVF was centrifuged at 540g for 5 min, the supernatant was discarded, and the cell pellet was resuspended and washed using Dulbecco’s phosphate-buffered saline (DPBS, ref.: 20-031-CV, Corning, Manassa, VA, USA). The cell pellet was resuspended in DPBS and filtered using a 100 µm mesh. The cell suspension was washed a second time with DPBS and centrifuged at 540g for 5 min. The cell pellet was resuspended using 2 mL of DPBS, transferred to a 5 mL polystyrene tube, and washed one last time before proceeding with the immunophenotyping protocol.

2.4. Immunophenotypic study of immune cells

2.4.1. Immune cell staining and acquisition

The immunophenotyping was performed by flow cytometry, at the Flow Cytometry Unit, CHUC, using a stain-lyse-wash protocol described elsewhere [16–18]. Briefly, for the 12-color tubes 1 and 2, 100 µL of peripheral blood or SVF, respectively, were incubated with 50 µL of Brilliant stain buffer (Becton Dickinson Biosciences (BD), San Jose, CA, USA) and the monoclonal antibodies (mAbs), described in Table 1, for 10 more minutes, under the same conditions. Then, the tube was centrifuged at 540 × g for 5 min, the supernatant was discarded, and the cell pellet was resuspended and washed using Dulbecco’s phosphate-buffered saline (DPBS, ref.: 20-031-CV, Corning, Manassa, VA, USA). The pellet was resuspended in DPBS and centrifuged at 540g for 5 min. The cell pellet was resuspended using 2 mL of DPBS, transferred to a 5 mL polystyrene tube, and washed one last time before proceeding with the immunophenotyping protocol.

2.4.2. Flow cytometry

The immune cells were acquired using a BD FACSAria III flow cytometer equipped with FACSsuite software (v1.5.0.925; BD). Furthermore, the T cell functional compartments were evaluated using a similar protocol, with some alterations. In summary, 100 µL of peripheral blood were incubated with the mAbs described in Tube 3 (Table 1), for 10 min at RT, in the dark. After incubation, 2 mL of FACSLysing Solution (BD) were added to the tube and incubated for 10 more minutes, under the same conditions. Then, the tube was centrifuged at 540 × g for 5 min, the FACSLysing solution was discarded, and the cell pellet was washed using 2 mL of DPBS. Finally, the cell pellet was resuspended in 500 µL of DPBS and immediately acquired in a FACSLysing Solution (BD) flow cytometer equipped with FACSsuite software (v1.5.0.925; BD). Furthermore, the T cell functional compartments were evaluated using a similar protocol, with some alterations. In summary, 100 µL of peripheral blood were incubated with the mAbs described in Table 3 (Table 1), for 10 min at RT, in the dark. After incubation, 2 mL of FACSLysing Solution (BD) were added to the tube and incubated for 10
more minutes under the same conditions. The tube was then centrifuged at 540 \( \times \) g for 5 min, the FACS lysing solution was discarded, and the cell pellet was washed using 2 mL of DPBS. Finally, the cell pellet was resuspended in 500 \( \mu \)L of DPBS and immediately acquired in a FACS Canto II™ (BD) flow cytometer, equipped with FACSDiva software (BD).

2.4.2. Identification and characterization of immune cell populations

The flow cytometry data were analyzed using the Infinicyt software (version 2.0; Cytognos SL, Salamanca, Spain), using the gating strategy described below and illustrated in Fig. 2 to identify the different immune cell populations. After cell debris and doublet removal, considering their properties on FSC-A and FSC-H, T lymphocytes were identified based on their expression of CD3, and their side scatter (SSC) properties. Then, T lymphocytes were subdivided into their CD4\(^{+}\), CD8\(^{+}\), CD4\(^{+}\)CD8\(^{+}\) and CD4\(^{+}\)CD8\(^{-}\) T cell populations. The identification of T lymphocytes within SVF required previous identification of the leukocytes based on the expression of CD45. A similar approach was applied to the three tissues, PB, SAT and VAT.

As depicted in Fig. 2, CD8\(^{+}\) regulatory T cells were identified based on their high expression of CD25 and low to negative expression of CCR5 and CCR6. These T cells were subdivided into CXCR5\(^{+}\) (CD8\(^{+}\)Tfr cells) and, according to their expression of CCR5, CCR6 and CCR7, into CD8\(^{+}\)Treg cells. Moreover, the expression of the immune-checkpoints molecules PD-1 and TIM-3 was also measured within the different CD8\(^{+}\) Treg cells functional compartments previously identified based on CD45RA and CD27 expression.
and effector (CD27+ CD45RA+ cells). Of note, TIM-3 expression and the CD8+ Tregs’ functional compartments were only evaluated in the PB.

2.5. Statistical analysis

Participants with obesity were divided into different groups using, as criteria, the obesity class, and the metabolic status. According to the obesity class [19], OB participants were divided into Class II (35 ≤ BMI < 40 kg/m²), Class III (40 ≤ BMI < 50 kg/m²) and Class IV (BMI ≥ 50 kg/m²) obesity. According to the metabolic status [20], OB participants were classified as insulin sensitive (IS), insulin resistant normoglycemic (IRn), pre-diabetic (Pre-T2D) and type 2 diabetic (T2D). Whenever appropriated, the group “insulin resistant (IR)”, comprising the IRn, Pre and T2D groups, was considered. The criteria used to assess the metabolic status of the OB participants were the HOMA-IR, fasting glucose plasma levels and HbA1c percentage, as detailed in Fig. 3.

The data are represented as median (1st quartile–3rd quartile) for all variables in the study. The Shapiro-Wilk normality test was performed to address the distribution of the data. The Wilcoxon rank sum test and Kruskal-Wallis test from package coin version 1.4.2 [21,22], followed by Dunn’s test from package rstatix version 0.6.0 [23], were applied when appropriate. For paired comparisons between the different tissues (PB, SAT and VAT), Wilcoxon-signed-rank test and Friedman’s test were performed [24]. Siegel and Castellan’s all-pairs comparisons test from package PMCMRplus version 1.9.7 as post-hoc [25] was used. Spearman’s correlations were performed using the function rcorr() from package Hmisc version 4.4-2 [26]. Differences were considered statistically significant when p < 0.05. All statistical analysis and data visualization were performed using R (version 4.0.2) [24,27].

3. Results

3.1. Physiologic and biochemical characterization

The study was performed in a cohort of people that integrates different obesity classes, and metabolic spectra. The metabolic spectra include insulin sensitive (IS), insulin resistant normoglycemic (IRn), pre-diabetic (Pre-T2D) and T2D patients (T2D) (Fig. 3; Table III). When comparing the OB group to an age- and gender-matched healthy non-obese group (nOB) (Table II), differences arise in metabolic parameters with the OB group displaying higher fasting insulin levels in circulation, and consequently an increased HOMA-IR value (≥2). HDL showed differences among groups, with significantly decreased levels in the OB group compared to the nOB. CRP and leptin levels were significantly increased in OB comparing to the nOB group.

When we stratified the OB group by obesity classes (II, III and IV), Class IV showed the lowest HDL levels in circulation (Class II: 45.0 (45.0–51.5) vs Class IV: 37.0 (32.0–45.0), p < 0.05). However, with the stratification of the participants according to their metabolic profile other differences arose (Table III). Despite the differences in metabolic characteristics used to separate the groups, IRn group were composed of a younger group of participants (IRn vs IS, p < 0.05; IRn vs T2D, p < 0.05). Regarding the lipid profile, IS showed similar levels when compared to nOB, while the remaining groups showed a reduction in circulating levels of HDL (p < 0.05). Of note, IS groups showed the highest levels of triglycerides (IS vs IRn and IS vs T2D, p < 0.05). Interestingly, Pre-T2D showed the highest levels of CRP in circulation among all groups (p < 0.05).

Analyzing the effect of bariatric surgery, we observed an amelioration of the anthropometric and biochemical parameters between T1 and T2 (Table IV). A reduction of BMI, WC, NC and HC was observed after
3.2. A higher percentage of circulating CD8 T regulatory T cells, with increased expression of CCR5, in people with obesity

In a first approach, the impact of obesity on the circulating CD25+IL-2Rα+CD127dimCD8 T regulatory T cell number and phenotype, was studied. An increased percentage of CD8 Treg cells, within CD8 T cells, was observed in OB group when compared to the healthy non-obese group (p < 0.05; Fig. 4.A), while no differences were found in their absolute number. To understand the impact of the degrees of obesity and metabolic status on this regulatory cell population, the OB group was subdivided into obesity classes (Fig. 4.B) and grouped according to their metabolic profile (Fig. 4.C). Participants with Class III obesity and Pre-T2D participants displayed the highest percentage of CD8 Treg cells within the circulating CD8 T cells (Fig. 4.B and C).

Interestingly, despite the sequential increase in the percentage of CD8 Treg cells with the worsening of metabolic status (Fig. 4.C), the T2D group displayed a lower percentage of these cells compared to Pre-T2D, however not significant.

Considering obesity as a potential trigger for the increased levels of inflammatory markers in circulation, the percentage of circulating CD8 Treg cells was weak and moderately correlated to plasma levels of leptin (rho = 0.38, p < 0.05) and CRP (rho = 0.40, p < 0.05), respectively (Fig. 4.D and .E). It is important to note that alongside the correlation with CRP, the Pre-T2D group was the one displaying the highest levels of CRP in circulation, as well as of CD8 Treg cells. Furthermore, the percentage of CD8 Tregs was moderately positively correlated to circulating insulin levels (rho = 0.42, p < 0.05) and HOMA-IR (rho = 0.43, p < 0.05), after excluding the participants taking medication for T2D from the analysis (Fig. 4.F and .G).

3.3. Circulating CD8 Treg cells polarize preferentially towards the Tc1/17-like phenotype in people with obesity

The polarization of circulating CD8 Treg cells was evaluated, as mentioned above. In the OB group, CD8 Treg cells were more prone to polarize into the Tc1-like and Tc17-like when compared to nOB group (Fig. 5.A), with a consequent reduction in the CCR5+CCR6+CD8 Treg cells (OB vs nOB, p < 0.05) (Fig. 5.A). Of note, the Tc1 (Fig. 5.B) and Tc17-like (Fig. 5.C) CD8 Treg cells from the OB group displayed a higher expression of CCR5 (measured as mean fluorescence intensity, MFI), when compared to the nOB group. No differences were found in the percentage of CD8 Tfr cells.

After distributing the OB group according to their specific obesity classes, there was a higher tendency for CD8 Treg cells to polarize towards Tc1-like in those with Class III obesity. Despite no statistical differences being observed regarding the percentage, the Tc1-like CD8 Treg cells from Class II and Class III obesity showed a higher expression of CCR5 (MFI) when compared to nOB group (p < 0.05) (data not showed). The CD8 Treg cells from Class II obesity showed a preference for Tc1/17-like polarization, displaying the highest percentage among groups (p < 0.05), Fig. 5.D. However, the expression of CCR5 by Tc1/17-like Treg cells was higher in Class III than nOB group (p < 0.05) despite no differences in their percentage. On the other hand, those with Class II obesity displayed the lowest percentage of CCR5+CCR6+CD8 Treg among the groups, especially compared to nOB and Class IV obesity (p < 0.05) (Fig. 5.D). The percentage of the CCR5+CCR6+CD8 Treg cells compartment was highest in the nOB group, within CD8 Treg cells.

Regarding the metabolic status, the IRn group showed a reduced percentage of the CD8 Treg with Tc17-like profile (p < 0.05 vs IS and vsT2D) (Fig. 5.E). Interestingly, the IRn displayed a similar percentage as the nOB group, regarding the CCR5+CCR6+CD8 Treg cells compartment, while a reduced percentage was observed among the IS, Pre-T2D and T2D (p < 0.05, vs IRn). The IS group showed the lowest percentage of CD8 Treg with Tfr-like phenotype (p > 0.05) (Fig. 5.E).

3.4. People with obesity display an increased percentage of CD8 Treg cells within the effector memory functional compartment

Regarding the functional compartments of the circulating CD8 Treg cells from the OB group, there was a tendency to decrease in the percentage of naïve and central memory compartments, compared to the nOB group (p > 0.05), accompanied by an increased percentage of the effector memory cells (p > 0.05), with no differences in the effector compartment (Fig. 6).

3.5. SAT displays higher a percentage of CD8 Treg cells than PB and VAT

Focusing on the OB group, differences in the CD8 Treg cell quantity and phenotype between PB and the different adipose tissue depots, (SAT and VAT) were investigated. CD8 Treg cells represent a small cell population within CD8 T cells. Therefore, we used two analytical

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Table II

<table>
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<th>Characteristics</th>
<th>nOB</th>
<th>OB</th>
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</tr>
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<td>Age (years)</td>
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</table>

nOB - participants without obesity; OB - participants with obesity; n - number of participants per analysis; BMI - Body mass index; WC - Waist circumference; HC - Hips circumference; WHR - waist-to-hip ratio; SBP - Systolic blood pressure; DBP - Diastolic blood pressure; HbA1c - glycated hemoglobin; HOMA-IR - Homeostatic model assessment of insulin resistance; HOMA-β - Homeostatic model assessment of beta cell function; LDL - Low-density lipoprotein; HDL - High-density lipoprotein; CRP - C-reactive protein.
approaches, in the first approach, we performed unpaired analysis, in all subjects from which at least one of the tissues was collected. The results showed that SAT displayed a higher percentage of CD8\(^+\) Tregs in comparison to VAT (p < 0.05) or PB (p < 0.05) (Fig. 7.A). In a second approach, the 11 participants for whom it was possible to measure the CD8\(^+\) Treg cells’ amount and phenotype in the three tissues, using paired analysis, were used. Despite no statistical differences being observed, these results were consistent with those obtained using the first approach. In fact, it shows that 7 out of 11 participants displayed a reduction in these cells is observed as the insulin resistance worsens compared to VAT (p < 0.05 vs T2D). Of note, a sequential decrease in CD8\(^+\) Tregs in comparison to VAT and plasma glucose levels (rho = -0.78, p < 0.05). No differences were found considering the obesity compartments. Noteworthy, the paired-sample analysis (Fig. 7.F), confirmed the results obtained with independent samples.

After grouping the OB group of participants according to their metabolic profile, the T2D group showed the lowest percentage of CD8\(^+\) Treg infiltrated in VAT (p < 0.05 vs IS and vs IRn). Of note, a sequential reduction in these cells is observed as the insulin resistance worsens (Fig. 8.A). In fact, a negative correlation was found between the percentage of CD8\(^+\) Treg infiltrated in VAT and plasma glucose levels (rho = -0.64, p < 0.05). No differences were found considering the obesity classes (Fig. 8.B).

3.6. Immune checkpoint molecules in CD8\(^+\) Treg cells

Besides the polarization of CD8\(^+\) Treg cells, we also evaluated the expression of two immune checkpoint proteins, namely programmed death cell (PD)-1 and T cell immunoglobulin and mucin domain containing protein (TIM)-3. The latter was only evaluated in CD8\(^+\) Treg cells from peripheral blood. When the percentage of circulating PD-1 CD8\(^+\) Treg cells (Fig. 9.A) and TIM-3 CD8\(^+\) Treg cells (Fig. 9.B) was compared between NOB and OB groups, a slight decrease was observed in OB group for both markers (p < 0.05). When evaluating the polarization of the circulating CD8\(^+\) Treg cells, a decreased percentage of the Tc1/7-like CD8\(^+\) Treg cells expressing PD-1 was detected in the OB compared to the NOB group (p < 0.05, Fig. 9.A). No major differences were found in the immune regulatory markers among the obesity classes studied (data not shown). On the other hand, when comparing metabolic groups, the percentage of cells expressing the immune checkpoint molecules was reduced in Pre-T2D, compared to the other groups. Namely, the percentage of circulating PD-1 CD8\(^+\) Treg cells (Pre-T2D vs IRn; p = 0.07)

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**Table III**

Physiologic and biochemical characterization of the OB group stratified by metabolic status.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>nOB</th>
<th>IS</th>
<th>IRn</th>
<th>Pre-T2D</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>12/4</td>
<td>6/1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>12</td>
<td>42  (35-51)</td>
<td>6</td>
<td>55 (44-56)</td>
<td>9</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>12</td>
<td>25.2 (22.8-27.5)</td>
<td>6</td>
<td>41.3 (38.5-44.5) (^A)</td>
<td>9</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>86</td>
<td>74-94</td>
<td>4</td>
<td>122 (120-129) (^A)</td>
<td>9</td>
</tr>
<tr>
<td>NC (cm)</td>
<td>6</td>
<td>39 (37-43)</td>
<td>8</td>
<td>41 (40-43)</td>
<td>9</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>99</td>
<td>97-101</td>
<td>10</td>
<td>129 (120-138) (^A)</td>
<td>9</td>
</tr>
<tr>
<td>WHR</td>
<td>0.85</td>
<td>0.74-0.90</td>
<td>1.00</td>
<td>98-101 (^A)</td>
<td>8</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>149.0 (130.0-153.0)</td>
<td>14.0</td>
<td>140.2 (130.0-153.0)</td>
<td>14.0</td>
<td>140.2 (130.0-153.0)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>82.0 (65.0-86.3)</td>
<td>12.0</td>
<td>80.0 (65.0-86.3)</td>
<td>12.0</td>
<td>80.0 (65.0-86.3)</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>6</td>
<td>109 (102-118)</td>
<td>5</td>
<td>84.0 (73.8-90.5)</td>
<td>9</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>6</td>
<td>7.5 (5.5-7.5)</td>
<td>9</td>
<td>5.4 (5.3-5.3)</td>
<td>9</td>
</tr>
<tr>
<td>HOMA-β</td>
<td>6</td>
<td>87.2 (60.9-112.0)</td>
<td>112</td>
<td>98.8 (94.1-101.9)</td>
<td>112</td>
</tr>
<tr>
<td>C-peptide (mg/mL)</td>
<td>3</td>
<td>1.47 (1.17-1.59)</td>
<td>1.97</td>
<td>1.70 (2.29-2.99)</td>
<td>3.6</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>7</td>
<td>98.8 (88.5-128.5)</td>
<td>123.6</td>
<td>87.9 (164.4)</td>
<td>110.6</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>6</td>
<td>66.0 (52.5-73.0)</td>
<td>50.5</td>
<td>46.0 (54.3)</td>
<td>42.0</td>
</tr>
<tr>
<td>Total cholesterol (mg/180</td>
<td>7</td>
<td>76.0 (71.0-111.0)</td>
<td>82.5</td>
<td>62.0 (105.3)</td>
<td>82.5</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>3</td>
<td>0.61 (0.36-1.88)</td>
<td>12.12</td>
<td>9.17 (13.88)</td>
<td>6</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>119.5</td>
<td>96.1-145.1</td>
<td>89.2</td>
<td>65.2 (110.4)</td>
<td>63.2</td>
</tr>
</tbody>
</table>

nOB: participants without obesity; IS: participants with insulin sensitivity; IRn: participants with insulin resistance and normoglycemic; Pre-T2D: participants with obesity and pre-diabetic; T2D: participants with type 2 diabetes; participants; n: number of participants per analysis; BMI: Body mass index; WC: Waist circumference; NC: Neck circumference; HC: Hips circumference; WHR: waist-to-hip ratio; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Hba1c: glycated hemoglobin; HOMA-IR: Homeostatic model assessment of insulin resistance; HOMA-β: Homeostatic model assessment of beta cell function; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CRP: C-reactive protein. Differences were considered when \(p < 0.05\) vs nOB; \(p < 0.05\) vs IS and vs IRn; \(p < 0.05\) vs PB. **A** \(p < 0.05\) vs PB and vs SAT. SAT was excluded from the paired-sample analysis because CD8\(^+\) Treg cells were not able to be detected in all SAT samples, since small amounts of SAT were collected in several participants. As represented in Fig. 7.E a different landscape was identified among CD8\(^+\) Treg cells from the different tissues. The great majority of CD8\(^+\) Treg cells from VAT displayed a Tc1-like phenotype (p < 0.05 vs PB and vs SAT). In turn, peripheral blood CD8\(^+\) Treg cells were evenly distributed among Tc1-like, Tc1/7-like and CCR5-CCR6- compartments, accompanied by a low representativeness of the Tc17-like Treg cells (Fig. 7.E). Interestingly, SAT seemed to be an intermediary step between PB and VAT regarding Tc1-like polarized CD8\(^+\) Treg cells (PB vs SAT, p < 0.05). In this tissue, the majority of CD8\(^+\) Treg cells were in the Tc1-like and CCR5-CCR6- compartments. Noteworthy, the paired-sample analysis (Fig. 7.F), confirmed the results obtained with independent samples. After grouping the OB group of participants according to their metabolic profile, the T2D group showed the lowest percentage of CD8\(^+\) Treg infiltrated in VAT (p < 0.05 vs IS and vs IRn). Of note, a sequential reduction in these cells is observed as the insulin resistance worsens (Fig. 8.A). In fact, a negative correlation was found between the percentage of CD8\(^+\) Treg infiltrated in VAT and plasma glucose levels (rho = \(-0.64\), p < 0.05). No differences were found considering the obesity classes (Fig. 8.B).
3.7. Effect of bariatric surgery on CD8+ Treg cells and their polarization profile

Metabolic surgery is one of the most effective treatments for obesity and obesity-associated comorbidities. Of 42 participants with obesity, 14 of them performed a second metabolic surgery approximately 11 ± 2.9 months after the first surgery. From those, we were able to perform the follow-up in 8 participants, at both the first (T1) and the second surgery (T2), with an elapsed time of 11 ± 2.9 months (ranging from 9 to 18 months).

Considering the analysis of results obtained at both time points, the effects of weight loss and the amelioration of the metabolic profile induced by metabolic surgery on CD8+ Treg cells can be identified. Interestingly, after surgery, participants showed a tendency to display a higher percentage of circulating CD8+ Treg cells (within CD8+ T cells) (p < 0.05; Fig. 11.A). On the other hand, the percentage CD8+ Treg cells (within CD8+ T cells) infiltrating the AT displayed a reduction, observed in both AT depots after surgery (p < 0.05) (Fig. 11.A). The follow-up of the same participant indicated an inter-individual heterogeneity with the percentage of circulating CD8+ Treg cells (within CD8+ T cells) increasing after surgery in 4 out of 8 participants (Fig. 11.B).

Regarding the polarization of the circulating CD8+ Treg cells, an increase in the percentage of Th17-like CD8+ Treg cells (p < 0.05) and a reduction in the percentage of Th1-like CD8+ Treg cells (p < 0.05) from T1 to T2 (Fig. 11.C), was observed by unpaired comparisons. Of note, the paired-sample analysis (n = 7) (Fig. 11.D) supports the results obtained for Th17-like CD8+ Treg cells observed by unpaired comparisons.

Although the CD8+ Treg cells infiltrating VAT are polarized towards Th1-like, at both T1 and T2, a decrease (p = 0.066) in the percentage of Th1-like cells was observed. This was accompanied by an increase of the Th17-like (p < 0.05) and CCR5+CCR6+ CD8+ Treg cells (p < 0.05) compartments, from T1 to T2. Furthermore, CD8+ Tfr also displayed an increase in VAT (p < 0.05) from T1 to T2 (Fig. 11.E). Due to the low number of CD8+ Treg cells, we were able to obtain paired data at both T1 and T2 in only a few individuals (Fig. 11.F).

Regarding the expression of immune-regulatory molecules, an increase in the overall percentage of circulating PD-1+ CD8+ Treg cells (p < 0.05) and TIM-3+ CD8+ Treg cells (p < 0.05) after surgery, was observed. This was due to an increase in the percentage of PD-1+ (p < 0.05) and TIM-3+ (p < 0.05) in Th1-like Tregs (Fig. 12.A and E). The same tendency was observed in paired samples, especially in the PD-1+ CD8+ Treg cells, where 6 out of 7 participants displayed an increase (p = 0.055) in the percentage of these cells after surgery (Fig. 12.B). A slight increase of PD-1+ CD8+ Treg cells in VAT was observed after surgery (Fig. 12.C).

4. Discussion

A detailed characterization of CD8+ Treg cells was performed in the peripheral blood, subcutaneous and visceral adipose tissues, of people with obesity displaying different metabolic profiles. Additionally, the effect of metabolic surgery on CD8+ Treg cells’ frequency and polarization was evaluated.

Obesity-associated low-grade inflammation has been associated with an increase in immune cell populations with a pro-inflammatory profile, such as neutrophils, M1 macrophages, Th1 and Th17 cells, and especially important are those infiltrating the adipose tissue depots [1,4]. This enhancement of a pro-inflammatory profile creates an imbalance in the homeostasis of the organism and may lead to a reduction in immune cells with anti-inflammatory function. This is particularly true for the CD4+ Treg cells, which are found at reduced number both in circulation and infiltrated on AT depots from individuals with obesity [1,3,4]. Furthermore, their function has also been described to be impaired in individuals with obesity displaying different metabolic profiles. Additionally, the percentage of circulating CD8+ Treg cells, an increase in the percentage of Th17-like CD8+ Treg cells (p < 0.05) and a reduction in the percentage of Th1-like CD8+ Treg cells (p < 0.05) from T1 to T2 (Fig. 11.C), was observed by unpaired comparisons. Of note, the paired-sample analysis (n = 7) (Fig. 11.D) supports the results obtained for Th17-like CD8+ Treg cells observed by unpaired comparisons.

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Fig. 4. Percentage of CD8\(^{+}\) Treg cells, within the CD8\(^{+}\) T lymphocytes, in nOB and OB groups (A) Percentage of CD8\(^{+}\) Treg in nOB and OB individuals; (B) Percentage of CD8\(^{+}\) Treg cells among the distinct obesity classes; (C) Percentage of CD8\(^{+}\) Treg cells in the OB grouped according to their metabolic status; (D–G) Spearman’s correlation between the percentage of CD8\(^{+}\) Treg cells, within CD8\(^{+}\) T cells, and (D) leptin (μg/mL), (E) CRP (mg/dL), (F) insulin (μU/mL) and (G) HOMA-IR. For (F) and (G) participants taking medication for T2D were excluded. Mann-Whitney, Kruskal-Wallis and Spearman’s correlation tests were used, as appropriate. Differences were considered significant when (*) \( p < 0.05 \).
Fig. 5. Polarization of circulating CD8^+ Treg cells in OB participants distributed according to their obesity class and metabolic status. (A) Percentage of CD8^+ Tfr (CXCR5^+), and CD8^+ Treg cells polarized towards Tc1-like (CCR5^+), Tc17-like (CCR6^+), Tc1/17-like (CCR5^+CCR6^+) and CCR5^+CCR6^+ in nOB (n = 12) and OB (n = 31–35); (B) Expression of CCR5 by Tc1-like Treg cells measured as mean of fluorescence intensity (MFI); (C) Expression of CCR5 by Tc1/17-like Treg cells, measured as MFI; (D) CD8^+ Treg cell polarization profile according to obesity classes (n = 6–14); (E) CD8^+ Treg cells polarization profile within metabolic groups (n = 4–13). Mann-Whitney, Kruskal-Wallis and Spearman’s correlation tests were used, as appropriated and differences were considered significant when * p < 0.05; ** p < 0.01.
Treg cells and a consequent reduction of the deleterious STZ effect, by P. Barbosa et al.

Impact on the percentage of CD8 highest levels of CRP. In fact, insulin and CRP levels appear to have more importance in obesity-related comorbidities such as insulin resistance, especially T2D. Moreover, in our study, the Pre-T2D status showed the worse the metabolic status, the higher the percentage of CD8 T cells that should be emphasized, in our study, the Pre-T2D group displayed the Pre-T2D status showed the percentage of CD8 T cells, in people with obesity. Within tissues, SAT showed to be a Treg cells reservoir. Interestingly, these cells showed a tendency to polarize towards the Tc1-like and the Tc1/17-like CD8 Treg in people with obesity. Although it was previously described that Tc1-like CD8 Treg cells are able to suppress the production of IFN-γ by CD8CCR3 T cells (in vitro) [29], further studies are needed for a broad understanding of their function and the differences between CD8+ Treg cells following different polarization paths. This is particularly important as we demonstrate that Tc1- and Tc1/17-like CD8+ Treg cells displayed higher levels of CCR5 on their surface, being more prone to migrate into inflamed tissues, such as the AT from individuals with obesity [30,31]. Our results agreed with a previous publication showing that CD8+ Tregs were increased in the cord blood units from mothers with overweight and obesity [12]. In line with this, it was previously reported a positive correlation between the percentage of CD8+Foxp3+ Treg cells and BMI [4].

Our data demonstrate that participants with pre-diabetes showed a higher percentage of CD8+ Treg cells in circulation. When looking closer into their biochemical characterization, we observed that the percentage of CD8 Treg was positively correlated to CRP levels, insulin, and HOMA-IR, especially in participants who were not taking T2D medication. It is noteworthy to observe that the Pre-T2D group displayed the highest levels of CRP. In fact, insulin and CRP levels appear to have more impact on the percentage of CD8 Treg cells than obesity by itself. It has been previously indicated that CRP increases in obesity and plays an important role in obesity-related comorbidities such as insulin resistance, corroborating our data [32].

In 2020, Shimokawa and collaborators reported CD8 Treg cells as a critical cell type in the prevention of type 1 diabetes (T1D) [10]. The authors induced T1D in male C57BL/6J mice with streptozotocin (STZ), previously infected with an intestinal nematode, Heligmosomoides polygyrus (Hp) [10]. The infection by Hp induced an increase in the CD8 Treg cells and a consequent reduction of the deleterious STZ effect, by protecting pancreatic β-cells. To address the involvement of CD8 Treg cells (identified as CD122+CD8+ T cells), the authors injected the mice with an anti-CD25 antibody to deplete the CD4+ Treg cells, concluding that the protective effect remained [10]. The same study also identified circulating CD8 Treg cells from children with T1D as CD8+CD122+CXCR3+ T cells and observed a reduction in this cell population compared to healthy volunteers [10]. An important aspect that should be emphasized, in our study, is the worse the metabolic status, the higher the percentage of CD8 Treg cells within CD8 T cells in circulation. However, the behavior of these cells infiltrated on VAT follows the inverse pattern, being reduced in individuals with IR, especially T2D. Moreover, in our study, the Pre-T2D status showed the highest percentage of circulating CD8 Treg cells, while their polarization displayed the lowest percentage of CCR5CCR6+CD8+ Treg compartment, which comprises mainly the naïve compartment. This could be indicative of a higher degree of differentiation of the circulating CD8 Treg cells from individuals with an unfavorable metabolic status.

From the analysis performed between tissues, PB, SAT, and VAT, the SAT displayed the highest percentage of infiltrated CD8 Treg cells within the CD8+ T cells. This higher percentage of SAT-infiltrated CD8+ Treg cells is corroborated by the analysis of the three tissues from the same participants (paired sample analysis). These results point to the role of SAT in the maintenance of metabolic homeostasis, as described by others [33], while VAT displays a higher pro-inflammatory status [33]. Furthermore, the increase of CD8+ Treg cells on AT, more marked on SAT, could be working as a compensatory mechanism to re-establish the homeostasis in the tissue. Among the various metabolic groups, the T2D group showed the lowest percentage of infiltrated CD8+ Treg cells in SAT. Interestingly, Wu and collaborators (2019) reported a reduction of CD4+ Treg cells in the VAT of T2D patients [34], while no differences have been observed by our group for this cell population (our unpublished data). In the present study, T2D group displayed lower levels of HDL compared to nOB and IS. Though HDL has been described has a modulator of the inflammatory function of macrophages in T1D and T2D [35,36], however, no correlations were found between HDL levels and CD8+ Treg cells and their subsets, in the present study.

When the phenotype of CD8+ Treg cells arising from the different tissues, of people with obesity, is compared, we observed that the percentage of cells expressing CCR5 infiltrated in VAT was higher than observed in PB or SAT, supporting the active role of this chemokine receptor in driving CD8+ Treg migration towards VAT. Interestingly, a higher percentage of CD8+ Tregs in the AT expressed PD-1, endowing these cells with a high suppressive potential [9,37]. CD8+ Treg cells are being described as a heterogeneous population with different subsets displaying immunoregulatory capacity [38]. Their immunosuppressive abilities involve the secretion of different molecules that lead to the inhibition of T cell proliferation and suppression of IFN-γ-producing T cells, as reviewed elsewhere [6,38]. Additionally, considering the pro-inflammatory profile associated with obesity and the increased percentage of CD8+ Treg cells in PB from people with obesity, it is imperative for future research to assess the immunosuppressive capacity of these cells in order to understand if they are increasing as a compensatory mechanism resultant from the reduction of CD4+ Tregs or if they are contributing to the pathogenic processes exacerbating obesity-related inflammation and the obesity-related insulin resistance.

Bariatric surgery is one of the most effective treatments for obesity, leading to weight loss, and ameliorating metabolic conditions [39]. Recent studies demonstrated that bariatric surgery induces changes in T and B cell subsets, modulating the characteristics of these cells to resemble the profile exhibited by lean controls [39]. We observed a tendency for an increase in the percentage of circulating CD8 Treg cells after surgery, while the percentage of these cells was reduced in SAT after surgery. Additionally, we also observed an increase in the percentage of CD8 Treg cells after surgery, as well as in the percentage of Tc1-like CD8 Treg cells expressing PD-1. This increased expression of PD-1 after surgery may be translated as an improvement in the immunosuppressive capacity of CD8 Treg cells [5,37]. However, the paired-sample analysis indicated a degree of heterogeneity among the OB participants and different responses to surgery, at least in CD8 Treg cells. This is an important observation and, based on it, bariatric surgery may impact the immune system differently in distinct individuals. These results should be validated in a bigger cohort in future studies.

To the best of our knowledge, this study describes for the first time the frequency, detailed phenotype, and expression of immune checkpoint proteins – PD-1 and TIM-3 – by CD8+ Treg cells in circulation, but also those infiltrated into SAT and VAT, in a context of obesity-related insulin resistance. However, our study is limited by the number of
Fig. 7. CD8\(^+\) Treg cell percentage (measured within CD8\(^+\) T cells) and polarization, in different tissues: peripheral blood (PB), subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). (A) Percentage of CD8\(^+\) Treg (within CD8\(^+\) T cells) in PB, SAT and VAT (unpaired/independent samples). (B) Percentage of CD8\(^+\) Treg (within CD8\(^+\) T cells) measured in the PB, SAT and VAT from the same participants (paired-sample analysis). (C and D) Spearman’s correlation between the percentage of CD8\(^+\) Treg (within CD8\(^+\) T cells) infiltrated on SAT and VAT, and within infiltrated immune cells, respectively. (E) Polarization of CD8\(^+\) Treg cells in circulation and infiltrated in SAT and VAT, from the same participants (paired-sample analysis, n = 3–15). Mann-Whitney and Kruskal-Wallis, and Wilcoxon Signed-rank test and Friedman’s tests were applied as appropriate. Differences were considered significant when \( ^* \ p < 0.05; \ ^{**} \ p < 0.01; \ ^{***} \ p < 0.001.\)
participants and tissue sample size, especially those regarding SAT samples, since we had limitations in the amount of SAT collected during laparoscopic bariatric surgery. Nevertheless, there is consistency in the results obtained from different tissues and among groups as indicated by the independent and paired analysis. This gives some degree of confidence regarding the direction of the described results. Nonetheless,
studies of this kind, analyzing CD8\textsuperscript{+} Treg cells with a high degree of detail, have not been previously reported before or after bariatric surgery.

5. Conclusion

In conclusion, the main results suggest that obesity is associated with an increased percentage of circulating CD8\textsuperscript{+} Treg cells, especially those displaying higher expression of CCR5. In fact, people with pre-diabetes
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or T2D display a more pronounced alteration in this regulatory T cell subset, compared to people without obesity. These alterations seem to be influenced by/or influence the circulating levels of CRP and fasting insulin. In addition, SAT appears to be a reservoir of CD8$^+$ Treg cells in people with obesity, while the VAT displayed a higher Tc1-like CD8$^+$ Treg cell polarization, as well as Tc1-like CD8$^+$ Treg cells expressing PD-1, thus with a high immunosuppressive potential. Future studies are needed to understand if the increase in this cell population works as a compensatory mechanism for the reduction of the CD4$^+$ Treg subset. In addition, further studies are needed to identify the function of CD8$^+$ Treg cells.

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CRediT authorship contribution statement


Declaration of competing interest

The authors declare that there are no conflicts of interest.

References


