Immunology Letters 211 (2019) 60–67

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Increased levels of adipose tissue-resident Th17 cells in obesity associated with miR-326

Mariela Vega-Cárdenas[b], Edith E. Uresti-Rivera[b], Juan D. Cortés-García[a], Margarita Briones-Espinoza[b], Víctor M. Ruíz-Rodríguez[b], Elizabeth Reynaga-Hernández[a], Alejandro Mendez-Mancilla[b], Diana P. Portales-Pérez[a,b,*]

[a] Laboratory of Immunology and Cellular and Molecular Biology, Faculty of Chemical Sciences, Autonomus University of San Luis Potosí, UASLP, Mexico
[b] Center for Research in Health Sciences and Biomedicine, Autonomus University of San Luis Potosí, UASLP, Mexico

ARTICLE INFO

Keywords:
Inflammation
Obesity
Transcription factors
miR-326
miR-155
Th17 cells
Adipose tissue
FOXP3 methylation

ABSTRACT

miRNAs are important immune regulators in the control of the CD4 + T cells phenotype. miR-326 regulates the differentiation towards Th17 cells and the inhibition of miR-155 is associated with low levels of Treg cells. However, miRNAs expression and transcription factors associated with these lymphocyte subsets in obesity-induced adipose tissue inflammation is still unknown. The aim of this work was to identify Th17 cells in subcutaneous adipose tissue (SAT), proinflammatory cytokine production and their association with the miRNAs and transcription factors involved. We collected SAT samples obtained by lipoaspiration from individuals with normal weight, overweight and obesity. We obtained the stromal vascular fractions and then a Ficoll gradient was performed to obtain adipose tissue mononuclear cells (ATMC). Th17 cells were evaluated by flow cytometry and the expression of miR-326, miR-155, RORC2 and FOXP3 by qRT-PCR. We also analyzed cytokines from the supernatants of the ATMC culture and measured the FOXP3 methylation percentage by bisulfite conversion by PCR. According to the results, the frequency of Th17 cells and RORC2 expression was higher in individuals with obesity and associated with miR-326 expression. The ATMC from this group secreted a proinflammatory cytokine profile by in vitro assay. In contrast, lower levels of mRNA FOXP3 expression was detected in ATMC from individuals with obesity that correlated with methylation percentage of FOXP3 gene but no association with miR-155 was detected. Our results suggested that miR-326 participates in the polarization towards Th17 promoting the inflammatory state in the obesity-induced adipose tissue.

1. Introduction

Obesity and overweight constitute a risk factor for the development of type-2 diabetes mellitus, cardiovascular diseases, hepatic steatosis and metabolic disorders [1]. Furthermore, obesity is characterized by a state of chronic low-grade inflammation in adipose tissue due to the release of adipokines, proinflammatory cytokines and chemokines such as, IL-6, IFN-γ, TNF-α IL-1β, RANTES, MCP-1 and SDF-1α, which promote the recruitment of immune cells into the adipose tissue [2]. The adipose tissue-infiltrating immune cells include M1 and M2 macrophages, natural killer and NKT cells, Foxp3+ T regulatory cells (Treg), and memory and effector T cells [3]. Several studies have shown that the number of CD4 + T cells are increased in adipose tissue from individuals with obesity and a diminished amount subset of Treg cells has been reported [4–6].

T cell development is given by lineage-specific transcription factors that regulate the expression of characteristic surface receptors and cytokines. The CD4+ effector T cells can differentiate into several subsets according to their phenotype and function [7,8]. The polarization towards Th17 cells requires IL-6 and TGF-β, also the stable expression of

Abreviations: APC, allophycocyanin; AT, adipose tissue; ATMC, adipose tissue mononuclear cells; ACTB, actin beta BMI= body mass index; FAM, fluorescein; Foxp3, forkhead box P3; gDNA, genomic DNA; IFN, Interferon; MethylS-qPCR, methylation Specific-qPCR; microRNAs, miRNAs; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PE, phycoerythrin; PMA, phorbol myristate acetate; RORC2, Retinoic Acid Receptor-Related Orphan Receptor C2; SAT, subcutaneous adipose tissue; SD, standard deviation; SVF, stromal vascular fraction; TGF, Transforming growth factor; Th17, IL-17-producing helper T cells; TNF, Tumor necrosis factor; Treg, regulatory T cells; VAT, visceral adipose tissue

* Corresponding author at: Laboratory of Immunology and Cellular and Molecular Biology, Faculty of Chemical Sciences, UASLP – Ave. Manuel Nava No. 6 78210 San Luis Potosí, SLP, Mexico.
E-mail address: dportale@uaslp.mx (D.P. Portales-Pérez).

https://doi.org/10.1016/j.imlet.2019.05.010
Received 21 February 2019; Received in revised form 30 April 2019; Accepted 24 May 2019
Available online 25 May 2019

RORC2 which regulates the production of the proinflammatory cytokines IL-17A, IL-17 F, IL-21 and IL-22 [9]. In contrast, the Treg cells maintain immunologic homeostasis and self-tolerance. FOXP3 is the transcription factor that regulates the differentiation of this subset which is characterized to produce IL-10, TGF-β and IL-35 [10]. Th17 cells participate in obesity-dependent inflammation and an increased frequency of these cells in peripheral blood of individuals with obesity and as well the levels of IL-17A [11] have been observed. Th17 cells can co-express IL-17A and IFN-γ or IL-10, thus the pathogenicity of Th17 cells have been associated with the production of IFN-γ and the elevated expression of T-bet and IL-22 [12,13]. Additionally, a study reported an elevated amount of Th17 cells on visceral adipose tissue from metabolically unhealthy obese subjects [14]. However, the mechanisms that are involved in the balance between Th17 and Treg cells in the inflammation process of obese subcutaneous adipose tissue, is still unknown.

miRNAs are non-coding RNAs that play key roles in regulating gene expression [15] by inhibiting translation or by targeting mRNA for degradation or deadenylation [16]. It has been shown that miRNAs can regulate the transcription factors that promote the differentiation of the Th phenotypes [17,18]. For instance, miR-326 targets Ets-1 and promotes polarization towards Th17, miR-155 targets SOCS1, inhibiting the negative regulation of STAT3, which induces the differentiation towards Treg cells by enhancing the expression of FOXP3 [19,20].

DNA methylation is an epigenetic mechanism that regulates gene expression [21], where a high methylation of promoters correlating with low or no transcription [22]. It is well described that FOXP3 is under epigenetic regulation. Particularly, several studies have shown that a high methylation in the FOXP3 gene is associated with autoimmune and inflammatory diseases such as apical periodontitis [23], thrombocytopenia [24], rheumatoid arthritis [25], coronary artery disease [26] and systemic lupus erythematosus [27]. In these diseases there is a decrease of Treg cells and a lower FOXP3 RNA transcript levels.

In obesity, chronic low-grade inflammation occurs with immune cells infiltration, where the percentage of Treg and Th17 cells is affected. The analysis of the microRNA expression and transcription factors associated with these lymphocyte subsets is relevant to know their function in obesity-induced adipose tissue inflammation. Therefore, the purpose of this study was to characterize the participation of Th17 cells in adipose tissue inflammation, through the evaluation of miRNAs expression and the methylation status of FOXP3 intron 1 (FOXP3i1); in order to establish a possible association of these mechanisms in T cell polarization in obesity.

2. Materials and methods

2.1. Study group and sample collection

Individuals who underwent liposuction by plastic surgeons were invited to participate. The female and male volunteers were classified in three groups based on their body mass index (BMI kg/m^2): normal weight, overweight and obesity. The study was approved by the Ethics Committee of the Faculty of Chemical Sciences at the Autonomous University of San Luis Potosi (project CEID2013006). Tissue samples, clinical data and the signed informed written consent were collected by the physician responsible of the surgery on the same day that the surgical process was performed.

2.2. Mononuclear cells isolation from adipose tissue samples

Adipose tissue was digested with 0.03% type I collagenase (GiBCO) solution in RPMI (Gibco, New York), supplemented with 1.5% bovine serum and antibiotics 200 µL/mL penicilin G and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO) for 1 h at 37 °C under constant stirring. After centrifugation at 1500 rpm for 10 min, the stromal vascular fraction (SVF) was obtained. The adipose tissue mononuclear cells (ATMC) samples were isolated by the ficoll-hypaque method from this SVF fraction using a method previously reported [28].

2.3. Flow cytometry analysis

Freshly isolated cells were intracellular-stained to detected IL-17A molecule. For the analysis of Th17 cells, these cells were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin for 3 h and brefeldin A was added for the last 2 h of the culture. Then, for the cell surface antigens, 5×10^5 cells were incubated with fluorescent labeled monoclonal antibody anti CD4-APC for 30 min. For the intracellular cytokine staining, 0.01% saponin was added to the cells and incubated for 10 min al 4 °C in the dark, then the cells were centrifuged for 300 *g* for 15 min. The antibody IL-17A-PE was used, and cells incubated for 30 min at 4 °C in the dark. After the wash step, the supernatant was discarded and the cells were fixed with 1% paraformaldehyde (PFA). Fluorescence positive cells were quantified by FACS Canto II cytofluorometer (BD Bioscience, San Diego, CA) and the FACS Diva software was used to analyze the data.

2.4. Total RNA extraction and quantitative real-time reverse transcription

Total RNA was extracted from ATMC using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA concentration and purity were determined using a spectrophotometer (Synergy HT, BioTek). A total amount of 100 ng of RNA was used to synthetize cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems) or TaqMan miR Reverse Transcription kit. A Total of 250 ng of cDNA was used as a template to perform qPCR using TaqMan® Universal Master Mix and TaqMan® MicroRNA Assay primers (Applied Biosystems™) for miR-326 and miR-155. To evaluate the mRNA expression of the transcription factors, iTaq™ Universal SYBR® Green Supermix and specific primers for RORC2 [sense: 5′-cagtcatgagaacacaaattgaagtg- 3′, antisense: 5′- caggtgataaccccgtagtggat- 3′] and FOXP3 [sense: 5′-gtggcatcatccgacaag- 3′, antisense: 5′-gtggaggaactctgggaa- 3′] were used. The analysis was performed on the CFX96 Touch™ Real-Time System (BioRad). The data obtained were analyzed with the $2^{-\Delta\Delta Cq}$ method against the level of the transcripts U6 and ACTB for miRNAs and mRNAs respectively.

2.5. Multiplex assays

The ATMC were stimulated with PMA and ionomycin at 37 °C for 5 h. Levels of cytokines IL-1β, IL-10, IL-17A, IL-2, IL-6, TNF-α and IFN-γ were measured from cell culture supernatants using a costume human ProcartaPlex multiplex Assays (Invitrogen). The assays were carried out following the manufacturer´s instructions. Data were analyzed using the software Bio-Plex Manager™ Software (BioRad).

2.6. Methylation specific-qPCR (MethylS-qPCR) for FOXP3i1

We used the method described by Muls et al [29]. Genomic DNA (gDNA) was prepared from frozen pellets containing ATMC and peripheral blood mononuclear cells (PBMC) from the same subject with the PureLink DNA Mini Kit (Invitrogen). This gDNA was treated with sodium bisulfite using the EpiTect Plus DNA Bisulfite Kit (Qiagen). Real-Time PCR was performed in a final reaction volume of 20 µL containing 200 nM each of methyl- [sense: 5′- ctcttctcttcctccgtaatatcg- 3′, antisense: 5´- gttattgacgttatggcggtc- 3′] or non-methyl- [sense: 5′-ccctcttctcttcctccataatatca-3′, antisense 5´- ttttgttattgatgttatggtggtt-3´] specific primers for FOXP3 intron 1 sequence, 10µM methyl- [aaacccgacgcatccgac], or non-methyl- [aaacccaacacatccaacca] specific FAM probe and 20 ng bisulfite-treated genomic DNA. Each sample was analyzed in triplicate using the CFX real-time PCR. Thermal cycling conditions consisted of a 95 °C preheating step for 10 min, followed by

44 cycles of 60 °C for 30 s and by 1 min at 61 °C. The percentage of methylated sequences was calculated by the following equation: $Ct_{meth} = 100/[1 + 2(Ct_{meth}\text{-}Ct_{demeth})]\%$, and it was expressed as the mean percent of methylation.

2.7. Statistical analysis

Data are shown as the mean ± the standard deviation (SD) or the median ± interquartile range. The distribution of each one of the variables was assessed by the Kolmogorov-Smirnov test. The assessment of differences in the expression of miR-155, miR-326 and the transcription factors RORC2 and FOXP3 was determined using both parametric and non-parametric tests. The differences in the expression of miRNAs between the groups were determined by ANOVA. The differences were considered significant at $p < 0.05$. The statistical analysis was performed using InStat GraphPad software (InStat GraphPad Inc., San Diego, CA, USA), version 5.0.

3. Results

3.1. Characteristics of the participants in the study groups

In accordance with body mass index (BMI kg/m^2), it was divided the total of 39 volunteers (female, n = 37 and male, n = 2), in three groups: 16 individuals who were normal weight (BMI < 25), 17 who were overweight (BMI 25-29.99) and 6 individuals with obesity (BMI ≥ 30). The age of the participants ranged from 20 to 63 years, with a mean age of 37 years. The main clinical and biochemical parameters of the 39 individuals assessed in this study are listed in Table 1. As expected, significant differences were observed in weight and BMI between the three groups.

3.2. Increased levels of Th17 cells and RORC2 expression in adipose tissue from individuals with obesity

Given the role of Th17 cells in proinflammatory diseases, we decided to evaluate whether the frequency of Th17 cells were altered in ATMC from study groups. Representative histograms for the identification of IL-17A + cells after defining the lymphocyte population, based on forward and side scatter parameters, are shown in Fig. 1A. Flow cytometry analysis showed significantly higher Th17 cell frequencies in ATMC from an individual with obesity than in normal weight and overweight (Fig. 1B). Retinoic acid-related orphan receptor C isoform 2 (RORC2) has been regarded as the master transcription factor in Th17 cells. Based on qRT-PCR analysis, we observed significantly higher expression levels from individuals with obesity compared with normal weight and overweight participants (Fig. 1C). Finally, we found a positive correlation between Th17 cell frequencies and the expression of RORC2 (Fig. 1D).

Table 1
Clinical characteristics of the study group. Samples from subcutaneous adipose tissue were obtained from thirty-nine individuals—men (n = 2) and women (n = 37)—who underwent liposuction surgery, the age ranged from 20 to 63 years old. The groups of individuals were divided according to their BMI: normal weight (BMI < 25 kg/m$^{2)}$, overweight (BMI 25–29.99 kg/m^2) and obese (BMI > 30 kg/m^2). Significant differences were found in weight and BMI from normal weight, overweight and individuals with obesity. Data are presented as the means ± SD, BMI: Body Mass Index, Upper letters indicate significant differences based on post-hoc testing.

Parameters n	Normal weight 16 (F)	Overweight 17 (F)	Obesity 6 (2 M/ 4 F)	p
Age	36.8 ± 8.3	37.7 ± 11.0	38.5 ± 10.1	0.9119
Weight	62.3 ± 7.3^a	69.6 ± 6.78^b	98.79 ± 14.06^c	< 0.0001
Height	1.62 ± 0.04	1.61 ± 0.07	1.73 ± 0.11	0.0009
BMI	23.7 ± 1.6^a	26.5 ± 2.7^b	32.6 ± 2.9^b	< 0.0001
Glucose	88.6 ± 9.6	94.0 ± 6.5	91.4 ± 9.6	0.3159
Creatinine	0.76 ± 0.15	0.79 ± 0.14	0.77 ± 0.13	0.9321
Hemoglobin	13.6 ± 0.9^a	14.3 ± 1.1^b	15.2 ± 1.7^c	0.0494
Cholesterol	210.7 ± 22.4	205.7 ± 43.8	210.5 ± 40.7	0.9604
Triglycerides	141.7 ± 20.9	99.6 ± 27.7	128.0 ± 55.1	0.4890
Leucocytes	5.87 ± 1.37	6.98 ± 0.89	6.43 ± 1.2	0.1056

3.3. Co-expression of high levels of miR-326 and RORC2 in mononuclear cells from adipose tissue in individuals with obesity

Previous reports have shown that miR-326 and miR-155 are important regulators of transcription factors that promote the differentiation of the Th17 or Treg phenotypes [17,18]. Therefore, this prompted us to test whether these miRNAs are involved in this differentiation process in ATMC. We found that ATMC from individuals with obesity expressed high levels of miR-326 when compared to normal weight and overweight subjects (Fig. 2A). Interestingly, those levels were positively correlated with the expression of RORC2 (Fig. 2B). On the other hand, the miR-155 expression levels were similar between groups and no association with FOXP3 mRNA levels was observed (Fig. 2C–D).

3.4. ATMC from individuals with obesity secrete altered levels of proinflammatory cytokines in vitro

Infiltrating immune cells produce cytokines that participate in cell signaling and immune regulation, affecting adjacent cells from tissues and organs. To determine the cytokines production, ATMC from individuals of the studied groups were stimulated with PMA or ionomycin as described in material and methods. High levels of the cytokines IL-1β, IL-17A, IL-6, TNF-α, and IFN-γ ($p < 0.05$) were observed in the supernatants of ATMC from obesity group when compared with the normal weight group (Fig. 3A–E). However, there were no significant differences observed in the levels of IL-2 and IL-10 in any of the studied groups (Fig. 3F–G).

3.5. High degree of FOXP3 methylation and low level of FOXP3 expression in ATMC from individuals with obesity

Methylation status in the FOXP3 gene has been shown associated with diverse inflammatory diseases, which also are characterized by a reduction of Treg cells and a lower FOXP3 mRNA levels. Therefore, we analyzed the methylation status of the FOXP3i1 sequence and the FOXP3 transcript levels (Fig. 4A) in ATMC and PBMC from individuals with a normal weight, overweight and obesity. The mean methylation level in the ATMC samples was not significantly different compared with PBMC samples from the same individual (Fig. 4B). Moreover, significant changes in the methylation percentage of FOXP3 were observed in the ATMC from the obesity group compared with the normal weight group (Fig. 4C). However, no significant associations were observed between relative expression of FOXP3 gene and methylation percentage (Fig. 4D).

4. Discussion

During obesity the excessive fat accumulation induces changes in the amount and activity of immune cells in the adipose tissue, which leads to activation of a local inflammatory response and subsequently systemic inflammation [30]. Th17 cells play an important role in the induction and maintenance of autoimmune diseases [31], and their prevalence in the adipose tissue is associated with the progression of obesity in humans [32]. It has been reported a high frequency of Th17 cells and a high level of expression of RORC2 in visceral adipose tissue (TAV) from morbidly obese subjects with metabolic abnormalities [33]. This data are in accordance with our present work where we detected an increase in CD4 + IL-17A + cell frequency related to RORC2

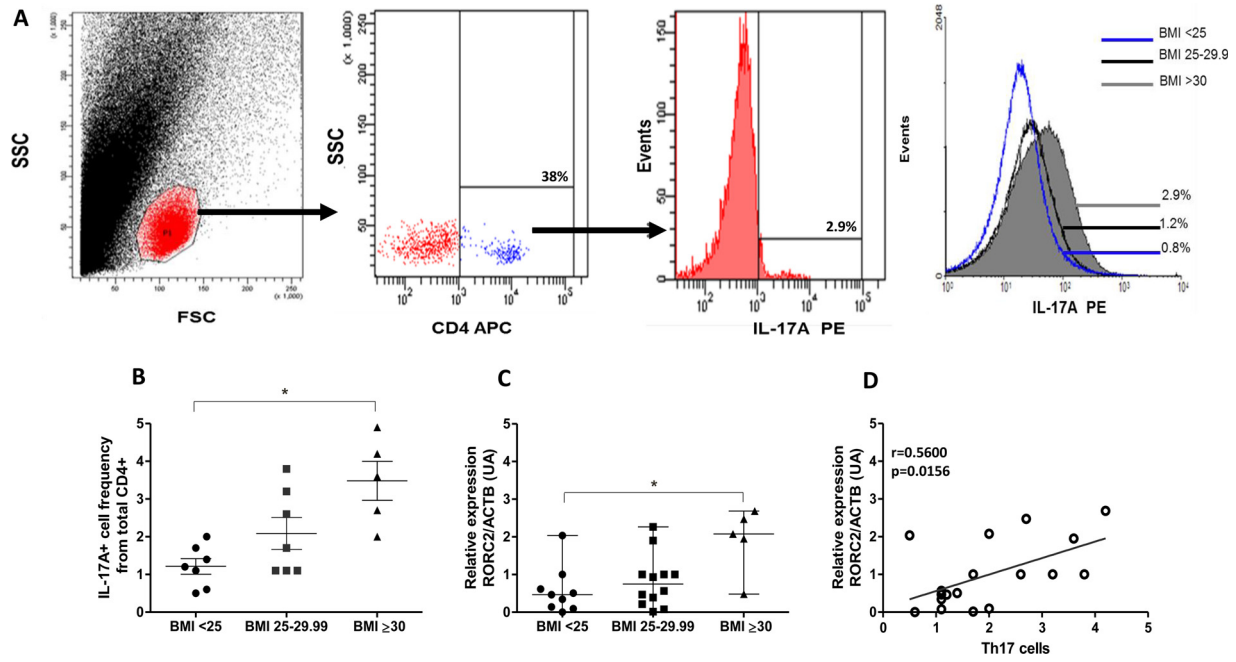

Fig. 1. Increased levels of adipose tissue- resident Th17 cells in obesity. The frequency of CD4 + IL-17A + cells in adipose tissue mononuclear cells (ATMC) from the individuals with normal weight, overweight and obesity was analyzed. A) Representative FACS profile showing the expression of CD4 + IL17A + cells and percentage of Th17 cells in ATMC from individuals with BMI < 25, BMI 25–29.99 or BMI ≥ 30. B) Percentage of Th17 cells from individuals with normal weight, overweight and obesity. Data are expressed as a percentage of all CD4 + T cells. C) Relative expression of RORC2 from individuals with normal weight, overweight and obesity D) Correlation between relative expression of RORC2 and percentage of Th7 cells in ATMC were analyzed using Pearson's (R) correlation. Graphs show means ± SEMs. Statistical significance is shown as $*p < 0.05$.

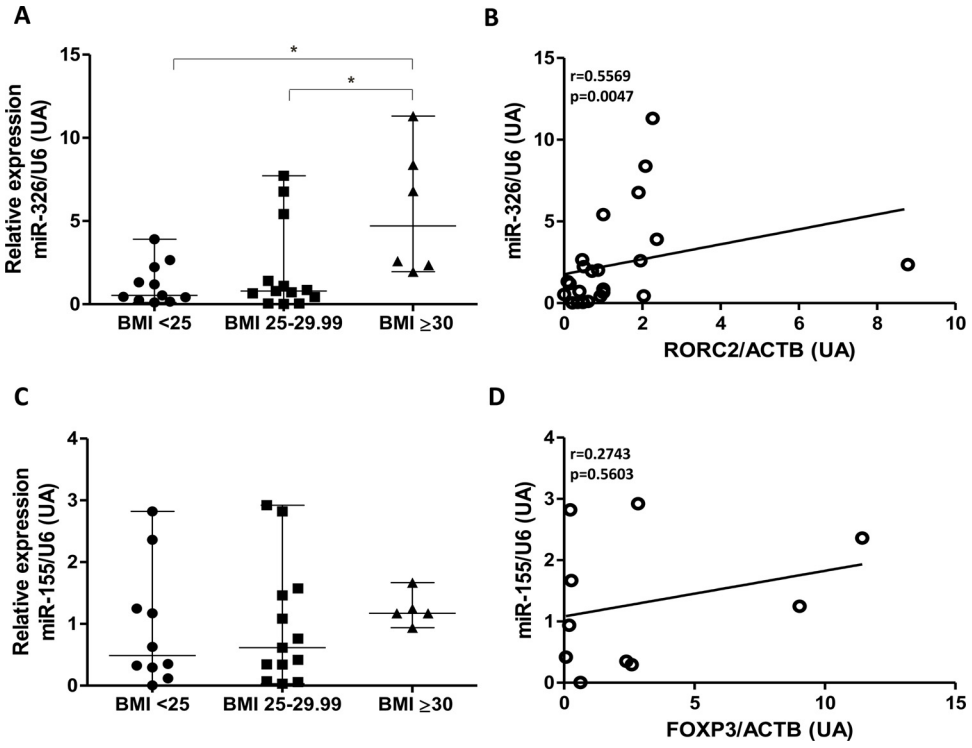

Fig. 2. Increased expression levels of miR-326 in individuals with obesity and associated with RORC2. Analysis of miRNAs (miR-326 and miR-155) expression in the adipose tissue mononuclear cells (ATMC) from individuals with normal weight (BMI < 25), overweight (BMI 25–29.99) and obesity (BMI ≥ 30) was performed. A) Relative expression of miR-326 from normal weight, overweight and individuals with obesity. B) Correlation between the RORC2 expression and miR-326. C) Relative expression of miR-155 from normal weight, overweight and obese group D) Correlation between the FOXP3 expression and miR-155. Graphs show medians with a range. Statistical significance is shown as $*p < 0.05$.

expression in ATMC from individuals with obesity. However, it has not been analyzed the presence of Th17 cells, as well as other factors as miRNAs or methylation status of FOXP3 gene, in subcutaneous adipose tissue (SAT) from individuals who did not present metabolic disorders or- obesity-associated diseases.

The hypertrophied adipose tissue becomes heavily infiltrated by a variety of immune cells. The polarization towards Th17 is regulated by the tissue microenvironment, mainly by the production of cytokines such as IL-6, TGF-β and IL-21. Also, the adipose tissue from individuals with obesity present a high production of IL-6 cytokine [34], which could be promoting the increase of Th17 cell markers in individuals with this condition. The Th17 cells as IL17A-producing CD4 + T cells, which were found to be increased in obesity, are well recognized as biomarkers of the inflammatory process. In addition, the in vitro

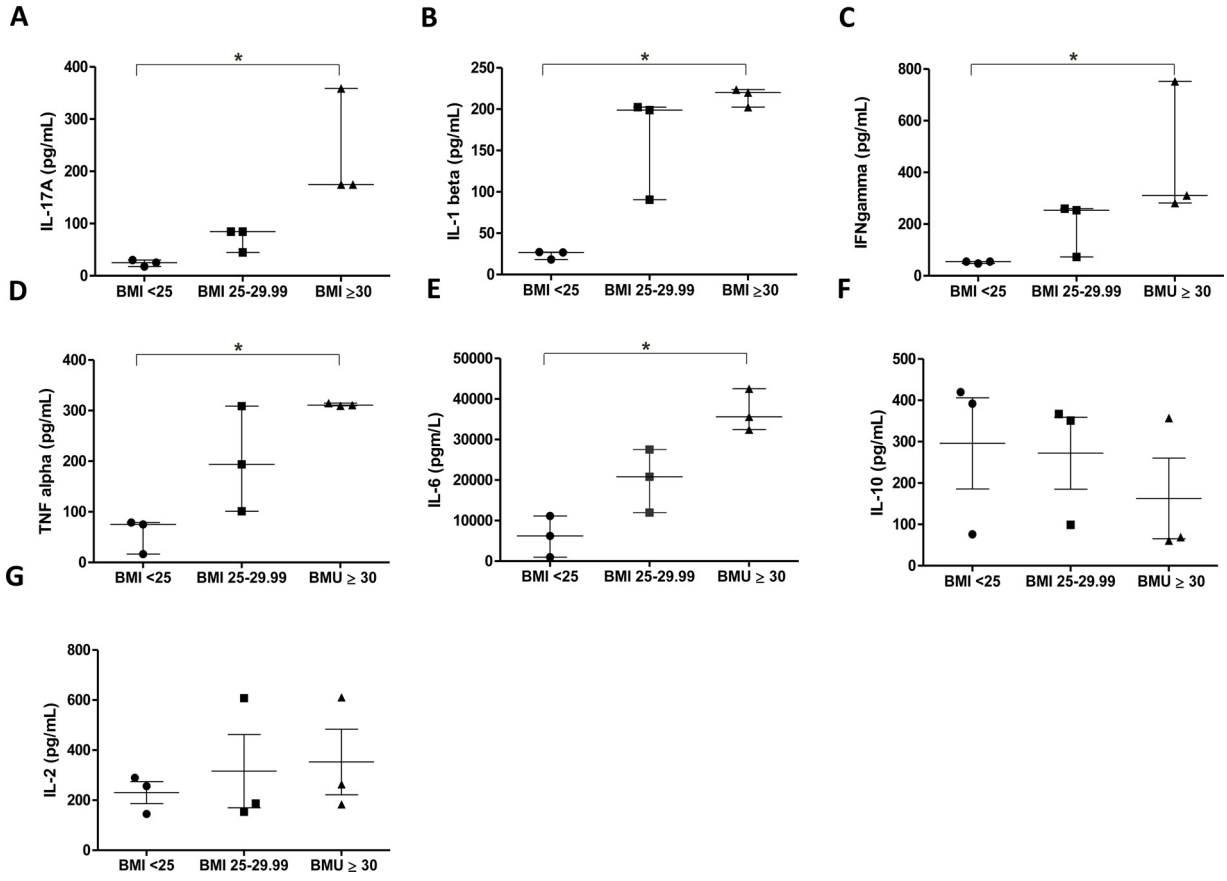

Fig. 3. Increased levels of proinflammatory cytokines in cell culture supernatants of adipose tissue mononuclear cells from obese subjects. The cell-free supernatant from adipose tissue mononuclear cells (ATMC) was analyzed for cytokine secretion by multiplex analysis using the BioPLex assay. A–E) The ATMC cells from the obesity group compared with a normal weight secreted higher amounts of generally inflammatory IL-17A, IL-1β, IFN-gamma, IL-6 and TNF-α, but not compared with overweight individuals. F–G) The ATMC cells from individuals with obesity compared with a normal weight or overweight secreted similar amounts of IL-10 or IL-2. Graphs show medians with range. Statistical significance is shown as $*p < 0.05$.

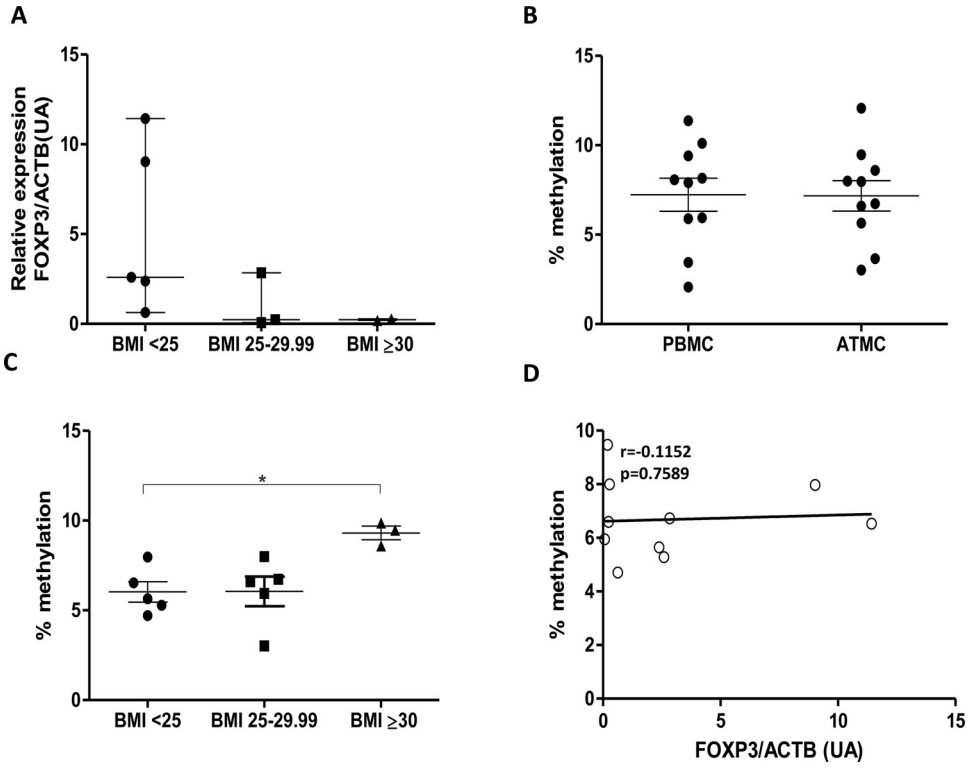

Fig. 4. Diminished expression levels of FOXP3 in adipose tissue mononuclear and FOXP3i1 methylation status in individuals with obesity. Comparison of the relative expression of FOXP3 and the methylation percentage between individuals with normal weight (BMI < 25), overweight (BMI 25–29.99) and obesity (BMI > 30) groups. A) Relative expression of FOXP3 RNAm transcripts from individuals with normal weight, overweight and obesity. B) Methylation percentage from adipose tissue mononuclear (ATMC) compared with PBMC from the same subject. C) Methylation percentage from normal weight, overweight and obese groups. D) Correlation between the FOXP3 expression and methylation percentage. Graphs show medians with range. Statistical significance is shown as $*p < 0.05$ or $**p < 0.01$.

cytokine production from ATMC was evaluated, finding a higher amount of proinflammatory cytokines associated with a pathogenic Th17 differentiation (IL-6 and IL-1β) [35], also increased in obesity. Those results suggest that the Th17 cells present in ATMC from individuals with obesity could be pathogenic Th17, but more studies are required to verify this hypothesis.

Excessive accumulation of body fat can lead to the development of inflammatory and metabolic disorders. The individuals with obesity vary their metabolic status. This has led to identify metabolically healthy and unhealthy subjects with obesity [36]. Our study group did not show differences with the mean of total cholesterol although it showed exceeded normal values in the three study groups. It was not possible to classify the study participants as metabolically unhealthy. Another characteristic in which subjects with obesity can vary is their body fat distribution. The subcutaneous and visceral fat depots present differences at the anatomical, cellular, molecular and physiological level. For example, VAT is a more vascularized tissue. Inflammatory cells are more prevalent in this depot and present greater percentage of mature adipocytes compared with SAT [37]. Previous studies indicated that Th17 cell frequency in visceral and subcutaneous adipose tissues are different, this may be due to the different functional and morphological differences of both tissues, for example the VAT secretes higher concentrations of inflammatory cytokines, compared to SAT.

The macrophage role as the key immune cells responsible for the obesity-associated inflammation in adipose tissue has been described [38–41]. However, the potential role of other immune cells, such as lymphocytes subsets in human adipose tissue [42] and the production of proinflammatory cytokines, is unknown. Our results for the first time showed a high production of proinflammatory cytokines in stimulated ATMC that are probably linked to the increase in the number of Th17 cells in individuals with obesity. This conclusion is also supported by previous studies which demonstrated a higher amount of proinflammatory cytokines in obesity-dependent adipose tissue inflammation [42,43]. We hypothesize that excessive accumulation of fat is enough to promote a pro inflammatory state on tissue prior to the occurrence of metabolic disorders. However, further studies are required to determine the mechanisms by which Th17 cells contribute to the inflammation present in SAT.

It is well known that specific miRNAs play an important role on function and differentiation of CD4 + T-cell subsets. One of the critical miRNAs that is involved in the frequency of Th17 cells and the production of IL-17A, is miR-326 [19]. Mahnaz Ghaebi et al. reported that this Th17 cell-associated miRNA, was up-regulated in peripheral blood leukocytes from patients with repeated implantation failure (RIF) who present high amount of Th17 cells [44]. Other studies have demonstrated the miR-326 participation in several autoimmune and inflammatory diseases, in which the patients profile showed a higher amount of IL-17A producing T cells, diminished frequency of Treg cells and a marked association with miR-326 expression [45,46]. miR-326 also plays an important role during the adipocyte differentiation process [47]; however, there are no reports regarding its participation during the inflammatory process in subcutaneous adipose tissue. In the present study, we found higher levels of miR-326 expression in ATMC from individuals with obesity compared to those with a normal weight and a positive correlation with RORC2 expression; the nuclear hormone receptor RORC2 is an important factor in transcriptional specific program of Th17 cells, promoting the generation of IL-17A and contributing to Th17 cell differentiation from naïve CD4 + T cells [48]. Therefore, we suggest that miR-326 participates in the polarization towards Th17 cells through RORC2 expression and contributing in the inflammatory process in the adipose tissue.

In contrast, miR-155 regulates different cellular processes and its expression is increased on Treg cells compared to other lymphocytes subsets [49]. Regarding adipose tissue, it has been reported that the proinflammatory cytokine TNF-α induces the expression of miR-155 in preadipocytes and adipocytes *in vitro* [50]. In addition, miR-155 expression has been reported to increase in subcutaneous adipose tissue and correlated with BMI from individuals with obesity [51].Those reports are in contrast with our results, since we found no association between miR-155 levels in ATMC and BMI, suggesting that the contribution of miR-155 in ATMC is low and probably more relevant in another cell type present in the adipose tissue, which may explain our findings. Although miR-326 and miR-155 are crucial in the immune system, it would be interesting to evaluate the presence of other miRNAs such as miR-21, which participates in the imbalance of Th17/Treg cells on inflammatory diseases [52].

It has been shown that the phenotype and genotype of T-cell subsets are altered in individuals with obesity, per example markers of T cell lineage are increased (CD3, CD4, CD8, FOXP3, GATA3) [6]. In contrast, a decreased frequency of Treg cells in subcutaneous and visceral adipose tissue from individuals with obesity has been reported [53,5]. It is well known that FOXP3 is a master transcription factor that regulates the pathway in the development and function of Treg cells [54]. However, the FOXP3 expression levels in ATMC is unknown. Hence, we evaluated the inter-relationship between the expression levels of FOXP3 and ATMC microenvironment and we found diminished levels of FOXP3 mRNA in overweight and in individuals with obesity (Fig. 4A). In this regard, it has been showed that in visceral adipose tissue from individuals with morbid obesity its characterized by decreased levels of FOXP3 mRNA transcripts [55], whereas others reported positive correlations [5,6]. Despite the central role of FOXP3 in Treg cell development and function; finding diminished transcript levels, it is not sufficient to conclude that Treg cells are decreased, due to FOXP3 expression may be transiently induced and cannot be assumed that Treg cells do not exhibit suppressor function [56–58]. Epigenetic mechanisms such as demethylation within the Foxp3 locus Treg-specific demethylated (TSDR) conferred a stable FOXP3 expression on Treg cells and suppressive function [59]. On the other hand, FOXP3 gene polymorphisms are associated with autoimmune diseases [60], the polymorphisms in the intronic region affect RNA processing [61] and specifically the polymorphism rs2232368 located in intron 1 is associated with unexplained recurrent spontaneous abortions [61] characterized with a low frequency of Treg cells and diminished suppressive function. DNA methylation has been shown to be involved in the regulation of FOXP3 expression; Cai-Xia Lü et al. reported that acute coronary syndrome patients showed lower demethylation of FOXP3i1 relative levels and pronounced reduction in the proportion of Treg cells [57] and for our knowledge the methylation pattern of intron 1 region in FOXP3 gene has not been evaluated in ATMC.

In the present study, we analyzed whether methylation of FOXP3 intron 1 is a reliable marker of Treg cells. Our results showed that there were significantly higher levels of methylation in individuals with obesity compared with normal weight, however, we did not detect differences on methylation percentage between ATMC and PBMC from the same subject. Also, we did not find a significant association between transcripts of FOXP3 gene and methylation percentage. Our data suggests that the reduction in FOXP3 RNAm transcripts levels may be related with the increased of FOXP3i1 methylation percentage in patients with obesity. Epigenetic suppression of FOXP3 might lead to a down-regulation of Treg cells and in turn exacerbate the inflammatory process in adipose tissue. However, it will be necessary to confirm this methylation percentage increasing the number of individuals and using other methodology, such as Bisulfite Sequencing.

5. Conclusions

We showed, for the first time, that the expression of RORC2 was associated with miR-326 expression levels in ATMC. The obesity group showed not only a higher frequency of CD4 + IL-17A + T cells, but also a higher expression of RORC2 and miR-326. These results supported the notion that miR-326 could be a reliable Th17 marker in the inflamed adipose tissue and the imbalance with the reduction in FOXP3 RNAm

transcripts levels related to the increase in FOXP3i1 methylation percentage associated with obesity. This work supports the importance of the participation of miR-326 in promoting a pro-inflammatory profile in adipose tissue in obesity.

Conflict of interest

The author declares no potential conflicts of interest related to this manuscript.

Acknowledgements

This work was supported by grant 251723 (to Portales-Pérez, DP) from FOMIX-CONACYT-SLP, México. As well as the master's fellowship awarded to MVC (621487) by Consejo Nacional de Ciencia y Tecnologia (CONACYT), Mexic (FMSLP-2014-02-251723).

References

[1] K. Ghoshal, Adiponectin: probe of the molecular paradigm associating diabetes and obesity, World J. Diabetes (2015), https://doi.org/10.4239/wjd.v6.i1.151.
[2] H. Yang, Y.-H. Youm, B. Vandanmagsar, A. Ravussin, J.M. Gimble, F. Greenway, et al., Obesity increases the production of proinflammatory mediators from adipose tissue t cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance, J. Immunol. 185 (3) (2010) 1836–1845, https://doi.org/10.4049/jimmunol.1000021.
[3] S. Sindhu, R. Thomas, P. Shihab, D. Sriraman, K. Behbehani, R. Ahmad, Obesity is a positive modulator of IL-6R and IL-6 expression in the subcutaneous adipose tissue: significance for metabolic inflammation, PLoS One (2015), https://doi.org/10.1371/journal.pone.0133494.
[4] E. Dalmas, N. Venteclef, C. Caer, C. Poitou, I. Cremer, J. Aron-Wisnewsky, et al., T cell-derived IL-22 amplifies IL-1beta-driven inflammation in human adipose tissue: relevance to obesity and type 2 diabetes, Diabetes 63 (2014) 1966–1977, https://doi.org/10.2337/db13-1511 (1939–327X (Electronic)).
[5] A. Núñez Ruiz, J.D. Cortés-Garcia, N. Cortez-Espinosa, P.I. Herrera-Rojas, V.M. Ruíz-Rodríguez, M. Salgado-Bustamante, et al., Diminished levels of regulatory T cell subsets (CD8 + Foxp3, CD4 + Foxp3 and CD4 + CD39 + Foxp3) but increased Foxp3 expression in adipose tissue from overweight subjects, Nutrition 32 (9) (2016) 943–954, https://doi.org/10.1016/j.nut.2016.02.006.
[6] R.L. Travers, A.C. Motta, J.A. Betts, A. Bouloumié, D. Thompson, The impact of adiposity on adipose tissue-resident lymphocyte activation in humans, Int. J. Obes. 39 (5) (2015) 762–769, https://doi.org/10.1038/ijo.2014.195.
[7] S.J. Szabo, S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, L.H. Glimcher, A novel transcription factor, T-bet, directs Th1 lineage commitment, Cell (2000), https://doi.org/10.1016/S0092-8674(00)80702-3.
[8] W. Zheng, Ra. Flavell, The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells, Cell 89 (4) (1997) 587–596, https://doi.org/10.1016/S0092-8674(00)80240-8.
[9] I.I. Ivanov, B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, et al., The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells, Cell 126 (6) (2006) 1121–1133, https://doi.org/10.1016/j.cell.2006.07.035 https://doi.org/S0092-8674(06)01105-6 [pii].
[10] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3, Science (New York, N.Y.) 299 (5609) (2003) 1057–1061, https://doi.org/10.1126/science.1079490.
[11] H.Y. Kim, H.J. Lee, Y.J. Chang, M. Pichavant, S.A. Shore, K.A. Fitzgerald, et al., Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity, Nat. Med. 20 (1) (2014) 54–61, https://doi.org/10.1038/nm.3423.
[12] Y. Kishi, T. Kondo, S. Xiao, N. Yosef, J. Gaublomme, C. Wu, et al., Protein C receptor (PROCR) is a negative regulator of Th17 pathogenicity, J. Exp. Med. (2016), https://doi.org/10.1084/jem.20151118.
[13] Y. Lee, A. Awasthi, N. Yosef, F.J. Quintana, S. Xiao, A. Peters, et al., Induction and molecular signature of pathogenic TH17 cells, Nat. Immunol. 13 (10) (2012) 991–999, https://doi.org/10.1038/ni.2416.
[14] J.B. Pandolfi, A.A. Ferraro, I. Sananez, M.C. Gancedo, P. Baz, L.A. Billordo, et al., ATP-induced inflammation drives tissue-resident Th17 cells in metabolically unhealthy obesity, J. Immunol. (2016), https://doi.org/10.4049/jimmunol.1502506.
[15] C. Xiao, K. Rajewsky, MicroRNA control in the immune system: basic principles, Cell (2009), https://doi.org/10.1016/j.cell.2008.12.027.
[16] K. Felekkis, E. Touvana, C. Stefanou, C. Deltas, MicroRNAs: a newly described class of encoded molecules that play a role in health and disease, Hippokratia (2010).
[17] B.J. Kroesen, N. Teteloshvili, K. Smigielska-Czepiel, E. Brouwer, A.M.H. Boots, A. van den Berg, J. Kluiver, Immuno-miRs: critical regulators of T-cell development, function and ageing, Immunology (2015), https://doi.org/10.1111/imm.12367.
[18] D.F. Steiner, M.F. Thomas, J.K. Hu, Z. Yang, J.E. Babiarz, C.D.C. Allen, et al., MicroRNA-29 regulates T-Box transcription factors and Interferon-γ production in helper t cells, Immunity (2011), https://doi.org/10.1016/j.immuni.2011.07.009.
[19] C. Du, C. Liu, J. Kang, G. Zhao, Z. Ye, S. Huang, et al., MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis, Nat. Immunol. 10 (12) (2009) 1252–1259, https://doi.org/10.1038/ni.1798.
[20] L. Fassi Fehri, M. Koch, E. Belogolova, H. Khalil, C. Bolz, B. Kalali, et al., Helicobacter pylori induces miR-155 in T cells in a cAMP-Foxp3-dependent manner, PLoS One (2010), https://doi.org/10.1371/journal.pone.0009500.
[21] M. Ehrlich, M.A. Gama-Sosa, L.H. Huang, R.M. Midgett, K.C. Kuo, R.A. Mccune, C. Gehrke, Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells, Nucleic Acids Res. (1982), https://doi.org/10.1093/nar/10.8.2709.
[22] H. Cedar, DNA methylation and gene activity, Cell (1988), https://doi.org/10.1146/annurev.bi.52.070183.000521.
[23] K. Campos, C.F. Franscisconi, V. Okehie, L.C. De Souza, A.P.F. Trombone, A. Letra, et al., FOXP3 DNA methylation levels as a potential biomarker in the development of periapical lesions, J. Endod. 41 (2) (2015) 212–218, https://doi.org/10.1016/j.joen.2014.10.003.
[24] Z. Chen, Z. Guo, J. Ma, J. Ma, F. Liu, R. Wu, Foxp3 methylation status in children with primary immune thrombocytopenia, Hum. Immunol. (2014), https://doi.org/10.1016/j.humimm.2014.09.018.
[25] A. Kennedy, E.M. Schmidt, A.P. Cribbs, H. Penn, P. Amjadi, K. Syed, et al., A novel upstream enhancer of FOXP3, sensitive to methylation-induced silencing, exhibits dysregulated methylation in rheumatoid arthritis Treg cells, Eur. J. Immunol. (2014), https://doi.org/10.1002/eji.201444453.
[26] X. Cheng, X. Yu, Y. Ding, Q. Fu, J. Xie, T. Tang, et al., The Th17/Treg imbalance in patients with acute coronary syndrome, Clin. Immunol. (2008), https://doi.org/10.1016/j.clim.2008.01.009.
[27] O. Ngalamika, G. Liang, M. Zhao, X. Yu, Y. Yang, H. Yin, et al., Peripheral whole blood FOXP3 TSDR methylation: a potential marker in severity assessment of autoimmune diseases and chronic infections, Immunol. Invest. (2015), https://doi.org/10.3109/08820139.2014.938165.
[28] J.D. Cortés-Garcia, M.J. Briones-Espinoza, M. Vega-Cárdenas, V.M. Ruíz-Rodríguez, A. Mendez-Mancilla, A.E. Gómez-Otero, et al., The inflammatory state of adipose tissue is not affected by the anti-inflammatory response of the A2a-adenosine system and miR-221/PTEN, Int. J. Biochem. Cell Biol. (2018), https://doi.org/10.1016/j.biocel.2018.04.020.
[29] N. Muls, H.A. Dang, C.J.M. Sindic, V. Van Pesch, Fingolimod increases CD39-expressing regulatory T cells in multiple sclerosis patients, PLoS One (2014), https://doi.org/10.1371/journal.pone.0113025.
[30] M. Mraz, M. Haluzik, The role of adipose tissue immune cells in obesity and low-grade inflammation, J. Endocrinol. (2014), https://doi.org/10.1530/JOE-14-0283.
[31] Y. Hu, F. Shen, N.K. Crellin, W. Ouyang, The IL-17 pathway as a major therapeutic target in autoimmune diseases, Ann. N. Y. Acad. Sci. (2011), https://doi.org/10.1111/j.1749-6632.2010.05825.x.
[32] M. Ahmed, S.L. Gaffen, IL-17 in obesity and adipogenesis, Cytokine Growth Factor Rev. (2010), https://doi.org/10.1016/j.cytogfr.2010.10.005.
[33] J.B. Pandolfi, A.A. Ferraro, I. Sananez, M.C. Gancedo, P. Baz, L.A. Billordo, et al., ATP-induced inflammation drives tissue-resident Th17 cells in metabolically unhealthy obesity, J. Immunol. 196 (8) (2016) 3287–3296, https://doi.org/10.4049/jimmunol.1502506.
[34] T.J. Guzik, D.S. Skiba, R.M. Touyz, D.G. Harrison, The role of infiltrating immune cells in dysfunctional adipose tissue, Cardiovasc. Res. (2017), https://doi.org/10.1093/cvr/cvx108.
[35] K. Ghoreschi, A. Laurence, X.P. Yang, C.M. Tato, M.J. McGeachy, J.E. Konkel, et al., Generation of pathogenic T H 17 cells in the absence of TGF-β 2 signalling, Nature (2010), https://doi.org/10.1038/nature09447.
[36] C.M. Phillips, Metabolically healthy obesity: definitions, determinants and clinical implications, Rev. Endocr. Metab. Disord. (2013), https://doi.org/10.1007/s11154-013-9252-x.
[37] A. Gómez-Hernández, N. Beneit, S. Díaz-Castroverde, et al., Differential role of adipose tissues in obesity and related metabolic and vascular complications, Int. J. Endocrinol. (2016), https://doi.org/10.1155/2016/1216783.
[38] R. Cancello, C. Henegar, N. Viguerie, S. Taleb, C. Poitou, C. Rouault, et al., Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss, Diabetes (2005), https://doi.org/10.2337/diabetes.54.8.2277.
[39] S.E. Shoelson, J. Lee, A.B. Goldfine, Inflammation and insulin resistance, J. Clin. Invest. (2006), https://doi.org/10.1172/JCI29069.
[40] B.M. Spiegelman, J.S. Flier, Obesity and the regulation of energy balance, Cell (2001), https://doi.org/10.1016/S0092-8674(01)00240-9.
[41] S.P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R.L. Leibel, A.W. Ferrante, Obesity is associated with macrophage accumulation in adipose tissue, J. Clin. Invest. (2003), https://doi.org/10.1172/JCI200319246.
[42] H. Wu, S. Ghosh, X.D. Perrard, L. Feng, G.E. Garcia, J.L. Perrard, et al., T-cell accumulation and regulated on activation, normal T cell expressed and secreted up-regulation in adipose tissue in obesity, Circulation (2007), https://doi.org/10.1161/CIRCULATIONAHA.106.638379.
[43] T. McLaughlin, L.F. Liu, C. Lamendola, L. Shen, J. Morton, H. Rivas, et al., T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans, Arterioscler. Thromb. Vasc. Biol. (2014), https://doi.org/10.1161/ATVBAHA.114.304636.
[44] M. Ghaebi, S. Abdolmohammadi-Vahid, M. Ahmadi, S. Eghbal-Fard, S. Dolati, M. Nouri, et al., T cell subsets in peripheral blood of women with recurrent implantation failure, J. Reprod. Immunol. (2019), https://doi.org/10.1016/j.jri.2018.11.002.
[45] G. Sebastiani, F.A. Grieco, I. Spagnuolo, L. Galleri, D. Cataldo, F. Dotta, Increased expression of microRNA miR-326 in type 1 diabetic patients with ongoing islet autoimmunity, Diabetes Metab. Res. Rev. (2011), https://doi.org/10.1002/dmrr.1262.

[46] X.G. Sun, J.H. Tao, N. Xiang, X.M. Li, G.S. Wang, X. Fang, et al., Negative correlation between miR-326 and Ets-1 in regulatory t cells from new-onset SLE patients, Inflammation (2016), https://doi.org/10.1007/s10753-016-0312-8.
[47] Y.-F. Tang, Y. Zhang, X.-Y. Li, C. Li, W. Tian, L. Liu, Expression of miR-31, miR-125b-5p, and miR-326 in the adipogenic differentiation process of adipose-derived stem cells, Omics A J. Integr. Biol. (2009), https://doi.org/10.1089/omi.2009.0017.
[48] X.F. Pei, L.L. Cao, F. Huang, X. Qiao, J. Yu, H. Ye, et al., Role of miR-22 in intestinal mucosa tissues and peripheral blood CD4+ T cells of inflammatory bowel disease, Pathol. Res. Pract. (2018), https://doi.org/10.1016/j.prp.2018.04.009.
[49] S. Kohlhaas, Oa Garden, C. Scudamore, M. Turner, K. Okkenhaug, E. Vigorito, Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells, J. Immunol. Baltimore, Md 1950 (2009), https://doi.org/10.4049/jimmunol.0803162.
[50] F.J. Ortega, M. Moreno, J.M. Mercader, J.M. Moreno-Navarrete, N. Fuentes-Batllevell, M. Sabater, et al., Inflammation triggers specific microRNA profiles in human adipocytes and macrophages and in their supernatants, Clin. Epigenetics (2015), https://doi.org/10.1186/s13148-015-0083-3.
[51] E. Karkeni, J. Astier, F. Tourniaire, M. El Abed, B. Romier, E. Gouranton, et al., Obesity-associated inflammation induces microRNA-155 expression in adipocytes and adipose tissue: outcome on adipocyte function, J. Clin. Endocrinol. Metab. (2016), https://doi.org/10.1210/jc.2015-3410.
[52] L. Dong, X. Wang, J. Tan, H. Li, W. Qian, J. Chen, et al., Decreased expression of microRNA-21 correlates with the imbalance of Th17 and Treg cells in patients with rheumatoid arthritis, J. Cell. Mol. Med. 18 (11) (2014) 2213–2224, https://doi.org/10.1111/jcmm.12353.
[53] M. Feuerer, L. Herrero, D. Cipolletta, Fat Treg cells: a liaison between the immune and metabolic systems, Nature (2009), https://doi.org/10.1038/nm.2002.
[54] M. Feuerer, L. Herrero, D. Cipolletta, A. Naaz, J. Wong, A. Nayer, et al., Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters, Nat. Med. (2009), https://doi.org/10.1038/nm.2002.
[55] J. Deiuliis, Z. Shah, N. Shah, B. Needleman, D. Mikami, V. Narula, et al., Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers, PLoS One (2011), https://doi.org/10.1371/journal.pone.0016376.
[56] M.A. Gavin, T.R. Torgerson, E. Houston, P. DeRoos, W.Y. Ho, A. Stray-Pedersen, et al., Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development, Proc. Natl. Acad. Sci. U. S. A. 103 (17) (2006) 6659–6664, https://doi.org/10.1073/pnas.0509484103.
[57] C.X. Lü, R. Xu, M. De Cao, G. Wang, F.Q. Yan, S.S. Shang, et al., FOXP3 demethylation as a means of identifying quantitative defects inregulatory T cells in acute coronary syndrome, Atherosclerosis (2013), https://doi.org/10.1016/j.atherosclerosis.2013.05.007.
[58] K.G. Schmetterer, A. Neunkirchner, W.F. Pickl, Naturally occurring regulatory T cells: markers, mechanisms, and manipulation, FASEB J. 26 (6) (2012) 2253–2276, https://doi.org/10.1096/fj.11-193672.
[59] E.K.A. Salazar, A. Cortés-Hernández, G.R. Alemán-Muench, J. Alberú, J.R. Rodríguez-Aguilera, F. Recillas-Targa, et al., Methylation of FOXP3 TSDR underlies the impaired suppressive function of tregs from long-term belatacept-treated kidney transplant patients, Front. Immunol. (2017), https://doi.org/10.3389/fimmu.2017.00219.
[60] N. Inoue, M. Watanabe, M. Morita, R. Tomizawa, T. Akamizu, K. Tatsumi, et al., Association of functional polymorphisms related to the transcriptional level of FOXP3 with prognosis of autoimmune thyroid diseases, Clin. Exp. Immunol. (2010), https://doi.org/10.1111/j.1365-2249.2010.04229.x.
[61] F. Naderi-Mahabadi, S. Zarei, R. Fatemi, K. Kamali, Z. Pahlavanzadeh, M. Jeddi-Tehrani, et al., Association study of forkhead box P3 gene polymorphisms with unexplained recurrent spontaneous abortion, J. Reprod. Immunol. (2015), https://doi.org/10.1016/j.jri.2015.04.001.