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FACULTAD DE MEDICINA

Centro de Investigación en Ciencias de la Salud y Biomedicina (CICSaB)

Evaluación cuantitativa y funcional de linfocitos Breg, Tr1 y Treg CD69^{+} en individuos con sobrepeso y obesidad

TESIS QUE PRESENTA

M. en C. NORMA ALEJANDRA MENDOZA PÉREZ

PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOMÉDICAS BÁSICAS

DIRECTOR DE TESIS
DR. ROBERTO GONZÁLEZ AMARO

FEBRERO 2024

CRÉDITOS INSTITUCIONALES

Esta tesis se llevó a cabo en la sección de Medicina Molecular y Traslacional del Centro de Investigación en Ciencias de la Salud y Biomedicina de la Universidad Autónoma de San Luís Potosí, bajo la tutoría del Dr. Roberto González Amaro, gracias al apoyo del Consejo Nacional de Ciencia y Tecnología que otorgó la beca con número 714854. Para la realización de este trabajo se utilizaron los recursos propios del Departamento de Inmunología de la Facultad de Medicina de la U.A.S.L.P.

Tesis que presenta:

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EN CIENCIAS BIOMÉDICAS BÁSICAS

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RESUMEN

La obesidad es una enfermedad de curso crónico y de etiología multifactorial que se caracteriza por un aumento en los depósitos de grasa corporal y ganancia de peso. La sobrecarga prolongada de nutrientes resulta en un estado de inflamación crónica de bajo grado en el tejido adiposo y sistémicamente, en particular en depósitos de grasa visceral. Al igual que cualquier estado inflamatorio, la inflamación crónica de bajo grado asociada con la obesidad debe estar sujeta a los mecanismos que controlan la actividad de las células inmunes. Recientemente se ha descrito que las células B reguladoras (Breg) ($CD19^{+}CD27^{+}CD38^{+}$) son una subpoblación adicional de linfocitos, las cuales se caracterizan por su capacidad de inhibir los fenómenos inflamatorios y contribuir a la homeostasis del sistema inmune. Por otra parte, las células T reguladoras (Treg) son también capaces de inhibir la activación y proliferación de linfocitos efectores y tienen un papel importante en la patogénesis de enfermedades autoinmunes y la inflamación crónica. Se han descrito diferentes subpoblaciones de linfocitos Treg, incluyendo a los $Foxp3^{+}$ ($CD4^{+}CD25^{high}CD127^{low}$), los Tr1 ($CD4^{+}CD49b^{+}LAG\text{-}3^{+}IL\text{-}10^{+}$) y los Treg $CD69^{+}$ ($CD4^{+}CD69^{+}Foxp3^{-}TGF\text{-}\beta^{+}$).

El objetivo de los trabajos que constituyen el documento de la tesis fue el de evaluar los niveles y la función de los linfocitos Tr1 y Treg $CD69^{+}$, así como de las células Breg en pacientes con obesidad y controles sanos.

En el primer manuscrito se analizó el número y la actividad inhibidora de los linfocitos Breg y Tr1 en pacientes con peso normal, sobrepeso u obesidad, asociados o no a disfunción metabólica. En este estudio, se encontró que los pacientes con obesidad presentan cambios en el número y función de linfocitos Breg ($CD19^{+}CD27^{+}CD38^{+}$), en tanto que en el caso de las células Tr1 no se observaron diferencias significativas entre los grupos de estudio.

En el segundo manuscrito se llevó a cabo un análisis cuantitativo y funcional de los linfocitos Treg $CD69^{+}$ y se observó una disminución del número de estas células en personas metabólicamente no sanas, con o sin sobrepeso. Además, se detectó que la función supresora de las Treg $CD69^{+}$ está disminuida en estos pacientes.

Los datos generados en estos dos trabajos de investigación sugieren que las anormalidades en el número y la función de los linfocitos Breg y Tr1 están involucradas en la patogénesis del fenómeno inflamatorio de bajo grado asociado a la obesidad y la disfunción metabólica.

Human Immunology

Analysis of B cell regulatory lymphocytes and Tr1 cells in patients with obesity

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Abstract

Overweight and obesity are usually associated with metabolic dysfunction and the presence of a low-grade inflammatory phenomenon. Several immune regulatory cell subsets have been described, which are able to suppress different immune phenomena and inflammation. The aim of this study was to analyze the number and activity of several regulatory lymphocyte subsets in individuals with overweight or obesity, accompanied or not of metabolic dysfunction. Blood samples from the following six groups of individuals were studied: normal weight metabolically healthy (NWH, n=11), normal weight metabolically unhealthy (NWU, n=6), overweight metabolically healthy (OWH, n=13), overweight metabolically unhealthy (OWU, n=10), obese metabolically healthy (OBH, n=6) and obese metabolically unhealthy (OBU, n=11). Tr1 cells ($CD4^+CD49b^+LAG3^+IL\text{-}10^+$) and regulatory B (Breg) lymphocytes ($CD19^+CD27^+CD38^+$ and $CD19^+CD71^-CD25^+CD73^+$ cell subsets) were analyzed by flow cytometry. The inhibitory activity of $CD19^+CD71^-$ $CD25^+CD73^+$ Br1 lymphocytes was also assessed. We detected an increased number of $CD19^+CD27^+CD38^+$ lymphocytes in obese patients, whereas a diminished inhibitory activity of Br1 cells was detected in these individuals. No apparent quantitative abnormalities of Tr1 cells were observed in the patients included in the study. Our data indicate that obese patients show different abnormalities in the number and function of Breg lymphocytes, which may contribute to the pathogenesis of the low-grade inflammatory phenomenon associated to obesity.

Keywords: Regulatory lymphocytes, Overweight, Metabolic syndrome, Tr1 cells.

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Introduction

The immune system has an important role in the pathogenesis of metabolic syndrome and different conditions associated to overweight and obesity, including the low-grade inflammatory phenomenon seen in patients with these conditions[1]. In this regard, it has been described the involvement of T regulatory (Treg) cells and B regulatory (Breg) lymphocytes in the inflammation associated to obesity[2-4]. Accordingly, different studies on the number and function of the Treg cell subset originally described by Sakaguchi S, et al. (with the phenotype $CD4^+CD25^{high}Foxp3^+$)[5] in both, the peripheral blood and adipose tissue from patients with overweight have been reported[6-8]. In addition, we have previously explored the levels and function of $CD69^+$ Treg lymphocytes in individuals with overweight[9].

B cells, which carry out the production of antibodies (along with plasma cells) and mediate the humoral immune response, exert other important functions in the immune system, including the presentation of antigens to T cells and the regulation of the immune response and inflammatory phenomena[10,11]. In this regard, those B lymphocytes characterized by the synthesis of the immunoregulatory cytokines IL-10, IL-35 and TGF-β, are able to inhibit different immune functions and thus they have been denominated as B regulatory cells[11,12]. Since its initial description, several subsets of Breg cells have been described, including immature B, B10 and Br1 lymphocytes as well as iBreg cells and regulatory plasmablasts[13]. The latter cells are characterized by the phenotype $CD19^+CD27^+CD38^+$ as well as by the synthesis of the immunoregulatory cytokine IL-10[11,13], whereas Br1 lymphocytes exhibit the phenotype $CD19^+CD71^-CD25^+CD73^+$[11,13]. It has been reported that all these regulatory B cell subsets seem to be involved in the pathogenesis of different immune-mediated conditions, including autoimmunity and allergy, as well as in the suppression of the immune response against tumoral antigens[14-16].

Type 1 T regulatory (Tr1) cells are inducible in the periphery and have a pivotal role in promoting and maintaining the immune tolerance towards different

antigens[17]. These $CD4^+$ T lymphocytes express high levels of the $\alpha 2$ chain of integrins (CD49b) as well as the LAG3 (CD223) molecule, which is a structural homolog of CD4 and can bind to MHC class II molecules with high affinity[18-20]. In contrast with the Treg cells described by Sakaguchi S[5], Tr1 lymphocytes does not show a constitutive expression of the transcription factor Foxp3[17,20]. These lymphocytes exert its immunoregulatory function through different mechanisms, including the release of IL-10, which inhibit the activity of macrophages and antigen-presenting cells[17]. As it has been reported, Tr1 cells exhibit the following phenotype: $CD4^+LAG3^+CD49b^+IL\text{-}10^+Foxp3^-$, which allows their identification and quantification by flow cytometry[18-20].

It has been widely described that most individuals with overweight or obesity show evidence of metabolic dysfunction (insulin resistance, etc.) and a low-grade inflammatory phenomenon, which is associated with an increased risk of atherosclerosis and ischemic cardiovascular disease, among others[21,22]. However, a small but significant proportion of individuals with overweight/obesity do not show evidence of metabolic dysfunction (insulin resistance, etc.) and tissue inflammation[21,22]. Conversely, it has been demonstrated that some normal weight individuals show a persistent metabolic dysfunction with evidence of a low-grade inflammatory phenomenon[21,22].

The aim of this study was to evaluate two subsets of Breg lymphocytes and Tr1 cells in patients with overweight/obesity, associated or not to metabolic dysfunction. Our results suggest that obesity is associated with abnormalities in the number or function of the Breg cells analyzed in this study.

Materials and Methods

Patients and controls. Fifty-seven individuals were included in the study, who were classified into six groups, according to their weight and the presence or not of metabolic dysfunction. Thus, the following groups of individuals were studied: normal weight metabolically healthy (NWH, n=11), normal weight metabolically unhealthy (NWU, n=6), overweight metabolically healthy (OWH, n=13), overweight metabolically unhealthy (OWU, n=10), obese metabolically healthy (OBH, n=6) and obese metabolically unhealthy (OBU, n=11). Overweight was defined as a body mass index (BMI) into the 25-29.9 range, obesity as a BMI>30, and normal weight into de range of 18.5-24.9. In addition, individuals were also classified according to their percentage of fat mass, as follows: normal weight, 21-33% (8-20% in men); overweight, 33-39% (20-25% in men), and; obesity, >39% (>25% in men). Moreover, those individuals with blood triglyceride levels higher than 150.0 mg/dL and high-density lipoprotein cholesterol concentrations lower than 40.0 mg/dL, insulin levels higher than 25 μUI/mL, waist circumference higher than 80.0 cm for women and 100.0 cm for men were considered as metabolically unhealthy. All individuals included in the study were apparently healthy (with no evidence of arterial hypertension or diabetes mellitus) and no record of chronic drug(s) intake was detected in none of them. All individuals signed a written informed consent and this study was performed according to the Declaration of Helsinki. This study was approved by the Bioethical Committee of the State of San Luis Potosí, México.

Cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) density-gradient centrifugation, and cellular viability was evaluated by trypan blue staining, which was always higher than 95%.

Flow cytometry analysis. Quantification of Tr1 cells was performed as described[23]. In brief, PBMC were labeled with the following mAbs: anti-CD4-PerCp (BD Biosciences, San Jose, CA), anti-CD49b-APC (BioLegend Inc, San Diego CA)

and anti-LAG-3-FITC (BioLegend). Then, cells were fixed and permeabilized with PFA 4% during 15 minutes at 4°C and saponine 0.1% during 10 minutes, and additionally stained with an anti-IL-10-PE mAb (BioLegend). Moreover, in the case of regulatory plasmablasts, PBMC were labeled with an anti-CD19-PerCp (BioLegend), an anti-CD38-PE (BioLegend) and an anti-CD27-FITC (BioLegend) mAb, whereas Br1 lymphocytes were identified as $CD19^{+}CD71^{-}CD25^{+}CD73^{+}$. In all cases, cell samples were acquired in a FACSCanto II flow cytometer (Becton Dickinson) and analyzed by using the Flow Jo software v10 (Tree Star Inc., Ashland, OR). Doublet discrimination was performed by analyzing FSC-A versus FSC-W dot plots from the lymphocyte gate.

Functional analysis of Br1 lymphocytes. The regulatory activity of Br1 cells was estimated by an assay of inhibition of interferon-γ (IFN-γ) release. In brief, $CD19^{+}CD25^{+}$ cells were purified from PBMC by using a B cell isolation kit II for magnetic cell separation (MAC System; Miltenyi Biotec, Germany), followed by the isolation of $CD25^{+}$ B cells with microbeads coupled with an anti-CD25 mAb (Miltenyi Biotec, Germany). Then, these $CD19^{+}CD25^{+}$ lymphocytes were co-cultured for 72 h at 37°C, 5% CO2, and 95% humidity with autologous naïve $CD4^{+}$ T cells in the presence of 10 ng/mL of human recombinant IL-12 and 10 μg/mL of an anti-human-IL-4. Then, the concentration of IFN-γ (and other cytokines) in the cell culture supernatant was analyzed by using a Cytometric Bead Array (BD Biosciences), an Accuri C6 cytometer (BD Biosciences) and the software FCAP Array v3.01 (BD Biosciences).

Statistical analysis. Data with normal distribution were represented as the arithmetic mean and SD, and those with a non-Gaussian distribution with the median and Q_1-Q_3 interquartile range. Comparisons of two groups were performed with the Mann-Whitney U test and analysis of more than two groups with the Kruskal-Wallis sum rank test. The possible associations between laboratory and clinical data were analyzed with the Spearman rank correlation test. Data were analyzed using the GraphPad Prism v8.1 software, and p values <0.05 were considered as significant.

Results

Analysis of Breg cells. We used flow cytometry analysis to evaluate the percentage of regulatory plasmablasts in PBMC from individuals with normal weight, overweight and obesity, healthy and unhealthy (Fig. 1A). According to these analyses, we observed no significant differences in the percent of $CD19^+CD27^+CD38^+$ cells in the six groups studied, classified according to the BMI ($p>0.05$, Kruskal-Wallis test, Fig. 1B). However, when the groups were classified according to the percent of fat tissue, those patients with obesity (either, metabolically healthy or unhealthy) showed higher levels of regulatory plasmablasts compared to healthy individuals with overweight ($p<0.05$ in both cases, Fig. 1C). In contrast, the percents of $CD19^+CD71^-CD25^+CD73^+$ Br1 cells were similar in the six groups studied, classified by both BMI or the percent of fat tissue ($p>0.05$ in both cases, Kruskal-Wallis test, data not shown).

When the regulatory function of $CD19^+CD25^+$ cells on the cytokine synthesis by autologous naïve T lymphocytes was analyzed, we found a significant diminished inhibitory activity in cells from obese patients compared to normal weight individuals, in the case of IFN-γ ($p<0.05$, Fig. 2, left panel). A similar result was observed in the case of IL-6, when overweight and obese patients were compared ($p<0.05$, Fig. 2, middle panel). In contrast, cell cultures from obese patients showed a significant higher release of IL-4 compared to overweight individuals ($p<0.05$, Fig. 2, right panel). No other significant differences among the three (or six) groups studied were detected ($p>0.05$ in all cases, data not shown).

Analysis of Tr1 lymphocytes. When the levels of Tr1 cells were determined by four-color flow cytometry analysis (Fig. 3A), the levels of $CD4^+CD49b^+LAG3^+IL\text{-}10^+$ lymphocytes were similar in individuals with normal weight, overweight and obesity (Fig. 3B, $p>0.05$, Kruskal-Wallis test). In addition, when metabolically healthy and unhealthy individuals were analyzed by separate, similar results were observed,

with no significant differences among the six groups (Fig. 3C, $p>0.05$, Kruskal-Wallis test).

Discussion

It has been widely described the low-grade inflammatory phenomenon that is associated with overweight and obesity[1]. Likewise, different studies have analyzed the number and/or function of immunoregulatory cells (mainly $CD4^+CD25^+Foxp3^+$ Treg lymphocytes), in both, peripheral blood and fat tissue, from individuals with obesity[2-4]. However, most of these studies have not taken into account that a significant proportion of individuals with overweight and obesity do not show evidence of metabolic dysfunction and low-grade inflammation[21,22]. Furthermore, there are also individuals without overweight or apparent excess of fat tissue that show evidence of metabolic dysfunction, associated or not to low-grade inflammation[21,22]. According to this, we decided to carry out a pilot quantitative and/or functional study of several immunoregulatory cells (different from $CD4^+CD25^+Foxp3^+$ Treg lymphocytes) in the peripheral blood from individuals classified according to their weight and the presence or not of metabolic dysfunction.In this regard, we have hypothesized that those individuals with metabolic dysfunction should show abnormalities in Tr1 cells and/or Breg lymphocytes.

Regulatory Tr1 cells seem to have a relevant role in different immune-mediated conditions, including type 1 diabetes mellitus, allergy, myasthenia gravis and multiple sclerosis[17,24-27]. In addition, we have previously observed that patients with autoimmune thyroid disease (Hashimoto´s thyroiditis and Graves' disease) show diminished levels of Tr1 lymphocytes as well as a defective function of these cells[23]. In contrast, in this study we have not observed apparent quantitative abnormalities of Tr1 cells associated to obesity or metabolic dysfunction. In this regard, we consider that it would be of interest to analyze the suppressive function of these regulatory cells in individuals with obesity, something that could not be done in this study due to the limited number of cells available.

An additional point of regulation of the activation of the immune system and the inflammatory phenomenon is exerted by different subsets of B lymphocytes[10,11].

In this regard, in this study we have observed no apparent quantitative differences in the case of $CD19^+CD71^-CD25^+CD73^+$ Br1 regulatory cells among the groups of individuals included in this study. However, our results indicate that these cells show a defective regulatory function in obese individuals, showing a diminished inhibitory activity on the release of the pro-inflammatory cytokines IFN-γ and IL-6 by T cells. In this regard, it is worth mentioning that it has been widely described that increased serum levels of IL-6 are tightly associated with an increased risk for ischemic cardiovascular disease[28].

Finally, the quantitative analysis of $CD19^+CD27^+CD38^+$ cells or regulatory plasmablasts showed that obese individuals tended to exhibit increased numbers of these lymphocytes. In this regard, it is of interest the study of Rueda CM, et al., showing that HDL promote the survival of $CD4^+CD25^+Foxp3^+$ Treg lymphocytes[29], indicating the relationship between metabolic and immune parameters. Moreover, it is also of interest the report of García-Hernández MH, et al., showing decreased levels of regulatory plasmablasts in individuals with obesity (compared to individuals with normal weight), but not in those with overweight[30]. We consider that these apparent contradictory results can be due to the different number of individuals studied as well as to the classification made in our study, in individuals with and without metabolic dysfunction.

In summary, we have explored, in this pilot study, the possible involvement of different subsets of regulatory lymphocytes in individuals with overweight/obesity, associated or not to metabolic dysfunction. Since we have detected quantitative and functional abnormalities in two out of three lymphocyte subsets analyzed, we consider that these results further indicate that there is a dysregulation in the homeostatic mechanisms of the immune system in obesity.

Authors contribution:

AM-P, performed experiments, collected and analyzed the data and wrote the draft; LG-B, collected the data and contributed data or analysis tools; MV-N, performed experiments, collected and analyzed the data; BH-C, analyzed the data and interpreted the data; SB-S, supervised the study; PN-M, supervised the study and wrote the paper; DPP-P, conceived and designed the study; EJN-G, interpreted the data and conceived and designed the study; RG-A, conceived and designed the study, supervised the study, approved the final version of the manuscript.

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Figure legends

Fig. 1. Quantitative analysis of B regulatory lymphocytes in individuals with overweight and obesity. Blood samples from individuals with normal weight, overweight and obesity, with metabolic dysfunction or not, were analyzed for the levels of regulatory plasmablasts ($CD19^+CD27^+CD38^+$), as described in Materials and Methods. **A)** Flow cytometry strategy for the analysis of lymphocytes with the $CD19^+CD27^+CD38^+$ phenotype. **B)** Percentages of regulatory plasmablasts (Breg cells) in individuals classified as normal weight, overweight and obese, according to their BMI, and further classified as metabolically healthy or unhealthy. **C)** Percentages of regulatory plasmablasts in individuals classified as normal weight, overweight and obese, according to their % fat tissue, and further classified as metabolically healthy or unhealthy. Data correspond to the arithmetic mean and SD. * $p<0.05$. NWH, normal weight metabolically healthy; NWU, normal weight metabolically unhealthy; OWH, overweight metabolically healthy; OWU, overweight metabolically unhealthy; OBH, obese metabolically healthy; OBU, obese metabolically unhealthy.

Fig. 2. Functional analysis of Br1 lymphocytes in individuals with overweight and obesity. Cytokine (IFN-γ, IL-6 and IL-4) release in co-cultures of Br1 cells with autologous naïve $CD4^+$ T lymphocytes in blood samples from individuals with normal weight (NW), overweight (OW) and obesity (OB). Data correspond to the median and Q_1-Q_3 interquartile range. * $p<0.05$.

Fig. 3. Quantitative analysis of Tr1 cells in individuals with overweight and obesity. Blood samples from individuals with normal weight, overweight and obesity, with metabolic dysfunction or not, were analyzed for the quantification of Tr1 cells, as described in Material and Methods. **A)** Flow cytometry strategy for the analysis of lymphocytes with the $CD4^+CD49b^+LAG3^+IL\text{-}10^+$ phenotype. **B)** Percentages of Tr1 cells in individuals classified as normal weight, overweight and obesity, according to their BMI. **C)** Percentages of Tr1 cells in individuals classified as normal weight,

overweight and obese, according to their BMI, and further classified as metabolically healthy or unhealthy. Data correspond to the median and Q_1-Q_3 interquartile range. No significant differences were detected. NWH, normal weight metabolically healthy; NWU, normal weight metabolically unhealthy; OWH, overweight metabolically healthy; OWU, overweight metabolically unhealthy; OBH, obese metabolically healthy; OBU, obese metabolically unhealthy.

Figures

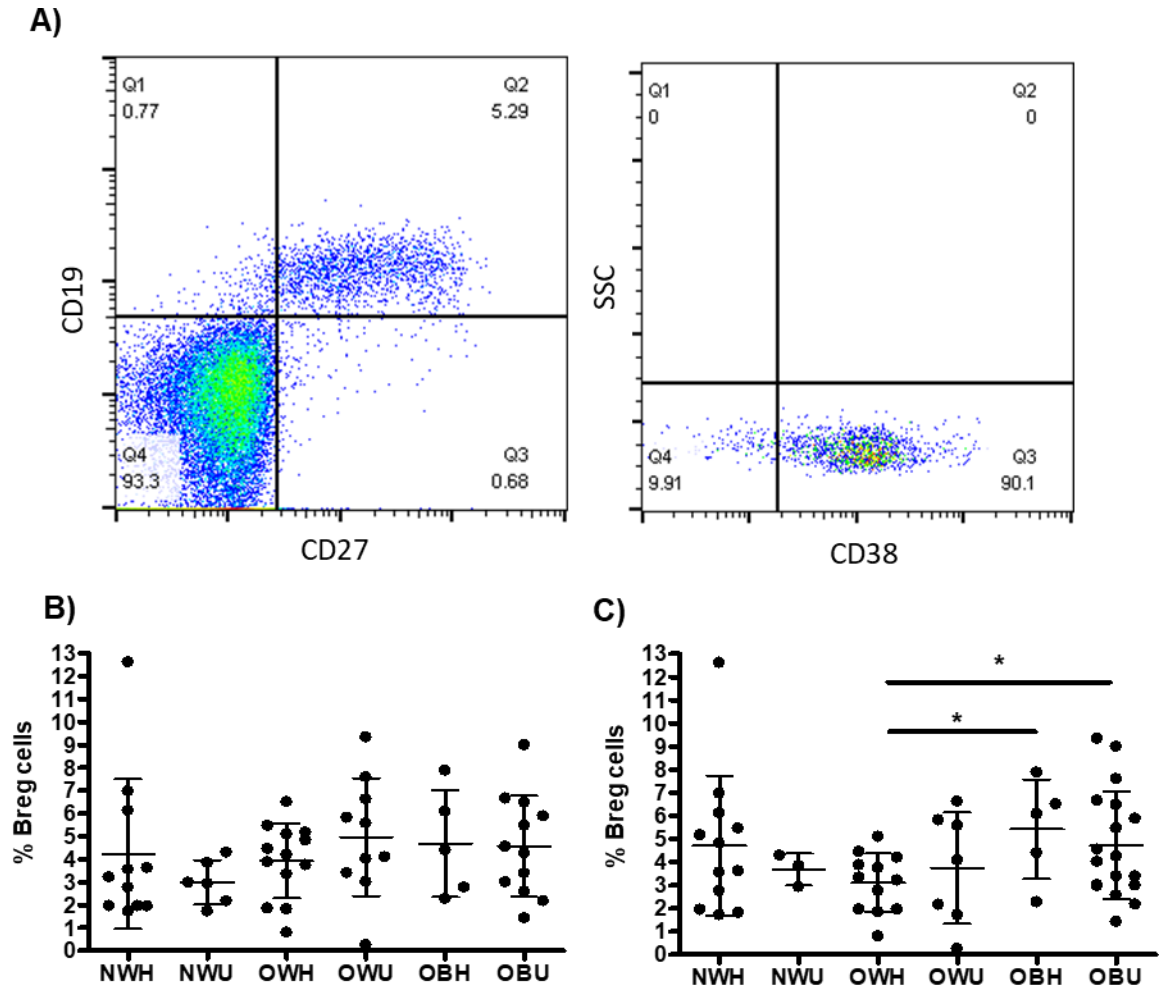
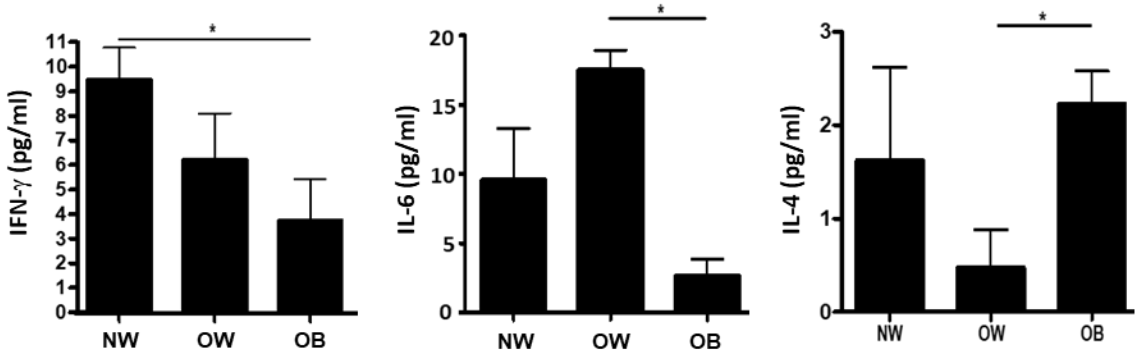


Fig. 1


IFN-γ (pg/ml)
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IL-6 (pg/ml)
20
15
10
5
0
*
NW
OW
OB
IL-4 (pg/ml)
3
2
1
0
*
NW
OW
OB

Fig. 2

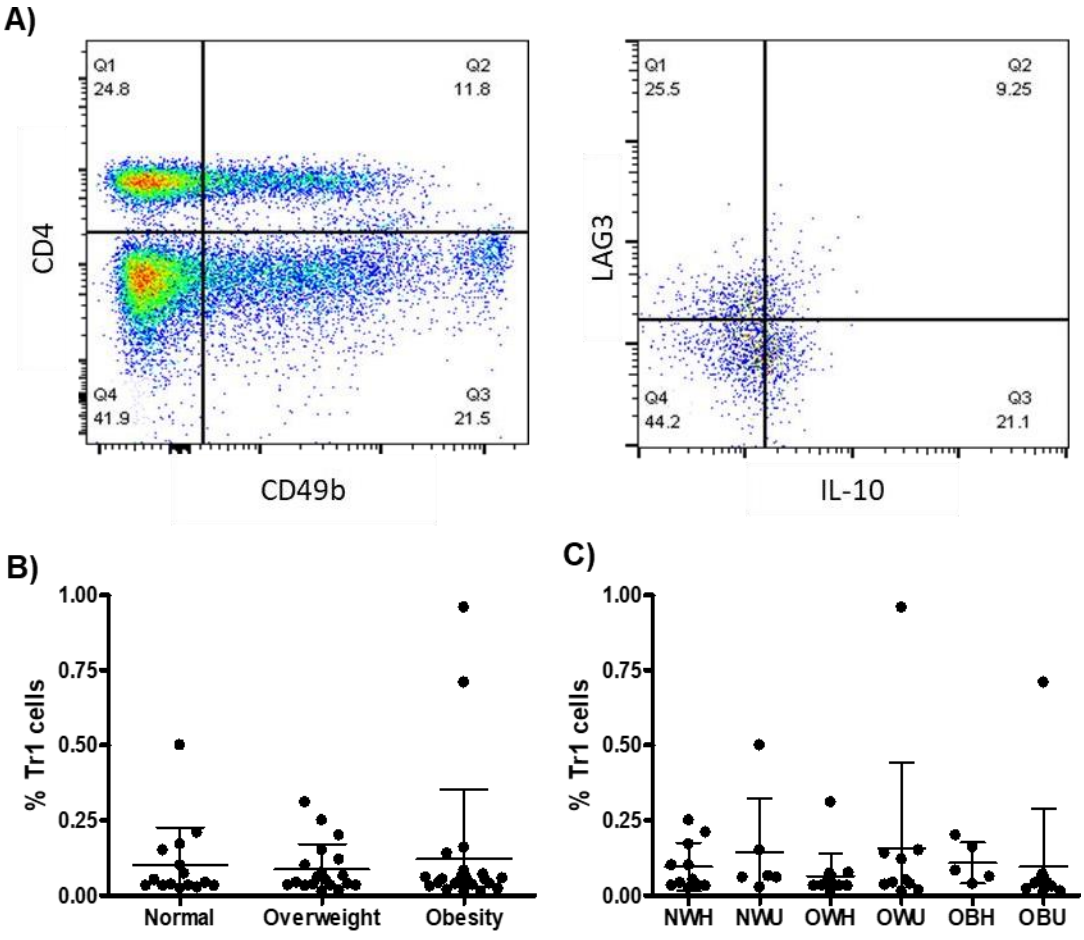
A)
Q1
24.8
Q2
11.8
Q4
41.9
Q3
21.5
CD4
CD49b
Q1
25.5
Q2
9.25
Q4
44.2
Q3
21.1
LAG3
IL-10
B)
% Tr1 cells
1.00
0.75
0.50
0.25
0.00
Normal
Overweight
Obesity
C)
% Tr1 cells
1.00
0.75
0.50
0.25
0.00
NWH
NWU
OWH
OWU
OBH
OBU

Fig. 3

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Research Article

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Research Article

Increased levels of pathogenic Th17 cells and diminished function of CD69⁺ Treg lymphocytes in patients with overweight

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Abstract

A low-grade inflammatory phenomenon is a feature of overweight and metabolic syndrome. The involvement of a pro-inflammatory Th17 lymphocyte subset and the CD69⁺ T regulatory (Treg) cell subtype in patients with metabolic dysfunction associated with or without overweight has not been fully elucidated. The aim of this study was to perform a quantitative and functional analysis of pathogenic Th17 lymphocytes and CD69⁺ Treg cells in patients with metabolic dysfunction (insulin resistance and dyslipidemia). The number of pathogenic Th17 cells and the levels and function of CD69⁺ Treg cells were analyzed in blood samples from individuals with metabolic dysfunction, associated with or without overweight. Pathogenic and non-pathogenic Th17 lymphocytes as well as Th22 cells were determined by eight-color flow cytometry analysis, whereas the levels and suppressive function of CD69⁺ Treg cells were also analyzed by multiparametric flow cytometry. We detected increased levels of pro-inflammatory Th17 pathogenic cells and Th22 lymphocytes in overweight unhealthy individuals ($P < 0.001$, compared to normal weight healthy). Conversely, diminished numbers of CD69⁺ Treg lymphocytes were observed in metabolically unhealthy individuals, with or without overweight. Likewise, the immunosuppressive function of CD69⁺ Treg cells was also defective in these patients. The increased levels of pathogenic Th17 cells along with a diminished number and function of CD69⁺ Treg lymphocytes may significantly contribute to the low-grade inflammatory phenomenon of metabolically unhealthy patients.

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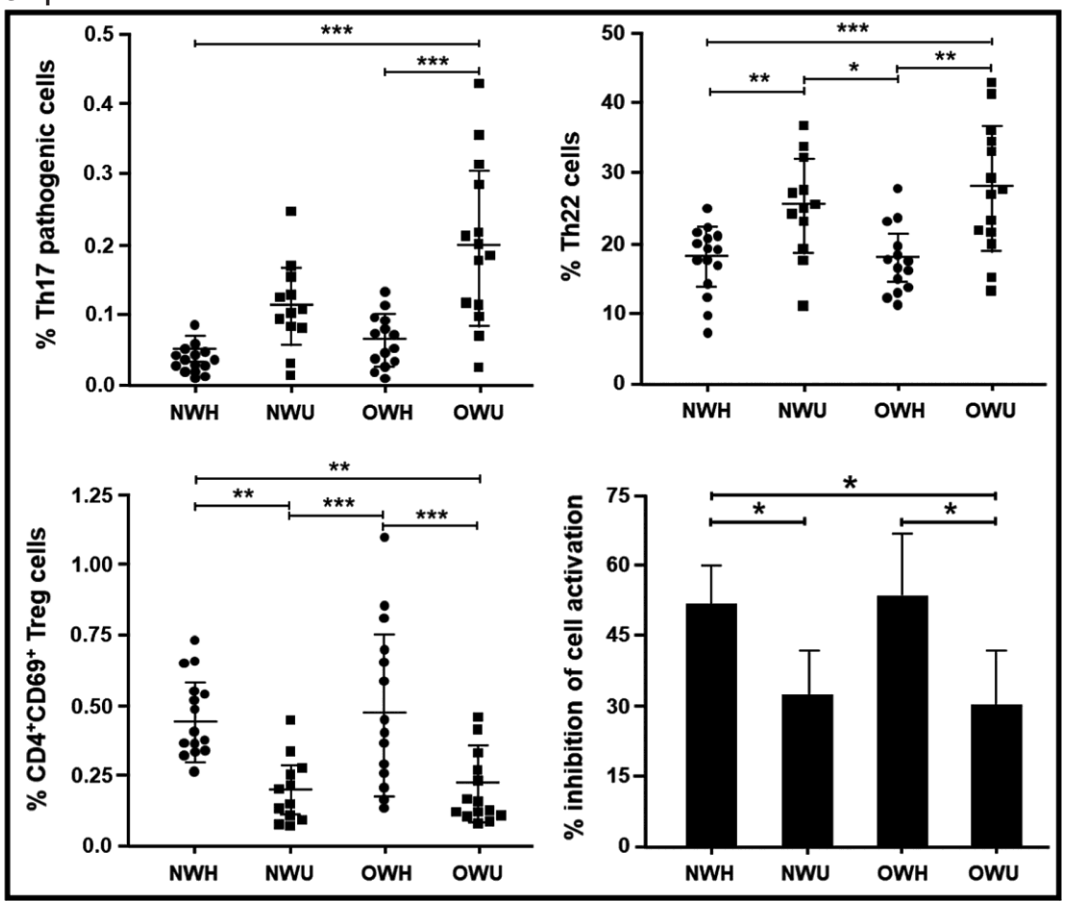
Graphical Abstract



Individuals who are metabolically unhealthy, with or without being overweight, show increased blood levels of pathogenic Th17 and Th22 cells. They also show decreased numbers of CD69⁺ T regulatory (Treg) lymphocytes, with a defective function of these cells. These immune abnormalities may contribute to the low-grade inflammatory phenomenon seen in patients with metabolic syndrome. NWH, normal weight healthy; NWU, normal weight unhealthy; OWH, overweight healthy; OWU, overweight unhealthy. *P < 0.05, ** P <0.01, *** P <0.001.

Keywords: metabolic syndrome, immune regulation, lymphocytes, interleukin-17

Abbreviations: BMI: body mass index; npTh17: non-pathogenic Th17; NWH: normal weight metabolically healthy; NWU: normal weight metabolically unhealthy; OWH: overweight metabolically healthy; OWU: overweight metabolically unhealthy; pTh17: pathogenic Th17; Th17: T helper-17; Th22: T helper-22; Treg: T regulatory

Introduction

The immune system has an important role in the pathogenesis of metabolic syndrome and different conditions associated with overweight and obesity, including the low-grade inflammatory phenomenon seen in patients with these conditions [1]. In this regard, the involvement of T regulatory (T_{reg}) cells and conventional effector T (T_{eff}) lymphocytes in the inflammation associated with overweight has been described [2,3]. Accordingly, different studies on the number, the function, and the presence of the Treg cells originally described by Sakaguchi *et al.* (with the phenotype $CD4^+CD25^{high}Foxp3^+$) [4] in the peripheral blood or the adipose tissue of patients with overweight have been reported [5–7]. However, other subsets of T_{reg} cells have been described and, to our best knowledge, they have not been studied in individuals with overweight or patients with obesity. In this regard, a $CD4^+$ T cell subset with regulatory function and a constitutive expression of the activation marker CD69 has been identified in healthy and diseased individuals [8–10]. This $CD69^+$ T_{reg} cell subset is characterized by the synthesis of IL-10, TGF-β andthe lack of the transcription factor Foxp3, with a variable level of expression of the alpha chain of IL-2 receptor (CD25) [8–10]. As expected, these Treg cells are able to inhibit the activation and proliferation of T_{eff} lymphocytes as well as suppress the synthesis and release of different cytokines [10,11]. Therefore, these $CD69^+$ T_{reg} lymphocytes should be able to downregulate the inflammatory phenomenon seen in patients with overweight and metabolic dysfunction.

T helper-17 (Th17) lymphocytes were originally described as $CD3^+CD4^+$ cells with the ability to synthesize IL-17, mainly the IL-17A and IL-17F isoforms [12]. In addition, these cells are able to release IL-22, a cytokine that may have a pro-inflammatory effect in the presence or not of IL-17A [13]. Accordingly, it has been reported that these lymphocytes may exert an important pro-inflammatory activity, playing a relevant role in the pathogenesis of different immune-mediated inflammatory diseases, including autoimmune thyroiditis, rheumatoid arthritis, psoriasis and inflammatory bowel disease [14,15]. Moreover, there is an additional T helper cell subset (Th22) that is able to synthesize IL-22, but not IL-17 [16]. These lymphocytes, in addition to their role in host defense against different infectious agents, may

also show a pro-inflammatory activity, participating in the pathogenesis of different immune-mediated conditions such as psoriasis, asthma, inflammatory bowel disease and sys- temic sclerosis [17].

Different Th17 lymphocyte subsets have been described, including pro-inflammatory or pathogenic (pTh17) and non-pathogenic (npTh17) cells [18]. In this regard, pTh17 lymphocytes are characterized by the expression of the chemokine receptor CXCR3 (CD183) and the cell differentiation molecules CD243 (MDR-1, P-glycoprotein 1) and CD161 as well as by the synthesis of interferon-γ (IFN-γ), tumor necrosis factor-α, and IL-17A/F. As expected, it has been described that this Th17 cell subset is involved in the pathogenesis of different immune-mediated inflammatory diseases [18,19], whereas the non-pathogenic subset participates in the host defense against fungi and different extracellular bacteria as well as in tissue repair and regulation of the immune response [20,21].

It has been widely described that most individuals with overweight and obesity show evidence of a low-grade inflammatory phenomenon, which is associated with an increased risk of atherosclerosis and ischemic cardiovascular disease, among others [22]. However, a significant proportion of individuals with overweight do not show evidence of metabolic dysfunction (insulin resistance, etc.) and tissue inflammation [23]. Conversely, some normal weight individuals show persistent metabolic dysfunction with evidence of a low-grade inflammatory phenomenon [23].

The aim of this study was to evaluate the number and function of CD69$^+$ Treg cells as well as the levels of Th22 and pathogenic/non-pathogenic Th17 lymphocytes in pa- tients with overweight, associated or not with metabolic dys- function. Our results indicate that the immune cell subsets analyzed in this study seem to be involved in the inflamma- tory phenomenon seen in metabolically unhealthy patients, whether overweight or not.

Materials and methods

Patients and controls

Fifty-five individuals were included in the study, who were classified in four groups according to their weight and the presence or absence of metabolic abnormalities. According to this, normal weight metabolically healthy (NWH, n = 15), normal weight metabolically unhealthy (NWU, n = 12), overweight metabolically healthy (OWH, n = 14), and overweight metabolically unhealthy (OWU, n = 14) groups of individuals were studied. Overweight was defined as a body mass index (BMI) in the 24.9–34.9 range, and no individuals with underweight (BMI < 18.5) or class III obesity (BMI > 34.9) were included in the study. Accordingly, individuals with a BMI between the 18.5 and 24.9 values were considered to have a normal weight. Moreover, those individuals with blood triglyceride levels higher than 150.0 mg/dl and high-density lipoprotein cholesterol concentrations lower than 40.0 mg/dl were considered as metabolically unhealthy. As expected, the arithmetic mean of Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and blood insulin levels were significantly higher in the metabolically unhealthy patients (HOMA-IR arithmetic mean = 3.2 mU/L in NWU, and 3.9 in OWU, respectively, n = 12 and 14) compared to healthy individuals (1.2 mU/L in NWH, and 2.1 in OWH, n = 15 and 14) (P < 0.05 in all cases). However, fasting glucose levels (as well as the age range and its arithmetic mean) were similar in the four groups analyzed, and patients with diabetes mellitus were excluded. Furthermore, all individuals included in the study were apparently healthy and no record of chronic drug(s) intake was detected in any of them. Additional characteristics of the individuals included in the study are shown in Table 1. Regarding the demographic background of the individuals analyzed, 52 (95%) of them had both the grandparents andthe parents were born in Mexico, and their surname(s) wereall of Hispanic origin. In the remaining three cases, no in- formation regarding parents and grandparents was available. These data, along with the skin color of the individuals in- cluded in the study (the great majority of whom fell into the categories III and IV in the scale of Fitzpatrick) [24], indicated that almost all of them corresponded to Mexican mestizos, with a variable admixture of Native American, European, and African genetic components [25]. This study was performed in accordance with the principles set out in the Declarationof Helsinki and was approved by the review board of our research center.

Flow cytometry analysis

To analyze the number of CD69$^+$ Treg lymphocytes, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare, Pittsburgh, PA) density-gradient centrifugation, and cellular viability was evaluated by trypan blue staining and it was always higher than 95%. These cells were incubated for 30 min at 4°C with monoclonal antibodies (mAbs) directed against CD4 (coupled to FITC, eBioscience, San Diego, CA, or to APC/Cy7, BioLegend Inc, San Diego, CA), CD25 (coupled to APC/Cy7, Becton-Dickinson, Franklin Lakes, NJ), NKG2D (labeled with FITC, eBioscience), anti-latency-associated peptide (LAP, a surrogate marker for TGF-β, labeled with PerCp/Cy5.5, BioLegend), and CD69 (coupled to APC, eBioscience). Then, cells were washed, fixed, and permeabilized with the Foxp3 Fix/Perm kit (eBioscience) for 30 min. Subsequently, cells

Table 1: Main characteristics of the individuals included in the study.

	NWH (n=15)	NWU (n=12)	OWH (n=14)	OWU (n=14)
Age	27.0 ± 3.9*	25.4 ± 4.8	29.1 ± 3.6	27.9 ± 5.0
Anthropometric				
Weight (kg)	59.4 ± 6.8	57.6 ± 5.8	74.7 ± 9.4	76.2 ± 12.3
Height (cm)	166.3 ± 8.6	161.7 ± 6.0	164.6 ± 7.7	164.4 ± 8.8
Body mass index	21.4 ± 1.9	22.0 ± 2.1	27.6 ± 3.9	28.2 ± 4.4
Waist circumference (cm)	72.5 ± 3.7	77.4 ± 3.8	92.9 ± 9.3	96.0 ± 14.8
Skinfold thickness (cm)	1.1 ± 0.2	1.2 ± 0.2	1.8 ± 0.4	1.9 ± 0.4
Daily physical activity				
Sedentary	1 (7%)**	3 (25%)	4 (29%)	5 (36%)
Light	5 (33%)	4 (33%)	7 (50%)	8 (57%)
Moderate	7 (47%)	5 (42%)	3 (21%)	1 (7%)
Vigorous	2 (13%)	0 (0%)	0 (0%)	0 (0%)

*Arithmetic mean and SD. **Number and percent of individuals in each category.

were incubated with mAbs against IL-10 (labeled with PE, BioLegend) and Foxp3 (tagged with PE/Cy7, eBioscience). Doublet discrimination was performed by analyzing FSC-A versus FSC-W dot plots from the lymphocyte gate. At least 1×10^6 events were analyzed, and gates were defined by using FMO (fluorescence minus one) controls and isotype- matched control mAbs. $CD4^+CD69^+$ and $CD4^+NKG2D^+$ cells were analyzed by separate, and data were acquired in FACSCanto II flow cytometer (Becton Dickinson) and analyzed using the Flow Jo software v10 (Tree Star Inc., Ashland, OR).

To analyze Th17 lymphocytes, PBMC were incubated with the following mAbs: CD4-Pacific Orange (Life tech- nologies, Carlsbad CA), CD183-APC-Cy7 (CXCR3, BioLegend), CD243-PerCp/Cy5.5 (MDR-1, Bio-Legend), and CD161-PE/Cy7 (BioLegend). Then, cells were washed, fixed and permeabilized with PFA 4% and saponine 0.1%, and further incubated with anti-IL-10-PE, anti-IFN-γ- FITC, anti-IL-17A-Pacific Blue (all from BioLegend), and anti-IL-22-APC (eBioscience Inc., San Diego, CA). Cell data were collected using a Becton–Dickinson FACSCanto flow cytometer and analysed with the Flow Jo software (TreeStar Inc.).

Functional analysis of $CD69^+$ Treg cells

The immune regulatory activity of $CD69^+$ Treg cells was analyzed by an assay of inhibition of cell activation [10], which compares the level of expression of the activation marker CD40L (CD154) by PBMC incubated in the presence or not of $CD69^+$ cells. Accordingly, PBMC were depleted or not from $CD69^+$ cells by negative selection with an anti-human CD69 mAb (eBioscience), rat anti-mouse IgG MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and MACS LD columns (Miltenyi). Then, PBMC (depleted and non-depleted of $CD69^+$ cells) were incubated in 24-well plates (Costar, Corning, NY) pre-coated with an anti-CD3 (OKT3 clone, 5.0 μg/ml) and an anti-CD28 (clone 28.2, 5.0 μg/ml) for 7 h at 37°C with 5% CO_2, in the presence of an anti-CD40L/ PE mAb (BD Pharmigen, San Jose, CA). Finally, cells were washed and analyzed for CD40L expression in a FACSCanto II flow cytometer (Becton Dickinson), using the Flow Jo software (Tree Star).

The regulatory function of $CD69^+$ Treg cells was additionally tested in an assay of inhibition of cytokine release. In brief, the suppressive effect of $CD69^+$ lymphocytes on the release of cytokines by autologous cells was assayed in PBMC cultures depleted or not of $CD69^+$ cells, as stated above. In this case, cells were cultured for 24 h, and at the end of incubation, supernatants were obtained and the concentration of IL-2, IL-6, IL-10, IL-17, interferon-γ (IFN-γ), and tumor necrosis factor- (TNF-) α was determined by a Cytometric Bead Array (BD Biosciences). Data were acquired in an Accuri C6 cytometer (BD Biosciences) and analyzed with the software FCAP Array v3.01 (BD Biosciences).

Statistical analysis

The arithmetic mean and SD were used to represent data with a normal distribution, while the median and Q1–Q3 interquartile range were used to represent data with a non-Gaussian distribution. Analysis of two groups was performed with the Mann–Whitney *U* test and comparisons of four groups with the Kruskal–Wallis sum rank test. The possible associations between laboratory and clinical data were analyzed with the Spearman rank correlation test. Data were analyzed using the GraphPad Prism v8.0.1 software, and *P* values <0.05 were considered as significant.

Results

Quantitative analysis of Th17 and Th22 cells

The levels of conventional non-pathogenic and pathogenic Th17 lymphocytes as well as those of Th22 cells were determined by multiparametric, eight-color flow cytometry analysis, according to the strategy shown in Fig. 1. In this regard, pTh17 cells were defined as $CD4^+CXCR3^+IL\text{-}17A^+IL\text{-}22^+MDR\text{-}1^+CD161^+IFN\text{-}\gamma^+IL\text{-}10^-$, whereas $CD4^+CXCR3^+IL\text{-}17A^+IL\text{-}22^-MDR\text{-}1^-CD161^-IFN\text{-}\gamma\text{-}IL\text{-}10^+$ cells correspondedto npTh17 lymphocytes, and the $CD4^+CXCR3^{+/-}IL\text{-}17A^-IL\text{-}22^+$ phenotype corresponded to Th22 cells, as previously reported [15,16,18]. According to these analyses, we observed a significant increase in the percent of Th17 pathogenic cells in OWU patients, compared to both, OWH and NWH individuals ($P < 0.005$ in both cases, Fig. 2A). In contrast, although the samples from NWU patients tended to show high percent levels of these pathogenic cells, no significant differences were observed when compared to the other three groups studied, including OWU patients ($P > 0.05$ in all cases, Fig. 2A). When the absolute numbers of these cells were analyzed, similar results were observed, showing the OWU patients significant increased levels of pathogenic cells, compared to NWH and OWH individuals ($P < 0.005$, in both cases, Fig. 2B), but not to NWU patients ($P > 0.05$).

When conventional non-pathogenic Th17 cells were analyzed, an increased proportion was detected in OWU patients compared to NWH individuals ($P < 0.01$), with no significant differences with NWU or OWH subjects ($P > 0.05$ in both cases, Fig. 2C). In contrast, data on the absolute number of these non-pathogenic lymphocytes showed no significant differences between OWU patients and NWH individuals ($P > 0.05$, Fig. 2D) as well as lower numbers of these cells in NWU patients compared to NWH individuals ($P < 0.05$, Fig. 2D).

Analysis of Th22 lymphocytes showed a higher proportion of these cells in NWU and OWU patients compared to NWH individuals ($P < 0.01$ in both cases, Fig. 3A), and similar levels in NWH and OWH individuals ($P > 0.05$). Accordingly, metabolically unhealthy patients (both, NW and OW) showed higher levels of these cells than OWH individuals ($P < 0.05$). Moreover, analysis of the absolute number of Th22 cells also showed higher levels in OWU pa- tients compared to both NWH and OWH ($P < 0.005$ and $P < 0.01$, respectively, Fig. 3B).

Analysis of $CD69^+$ Treg cells

As shown in Fig. 4, the levels of $CD69^+$ Treg cells were analyzed in peripheral blood samples by multiparametric, six-color flow cytometry, as previously described [8–10]. According to these analyses, we observed significant decreased levels of $CD4^+CD69^+$ Treg lymphocytes in metabolically unhealthy patients, with or without overweight, compared to normal weight healthy individuals ($P < 0.01$, in both cases, Fig. 5A). A similar finding was observed when metabolic- ally unhealthy patients (NWU or OWU) were compared to OWH individuals ($P < 0.01$, in both cases). Moreover, when the levels of $CD69^+$ Treg cells expressing the marker NKG2D

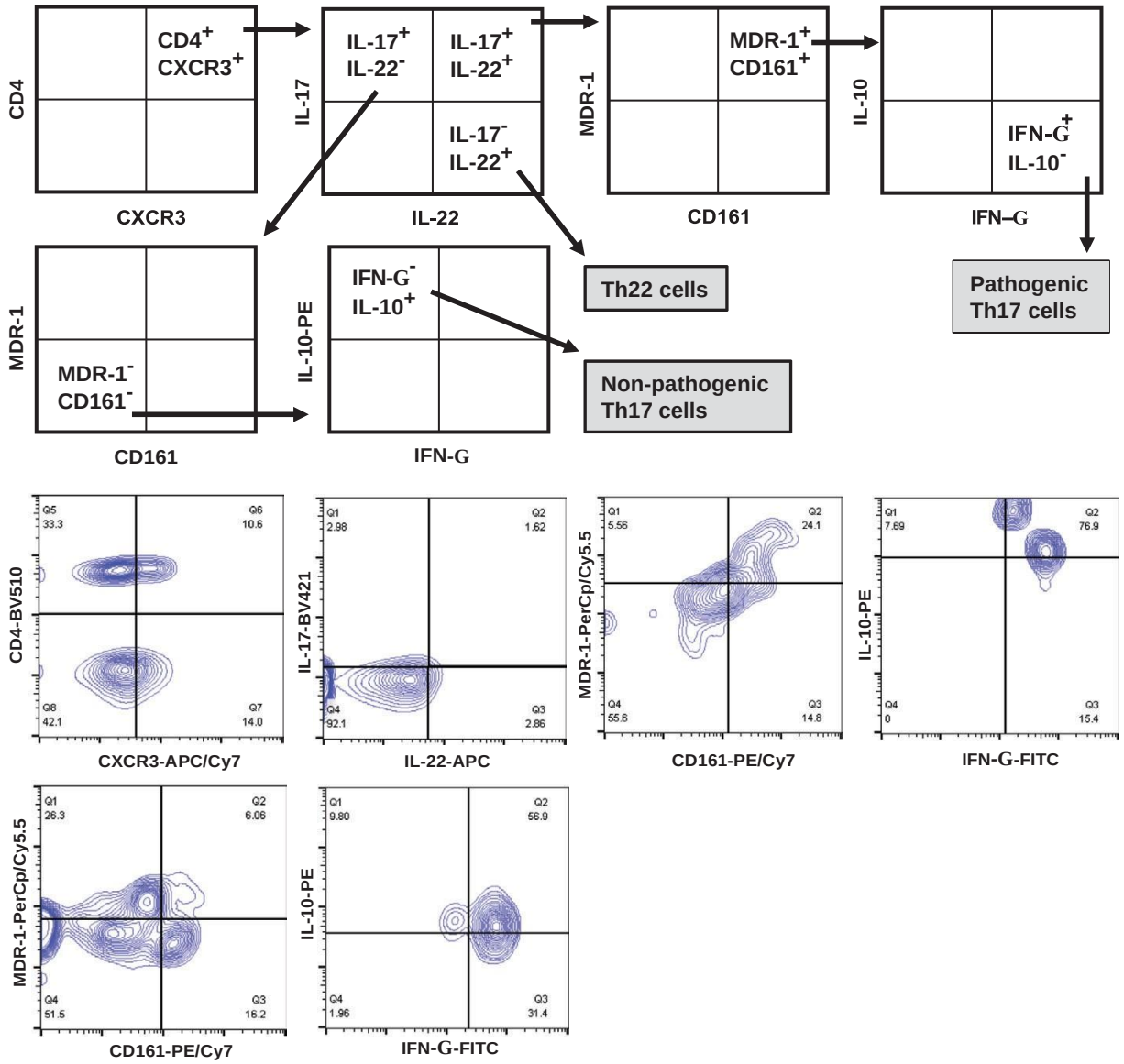


Figure 1: Flow cytometry strategy for the analysis of Th17 cells. PBMC were labeled with the indicated mAbs and analyzed by multiparametric flow cytometry, as stated in materials and methods. Th17 and Th22 cell levels were determined by eight-color flow cytometry analysis and, according to this, pathogenic Th17 lymphocytes were defined as $CD4^+CXCR3^+IL\text{-}17A^+IL\text{-}22^+MDR\text{-}1^+CD161^+IFN\text{-}\gamma^+IL\text{-}10^-$, whereas non-pathogenic Th17 cells as $CD4^+CXCR3^+IL\text{-}17A^+IL\text{-}22^-MDR\text{-}1^-CD161^-IFN\text{-}\gamma^-IL\text{-}10^+$. Lymphocytes with the phenotype $CD4^+CXCR3^{+/-}IL\text{-}17A^-IL\text{-}22^+$ were considered as Th22 cells. Flow cytometry images of a representative analysis are shown.

were analyzed, a significant increased proportion of these lymphocytes was detected in samples from OWH individuals, compared to the other three groups studied ($P < 0.01$ in all cases, Fig. 5B). Likewise, NWH individuals showed higher levels of $CD4^+CD69^+NKG2D^+$ Treg cells compared to NWU ($P < 0.01$).

When the numerical ratio between $CD69^+$ Treg cells and pathogenic Th17 lymphocytes was analyzed, very significant differences were observed between metabolically healthy and unhealthy patients (Fig. 5C). Accordingly, significant differences were observed among NWU or OWU patients and NWH or OWH ($P < 0.005$ in all comparisons, Fig. 5C).

Functional analyses of $CD69^+$ Treg cells

The suppressive function of $CD69^+$ Treg cells was analyzed in two types of assays, the inhibition of activation of autologous T effector or conventional lymphocytes (Fig. 6A) and the suppression of cytokine synthesis/release (Fig. 7). As shown in Fig. 6B, metabolically unhealthy patients, either with or without overweight, showed a significant diminished immunoregulatory activity compared to NWH individuals ($P < 0.05$, in both cases). In addition, OWU patients also showed defective regulatory activity compared to their metabolically healthy counterparts ($P < 0.05$, Fig. 6B). Moreover, the $CD69^+$ Treg cells from metabolically unhealthy patients (both, NW and OW) also showed a diminished inhibitory activity on the synthesis and release of IL-17A and IL-2, compared to NWH individuals ($P < 0.05$ in all comparisons, Fig. 7A and B). In addition, cells from OWU patients showed a lower inhibition of IL-17A release than OWH individuals ($P < 0.05$, Fig. 7A). In contrast, no significant differences were observed in the suppressive effect of $CD69^+$ Treg cells on the synthesis/

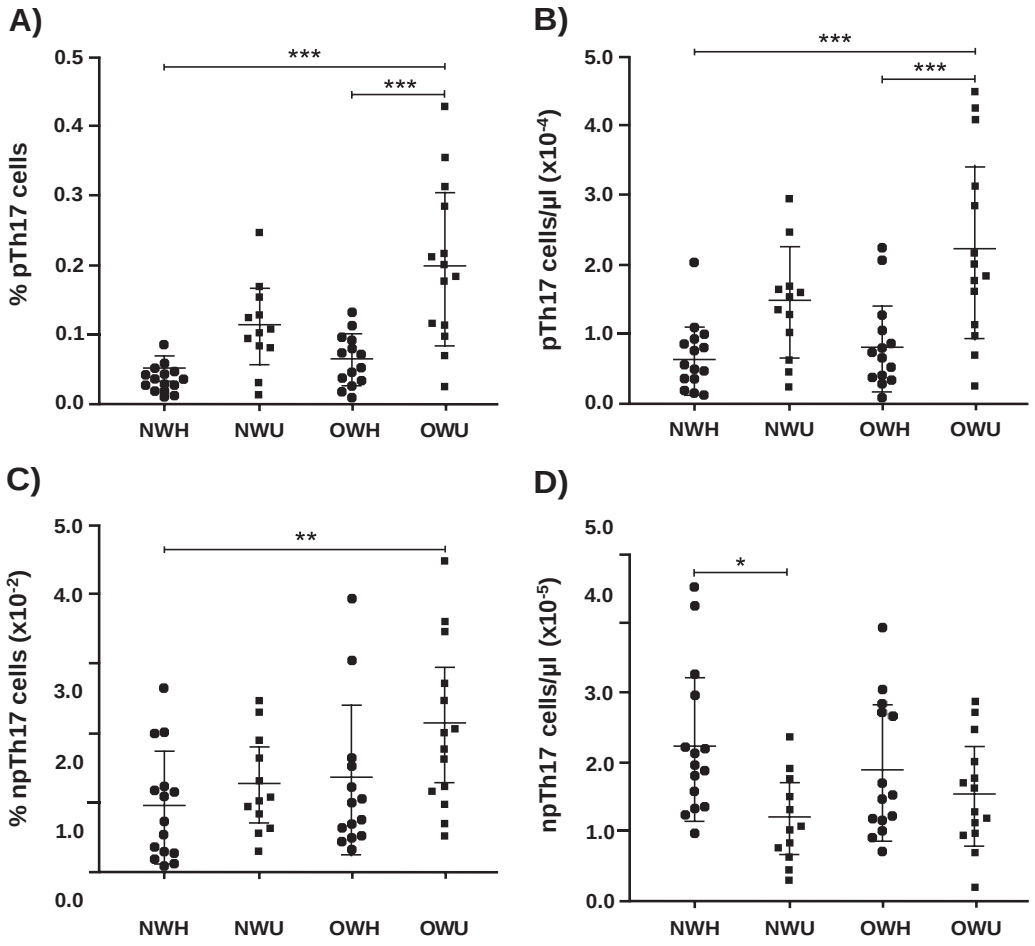


Figure 2: Levels of Th17 cells in individuals with overweight and controls. Blood samples from individuals with normal weight metabolically healthy (NWH), normal weight metabolically unhealthy (NWU), overweight metabolically healthy (OWH), and overweight metabolically unhealthy (OWU) were analyzed by multiparametric flow cytometry. (A) Percentages of pathogenic Th17 (pTh17) cells in the four groups studied. (B) Absolute number of pTh17 cells. (C) Percentages of non-pathogenic (npTh17) Th17 cells in the four groups studied. (D) Absolute number of np Th17 cells. The arithmetic mean and SD of the mean are indicated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

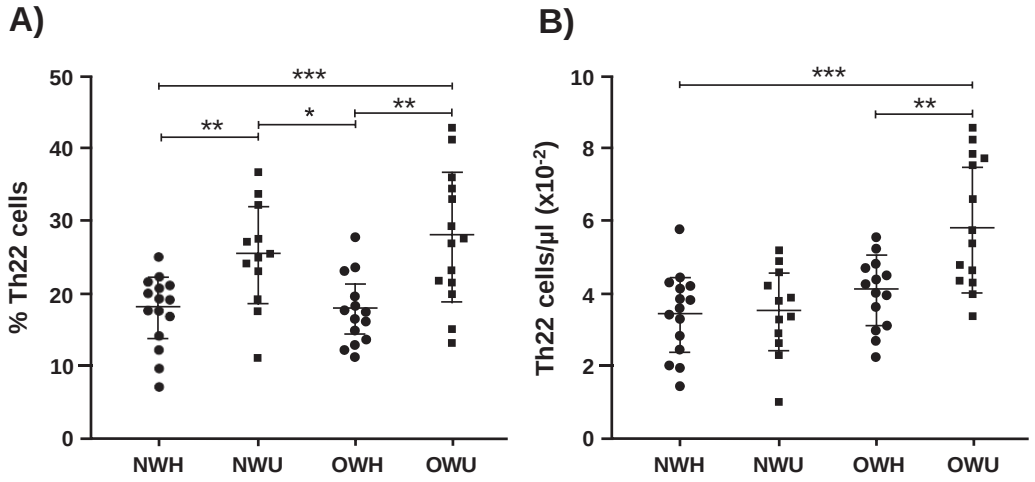


Figure 3: Levels of Th22 cells in individuals with overweight and controls. Blood samples from individuals with normal weight metabolically healthy (NWH), normal weight metabolically unhealthy (NWU), overweight metabolically healthy (OWH), and overweight metabolically unhealthy (OWU) were analyzed by multiparametric flow cytometry. (A) Percentages of Th22 cells in the four groups studied. (B) Absolute number of Th22 cells. The arithmetic mean and SD of the mean are indicated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

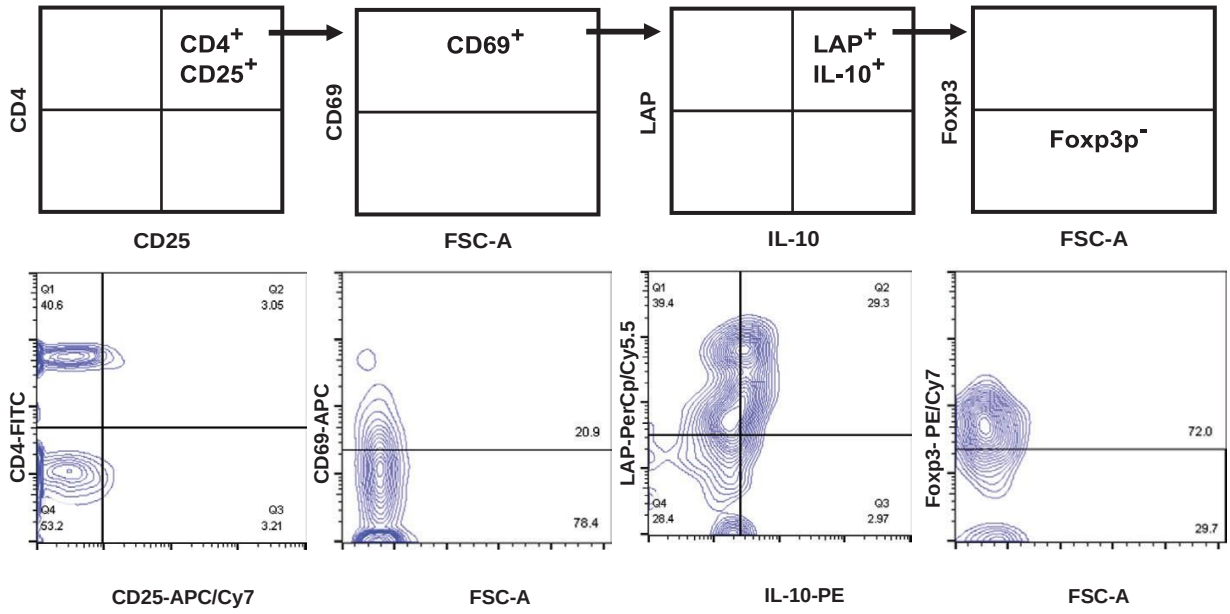


Figure 4: Flow cytometry strategy for the analysis of CD69⁺ Treg cells. PBMC were labeled with the indicated mAbs and analyzed by multiparametric flow cytometry, as stated in materials and methods. The CD69⁺ subset of Treg cells was analyzed by six-color flow cytometry analysis, and their phenotype corresponded to $CD4^+CD25^{var}CD69^+LAP^+IL\text{-}10^+Foxp3^-$. Flow cytometry images of a representative analysis are shown.

release of the anti-inflammatory cytokine IL-10 among the four groups studied ($P > 0.05$ in all cases, Fig. 7C).

Correlation analyses

Finally, we explored the possible association between different clinical and laboratory variables and the immune parameters analyzed in this study. In NWU patients, a significant posi- tive correlation was observed between HOMA-IR values and the percent of pTh17 cells ($r = 0.57$, $P = 0.026$, data not shown). Likewise, a significant negative association between HOMA-IR values and the levels of CD69⁺ Treg lymphocytes was detected in these individuals ($r = 0.59$, $P = 0.022$, data not shown). In addition, we observed in OWU patients a signifi- cant positive correlation between HOMA-IR values and the percent of pTh17 cells, and a negative association with the levels of CD69⁺ Treg cells ($r = 0.72$, $P = 0.003$ and $r = -0.57$, $P = 0.015$, respectively, Fig. 7D, and data not shown). However, no additional significant associations were detected, including those of BMI, weight, triglyceride, or cholesterol levels with the immune parameters analyzed in this study (data not shown).

Discussion

Although there are several studies on the role of Foxp3⁺ Treg cells and Th17 lymphocytes in the inflammatory phenomenon associated with overweight and obesity, the possible involvement of CD69⁺ Treg cells as well as the levels of pTh17 and Th22 lymphocytes have not been previously explored, to our best knowledge. Thus, in this study, we have hypothesized that patients with metabolic dysfunction, associated or not with being overweight, have an abnormal number and a defective function of CD69⁺ Treg cells, which is accompanied by abnormal levels of pTh17/Th22 lymphocytes. For this purpose, we analyzed blood samples from individuals with no evidence of metabolic dysfunction, with or without overweight, as well as those from patients with metabolic dysfunction associated with or without overweight.

According to our hypothesis, we have found that OWU patients exhibit both, increased percentages and absolute numbers of peripheral blood pTh17 cells, compared to metabolically healthy individuals (with or without overweight). However, although a similar trend was observed in NWU patients, in this case the differences in the percent or absolute number compared to metabolically healthy individ- uals did not reach significant differences, likely due to the small number of individuals studied. In this regard, it is of interest that in both NWU and OWU patients we observeda positive significant association between HOMA-IR values and the percent of pTh17 cells, a correlation that was not detected in metabolically healthy individuals. These patho- genic cells are characterized, among others, by the synthesis of proinflammatory cytokines (mainly IFN-γ, TNF-α and GM-CSF) with no production of the anti-inflammatory cyto- kine IL-10 as well as by their involvement in the pathogenesis of different autoimmune conditions, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and multiple sclerosis [18,21]. In addition, we have previously reported that patients with autoimmune thyroid diseases show enhanced levels of pTh17 cells in their peripheral blood and thyroid tissue, a phenomenon that significantly correlates with different clinically relevant parameters [15]. Accordingly, this study also suggests that pTh17 has a role in an additional chronic inflammatory condition, the metabolic syndrome. In this regard, different factors detected in patients with overweight could be involved in the induction and pathogenicity of Th17 cells, including the increased synthesis of different cytokines (mainly IL-1β and IL-23), gut dysbiosis, enhanced activity of the acetyl-CoA carboxylase 1, high levels of saturated fatty acids and cholesterol, hypoxia and, likely, increased sodium chloride intake [13,14]. In this regard, we consider of interest that our data suggest that an important factor for the induction of pTh17 cells is metabolic dysfunction rather than being overweight, since the levels of these lymphocytes in OWH individuals are similar to those observed in NWH subjects. Moreover, although the increase in the levels of

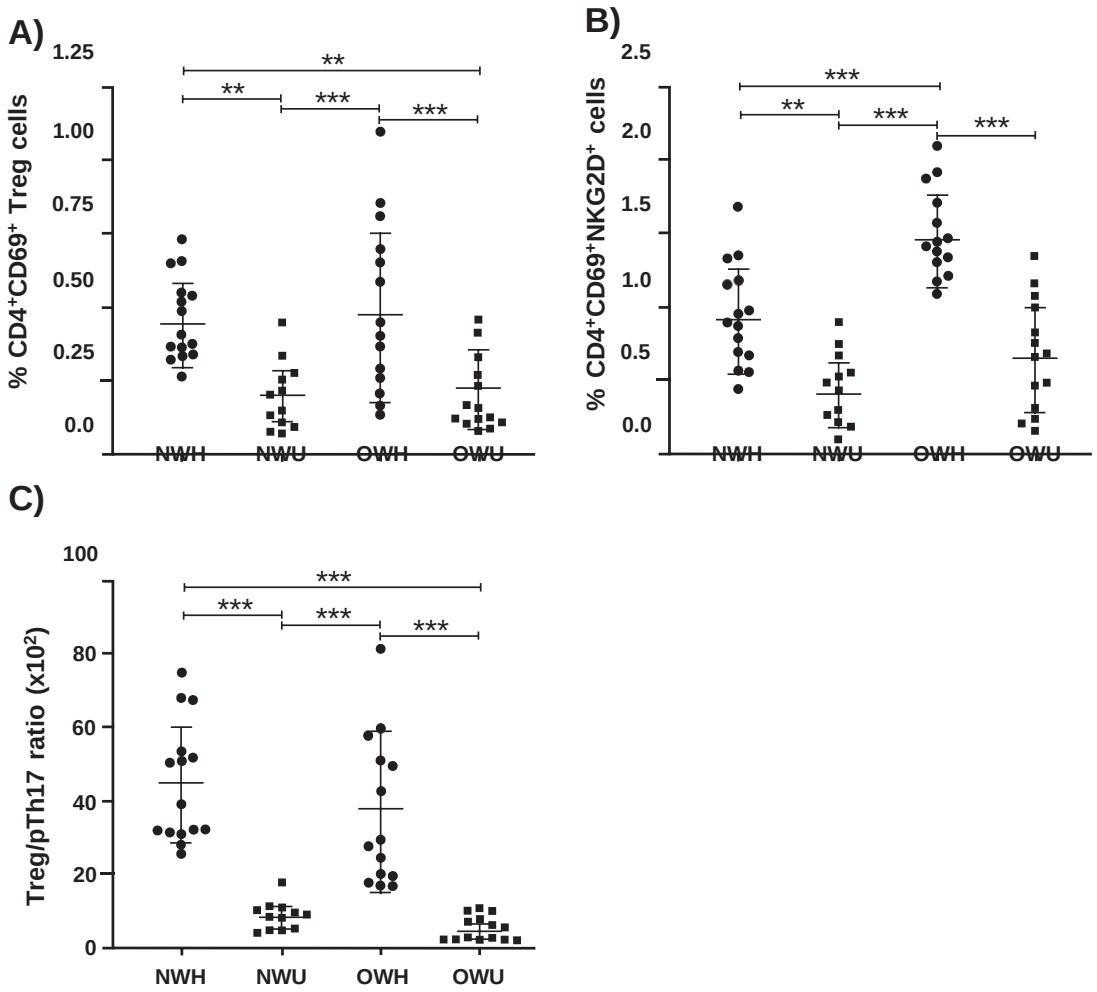


Figure 5: Levels of CD69⁺ Treg cells in individuals with overweight and controls. Blood samples from individuals with normal weight metabolically healthy (NWH), normal weight metabolically unhealthy (NWU), overweight metabolically healthy (OWH), and overweight metabolically unhealthy (OWU) were analyzed by six-color flow cytometry, and the results were expressed as the percent of $CD4^+CD25^{var}CD69^+LAP^+IL\text{-}10^+Foxp3^-$ lymphocytes (A) or their absolute number (B). The ratio between the percent of CD69⁺ Treg cells and pathogenic Th17 (pTh17) in the four groups studied are shown (C). Horizontal lines correspond to the arithmetic mean and SD of the mean. $^{**}P < 0.01$; $^{***}P < 0.005$.

conventional Th17 cells detected in OWU patients, compared to NWH individuals requires further investigation, it is feasible that this phenomenon indicates that overweight, in addition to metabolic dysfunction, is a strong condition for the differentiation of Th0 into Th17 cells, a step previous to the generation of pathogenic Th17 lymphocytes in these patients.

Th22 lymphocytes, which have the phenotype $CD4^+IL\text{-}17^-IL\text{-}22^+$, are primarily activated by a combination of three pro-inflammatory cytokines, namely IL-6, TNF-*α*, and IL-*β* TNF-*α* and IL-*β* [26]. Under physiological conditions, Th22 cells mainly exert their functions through the release ofIL-22, which binds to the heterodimeric IL-22R1/IL10R2 receptor, expressed only by nonhematopoietic cells [26–28]. Thus, Th22 lymphocytes promote antimicrobial immunity, tissue repair, and inflammation through the effect of IL-22 on, among others, epithelial cells, inducing the release of chemotactic cytokines and anti-microbial peptides [28]. Accordingly, Th22 cells are involved in the pathogenesis of different inflammatory conditions, including psoriasis, atopic dermatitis, asthma, and rheumatoid arthritis [17]. In this regard, our data, showing increased levels of $CD4^+IL\text{-}17^-IL\text{-}22^+$ in patients with metabolic dysfunction, suggest that Th22 cells may also participate in the low-grade inflammatory phenomenon associated to this condition. These data also suggest that metabolic dysfunction by itself (i.e., in the presence or absence of overweight) is a condition that favors the differentiation of Th22 lymphocytes. However, further investigation is needed to determine the exact role of IL-22 and Th22 cells in the complex pathogenesis of the metabolic syndrome.

The decreased levels of CD69⁺ Treg cells detected in the metabolically diseased patients included in this study add an additional piece to the pathogenesis of the immune dysregulation associated with the metabolic syndrome. This point is reinforced by the very low Treg/Th17 ratio observed in these patients as well as by the negative association be-tween the levels of insulin resistance and the percent of CD69⁺ Treg cells. We consider that all these data indicate a predominance of differentiation of effector pro-inflammatory cells over those lymphocytes with a regulatory function in metabolically unhealthy individuals. In addition, the signifi- cant low immunosuppressive function of CD69⁺ Treg cells observed in these patients further suggests that these lympho- cytes, in addition to $CD4^+Foxp3^+$ Treg cells, are involved in the pathogenesis of metabolic syndrome. Accordingly, it

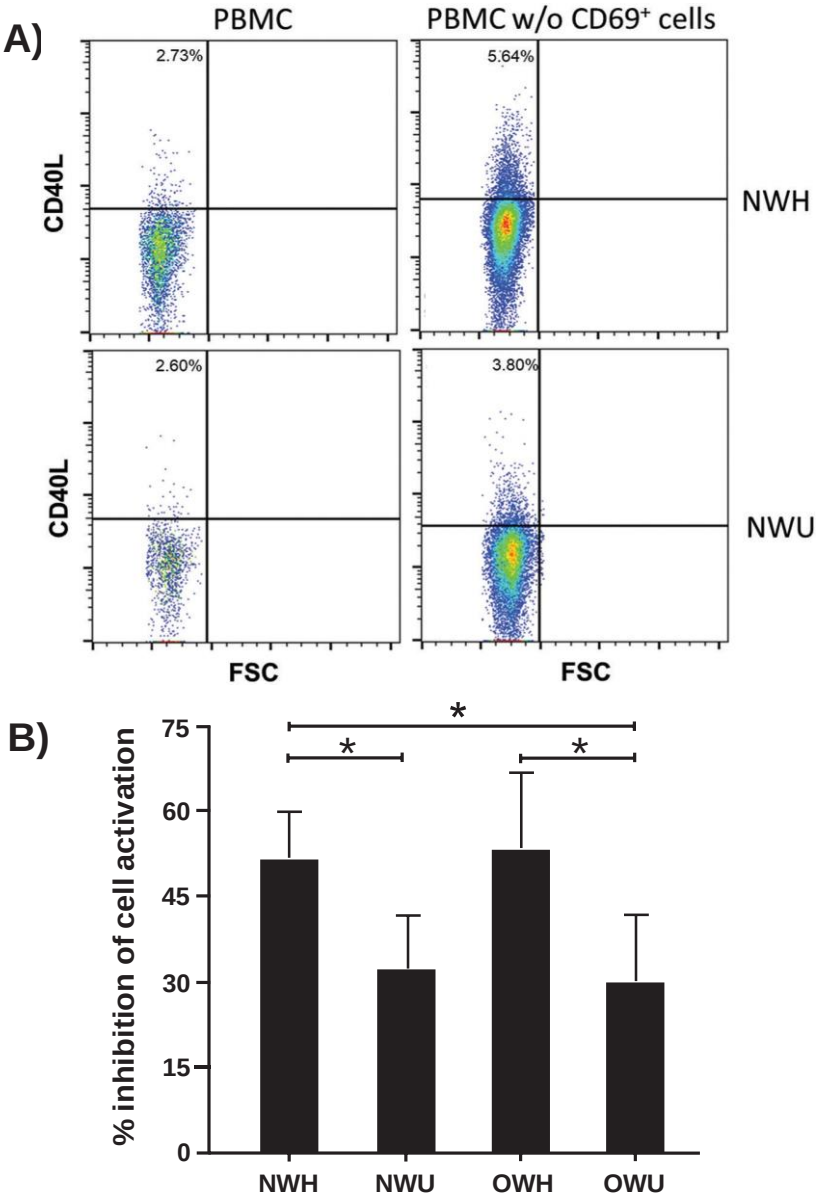


Figure 6: Functional analysis of CD69$^+$ Treg cells in individuals with overweight and controls. Blood samples from individuals with normal weight metabolically healthy (NWH), normal weight metabolically unhealthy (NWU), overweight metabolically healthy (OWH), and overweight metabolically unhealthy (OWU) were obtained and the suppressive function of CD69$^+$ Treg cells on the activation of effector lymphocytes was tested by an assay of inhibition of expression of CD40L, as described in material and methods. (A) Representative dot plots of CD40L expression in unfractionated PBMC and PBMC depleted from CD69$^+$ lymphocytes of blood samples from representative NWH and NWU individuals are shown. Percentages of CD40L$^+$ cells are indicated. (B) Analysis of the negative regulatory effect of CD69$^+$ Treg cells, expressed as the percent of inhibition of cell activation. Data correspond to the arithmetic mean and SD of the mean. $^*P < 0.05$.

has been reported that patients with other chronic inflammatory conditions, including systemic lupus erythematosus, rheumatoid arthritis, periodontal disease, and autoimmune thyroiditis also show numerical and functional abnormal- ities of CD69$^+$ Treg lymphocytes [8–10]. In this regard, we consider that it would be of interest to explore the possible involvement of other regulatory cell subsets in the metabolic syndrome, including the type 1 regulatory (Tr1) lympho- cytes, which seem to have a relevant role in the pathogenesis of type 1 diabetes mellitus [29].

We consider that our data further suggests that novel adjunctive therapies might be useful in patients with low-grade systemic inflammation associated with metabolic dysfunction, which in turn is strongly linked to serious conditions, including atherosclerosis and diabetes mellitus. Thus, the correction of the gut dysbiosis associated with obesity would favor the generation of conventional Th17 cells with no further differentiation into pTh17 lymphocytes [19,20]. Moreover, the induction of Treg cells [30], either CD4$^+$CD25^{high}Foxp3$^+$ or CD4$^+$CD69$^+$Foxp3$^-$, might be another strategy to correct the immune dysregulation observed in these patients.

We conclude that our data further suggest that patients with metabolic dysfunction (associated with or without overweight) show different abnormalities in their im- mune regulatory mechanisms that favor the differentiation of pathogenic Th17 and Th22 cells, which in turn may

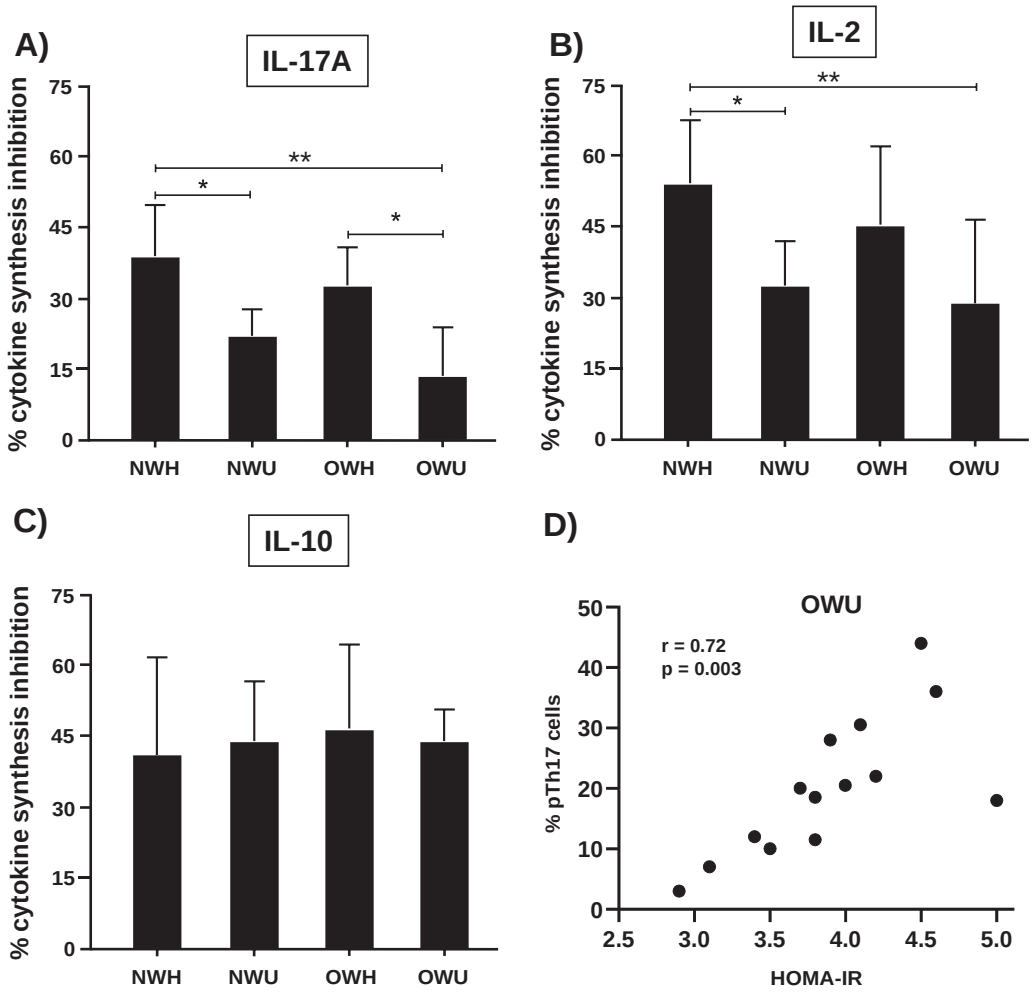


Figure 7: (A–C) Analysis of the suppressive activity of CD69⁺ Treg lymphocytes on the synthesis of cytokines by autologous cells. Blood samples from individuals with normal weight metabolically healthy (NWH), normal weight metabolically unhealthy (NWU), overweight metabolically healthy (OWH), and overweight metabolically unhealthy (OWU) were obtained and the suppressive function of CD69⁺ Treg cells on the release of IL-17A, IL-2, and IL-10 by autologous PBMC was assayed as described in material and methods. Data correspond to the arithmetic mean and SD of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$. (D) Correlation analysis. The association between the values of HOMA-IR and the percent of pathogenic Th17 (pTh17) cells in blood samples from overweight metabolically unhealthy patients (OWU) was analyzed by using the Spearman rank correlation test; r and P values are shown.

significantly contribute to the systemic low-grade inflammatory phenomenon observed in this condition. However, we consider that a limitation of this study is the small numberof individuals included in each group and that it would be interesting to perform a larger study in the near future.Likewise, the study of the lymphocyte subsets analyzed by us in samples of fat tissue is another relevant point to be addressed. Finally, it would be interesting to explore the possible role of the genetic background of the individuals studied [25] on the behavior of their Treg and Th17 lympho- cytes and their subsets.

Funding

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Conflicts of interest

AM-P, MV-N, LG-B, CA-Q, BH-C, AM-U, LB, PN-M, GH, RS-G and RG-A have nothing to declare.

Author contribution

AM-P, investigation; MV-N, investigation; LG-B, resources; CA-Q, investigation; BH-C, investigation; AM-U, methodology; LB, resources; PN-M, methodology; GH, conceptualization, methodology; RS-G, investigation; RG-A; conceptualization, writing—reviewing and editing.

Ethical approval

This study was approved by the institutional bioethical committee.

Patient consent

This study was performed in accordance with the principles set out in the Declaration of Helsinki, and all participants signed an informed consent statement.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Anexos

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UTILIDAD CLÍNICA DE ÍNDICES ANTROPOMÉTRICOS Y PARÁMETROS METABÓLICOS COMO MARCADORES DEL ESTADO FUNCIONAL DEL TEJIDO ADIPOSO

Clinical utility of anthropometric indices and metabolic parameters as markers of the functional state of adipose tissue

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RESUMEN

El estudio de la composición corporal es un tema de interés en los profesionales de la salud, ya que puede llevarse a cabo tanto para fines de investigación, en la clínica y para la realización del análisis de las condiciones funcionales de determinado sujeto o grupo de sujetos. Si bien son importantes las medidas antropométricas y de composición corporal, es aún más relevante la información del estado funcional de los tejidos metabólicos como el adiposo y muscular, los cuales se pueden obtener de los diferentes índices de composición corporal y bioquímicos. El índice de masa corporal (IMC) se desarrolló para clasificar tanto la obesidad como la desnutrición y se utiliza ampliamente como expresión de adiposidad en estudios poblacionales, sin embargo, no tiene la capacidad de diferenciar la masa grasa (FM) de la masa libre de grasa (FFM), en particular a nivel individual. Por lo que, el objetivo del presente trabajo es identificar el uso de parámetros indirectos de la composición corporal como el índice cintura-talla, índice de masa grasa e índice de masa libre de grasa por su utilidad en la evaluación de la acumulación y distribución del tejido adiposo asociado con alteraciones en el metabolismo de la glucosa y los lípidos, para conocer su uso en el riesgo de desarrollo de enfermedades crónicas.

Palabras clave: Composición corporal, antropometría, índice de masa corporal.

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