Differentiation of circulating monocytes into macrophages with metabolically activated phenotype regulates inflammation in dyslipidemia patients.

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September 13, 2021

Introduction

Dyslipidemia is one of the main modifiable risk factors for the development of cardiovascular diseases (CVD), being the ischemic heart disease the leading cause of mortality in the world. Every year, more people die from CVD than from any other reason, according to data from the World Health Organization, it is estimated that 17.9 million people died from this cause in 2019, which represents 32% of all registered deaths in the world . In addition to high blood pressure, diabetes, obesity, and smoking, dyslipidemia is one of the main cardiovascular risk factors. The latter it is defined as disorders in blood lipids characterized by an increase in cholesterol and/or triglyceride levels called hypercholesterolemia and triglyceridemia, respectively .

While most of the triglyceride and cholesterol content is obtained from dietary sources, *de novo* lipogenesis contributes significantly to serum lipid content in people who have a high-carbohydrate diet. The metabolic pathways by which the macromolecules obtained through the diet are processed, such as glycolysis, Krebs cycle, oxidative phosphorylation, beta oxidation, among others, have as their main function the generation of energy, and an imbalance in these can promote pathological processes, such as dyslipidemia . These metabolic pathways not only provide energy for cellular homeostasis, but also control immune cell functions. Immune cells at rest, use processes such as Krebs cycle and oxidative phosphorylation for ATP generation, but cells with pro-inflammatory phenotype such as M1 macrophages and activated T lymphocytes tend to change to aerobic glycolysis, while M2 macrophages and regulatory T lymphocytes induced in the periphery continue with oxidative phosphorylation. Reprogramming of the metabolic state of immune cells influences the generation of epigenetic changes which lead to functional changes. This cellular metabolic state is affected by systemic metabolism, either by nutrients availability or by signalling pathways induced by each metabolite . These concepts are the basis of innate immunological memory, this phenomenon, also called immune training, is defined by metabolic changes originated after priming with pathogens or sterile stimuli that lead to sustained functional changes orchestrated mainly by epigenetic reprogramming, which are sustained changes in gene expression and cellular physiology, which does not imply permanent changes .

An immune cell type that attracts more attention in the immunometabolism area is the macrophages population. Macrophages are phagocytic cells of innate immunity with a broad functional spectrum, from pro-inflammatory to anti-inflammatory phenotypes representing the extremes. Monocytes, cells that develop from bone marrow precursors, travel in bloodstream for a few days, then they migrate to tissue and become macrophages with different phenotypes . Tissue-resident macrophages are long-lived cells derived

mostly from erythro-myeloid progenitors that emerge from the yolk sac . The first to emerge are the primitive macrophages, which are not derived from monocytes and seed every tissue. When erythro-myeloid progenitors seed the fetal liver, they generate fetal monocytes that differentiate into macrophages, and represent the most abundant tissue-resident macrophage population . Furthermore, monocytes derived from hematopoietic stem cells emerge from the fetal liver and differentiate into long-lived macrophages, while adult hematopoiesis begins in the bone marrow. Bone marrow -derived monocytes contribute to the different populations of postnatal tissue resident macrophages .

Monocytes/ macrophages are recognized because their important roles in regulating homeostasis and immune defense through their inflammatory or tissue repair properties . The importance of metabolism in immune cells for the programming of macrophages with their different functional spectra suggests that metabolic pathways may play a role for long-term functional changes in monocytes and macrophages during immune training . The role of these cells is widely described in obesity, being the main population present in the adipose tissue stromal vascular fraction, where there is an increase in the proliferation of macrophages coupled with the recruitment of circulating monocytes to this tissue. Due to the production of cytokines such as IL-1β, IL-6 and TNF-α, M1 macrophages participate in the low-grade chronic inflammation that characterizes obesity .

In past years, it has been shown that when treating monocyte derived macrophages with high concentrations of insulin, glucose and palmitate, characteristic of metabolic syndrome, a different pro-inflammatory phenotype is induced. These cells present surface markers and transcription factors different from classical macrophages and were called metabolically activated macrophages (MMe) . MMe present surface molecules such as CD36, which binds to long chain fatty acids and facilitates their transport in the cell, participating in the use of lipids in muscle, storage of adipose energy and absorption of intestinal fat ; ABCA1 is a cholesterol efflux pump in the elimination pathway of cellular lipids that are then collected by apoA-I, forming high-density lipoproteins (HDL) ; and PLIN2, which is a protein expressed on the lipid droplet membrane . These MMe have been described in metabolic syndrome and have been found in adipose tissue during obesity, performing beneficial and detrimental functions during diet-induced obesity in mice , and in mammary adipose tissue promoting tumorigenesis during obesity . MMe produce pro-inflammatory cytokines, although in a lesser extent than classic M1 macrophages. The expression of their characteristic surface markers, as well as the attenuated inflammatory response, is mainly mediated by the transcription factor PPAR-γ , that could be contributing to the chronic low-grade inflammatory state present in metabolic syndrome and obesity.

Due to dietary overload, the metabolites produced by the different metabolic pathways can be used for alternative pathways in organs and tissues, such as adipose tissue, modifying and defining systemic metabolic responses It is not completely clear whether the change from a healthy systemic metabolic state to a pathological one, such as the dyslipidemic state, lead to changes causing immune training influencing polarization to different cell types.

The aim of this study was to evaluate if high cholesterol and triglycerides levels, main feature of dyslipidemia, are promoting immune training in peripheral blood cells, functioning as a first stimulus, conditioning monocytes to present a metabolic phenotype and leading them to polarization into metabolically activated macrophages. We found that monocytes with metabolic phenotype expressing CD36, ABCA1 and PLIN2, are present in systemic circulation. *In vitro* stimulation showed that MMe from patients with dyslipidemia, play a dynamic role with production of pro- and anti-inflammatory cytokines.

Materials and methods

Patients

Thirty-two patients with diagnosis of dyslipidemia were included. Dyslipidemia was defined by lipid serum levels: Cholesterol >200 mg/dl and/or triglycerides >150 mg/dl. All patients were without pharmacological treatment at the time of the study. Twenty-two healthy individuals with normal lipid levels were also included (cholesterol <200 mg/dl and triglycerides <150 mg/dl). All subjects were recruited from the University Health Center of the Universidad Autónoma de San Luis Potosí. None of our included subjects showed

evident infectious or autoimmune disease, previous acute myocardial infarction, hypertension, diabetes or cancer. Clinical data were collected for all patients, including sex, age, and tobacco use.

Clinical and demographic data were collected and are summarized in table 1. The Research Ethics Committee of the Faculty of Stomatology of the UASLP approved this study (CEI-FE-014-019). This work was carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

Peripheral blood mononuclear cells isolation

Human peripheral blood (20 ml) samples were collected from patients and healthy donors in vacutainer tubes (BD Vacutainer tubes with EDTA K2; BD Vacutainer RST tube) through venepuncture; complete blood and serum were obtained. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral venous blood by density gradient centrifugation with Ficoll-Hypaque (Sigma Aldrich). Cellular viability was evaluated by staining with trypan blue. PBMC were used for staining and monocyte isolation.

Monocyte isolation

Monocytes were isolated from PBMC by incubation with anti-CD14 antibody coupled to magnetic microspheres (EasySep Human CD14 Positive Selection Kit II), the tube with the mixture was placed on an EasySep magnet for immunomagnetic positive selection of $CD14^+$ cells (StemCell). Monocytes were used for cell culture and an aliquot stored in RNAlater (Sigma Aldrich) for RNA isolation.

Cell culture

For macrophage differentiation, monocytes were cultured at 37°C with 5% CO_2 in 24-well plates (Costar) for 6 days in supplemented RPMI medium (10% fetal bovine serum, 10nM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin) and 50 ng/ml GM-CSF for M1 macrophages, then incubated for 24 hours with IFN-γ (100 ng/ml) and LPS (100 ng/ml). For MMe and M2 macrophages, monocytes were incubated for 6 days with M-CSF (50 ng/ml), and then incubated for 24 hours with 15mM glucose (Sigma Aldrich), 10nM insulin (Sigma Aldrich) and 0.4mM palmitate (Sigma Aldrich) to polarize to MMe, or with IL-4 (20 ng/ml) to obtain M2 macrophages. Cells were harvested using EDTA and further evaluation by flow cytometry was performed. Culture media were collected for cytokine measures.

Flow cytometry

500,000 to $1x10^6$ PBMC or macrophages were labelled after Fc receptor blockade with 10% human AB serum by 15 minutes. Then, cell surface staining was performed with the following monoclonal anti-human antibodies labelled with different fluorochromes: CD14-FITC (BioLegend), CD80-PE (BioLegend), CD163-PE (BD Pharmigen), CD206-APC (Invitrogen), CD36-PerCp (Novus) and ABCA1-PE (Novus). After incubation and washing, cells were fixed and permeabilized with Foxp3 Fix/Perm kit (eBioscience) and stained with antibodies against CD68-APC (Invitrogen), PLIN2-APC (Novus) or IL-10-PE (BioLegend). Cells were analysed with the FACSCanto II flow cytometer (BD Biosciences, Becton Dickinson) and the FlowJo software (Tree Star, Ashland, OR).

Soluble cytokine quantification

To measure multiple soluble cytokine levels in serum and culture supernatants from dyslipidemia patients and control subjects, we performed a cytometric bead assay (BD CBA, inflammatory cytokine array). Briefly, serum and supernatants samples were incubated with capture beads and detector antibodies; then, sandwich complexes were formed with these reagents and the analyte. These complexes were measured by flow cytometry, according to size and fluorescence characteristics of both the beads and the detector antibody, in an Accuri C6 cytometer using FCAP array software for analyses.

Real-Time Quantitative PCR

To extract total RNA from monocytes, a RNeasy Mini Kit (Qiagen) was used. Once obtained RNA, cDNA was synthesized starting off from 0.5 µg of total RNA using high-capacity cDNA reverse transcription kit

(Applied Biosystems). For real-time qPCR (real-time quantitative polymerase chain reaction), a SYBR Green master kit (Applied Biosystems) was used to evaluate PPAR-γ and β-actin mRNA relative levels in monocytes. Relative PPAR-γ mRNA expression was normalized against endogenous gen β-actin mRNA. The amounts of PPAR-γ mRNA relative to control were calculated with ΔC_T method.

Statistical analysis

Data were analysed with GraphPad Prism, 5.01 software. Data were analysed for normality with the Pearson test, Kolmogorov-Smirnov test, D'Agostino, and Shapiro-Wilk test. Flow cytometry data were evaluated by paired or unpaired Student t-test in case of a normal distribution; otherwise, we used the Mann-Whitney U test or Wilcoxon signed-rank sum-test. In some cases, data were analysed by one-way ANOVA, Friedman test, or two-way ANOVA test. The analysis of correlations between variables was based on Spearman's rank test. $P < 0.05$ was considered statistically significant. The data are shown as mean and SD, or median with interquartile range (IQR).

Results

Identification of circulating monocytes with a metabolic phenotype.

Peripheral blood was obtained from healthy individuals to analyse metabolic markers in circulating monocytes. Cell staining was performed as described in materials and methods. Cells were analysed by multiparametric flow cytometry. We used ABCA1, CD36 and PLIN2 to define the metabolic population (Fig 1A). Interestingly, with this strategy, we proved by the first time the presence in bloodstream of monocytes with metabolic phenotype (MoMe). We could identify two populations according to the ABCA1 and CD36 expression (3.4%, 1.93%-9%, median and IQR) or ABCA1, CD36 and PLIN2 expression (2.05%, 0.95%-4.17%, median and IQR respectively) (Fig 1B). Since a common marker for the study of monocytes is CD14, we decided to evaluate this molecule expression in metabolic monocytes (Fig 1C), we found that in each subject, there are metabolic monocytes with different CD14 expression levels. We found a range from a small population that does not express this marker (6.83%, 3.33%-14.08%median and IQR), to CD14 positive metabolic monocytes with dim (31.05%, 20.48%-40.78%, median and IQR) and high expression, but the most predominant fraction was the one with high expression of CD14 ($p<0.0001$, 58.75%, 43.13%-75.33%, median and IQR).

Healthy individuals and dyslipidemic patients show similar levels of metabolic monocytes.

One common characteristic of obesity and metabolic syndrome is the chronic inflammatory state . It has been documented the macrophages in these pathologies undergo a phenotypic switch from anti-inflammatory to inflammatory phenotype. To determine whether diseases such as dyslipidemia are conditioning circulating monocytes to express metabolic markers, we evaluated the percentages of these cells from subjects with elevated lipid levels (triglycerides >150 mg/dl, total cholesterol >200 mg/dl) and compared them with the percentages from healthy subjects. We evaluated the cell populations with 2 (ABCA1, CD36) and 3 (ABCA1, CD36, PLIN2) phenotypic markers and found that metabolic monocytes percentages do not differ between healthy individuals and those with dyslipidemia (not statistically significant (NS); Fig 2A), and CD14 expression seems not to have influence in this context (NS; Fig 2B). Additionally, relative expression of PPAR-γ mRNA in monocytes from healthy individuals and patients with dyslipidemia was similar (NS; Fig 2A). Furthermore, we did not find a relationship between the percentages of metabolic monocytes and lipid levels (NS; Fig 2C) or body mass index (NS; Fig 2D) in subjects with dyslipidemia.

Dyslipidemia patients display higher levels of pro-inflammatory monocytes than healthy subjects.

We also determined the levels of M1- and M2-like monocytes in healthy subjects and those with dyslipidemia based on the CD14 expression (Fig 3A). There is a higher percentage of M1-like monocytes expressing CD14 high ($^{++}$) ($p= 0.0003$, 45.99% ± 23.23% vs 57.52% ± 26.47%, arithmetic mean and SD; Fig 3B), and a higher percentage of M2-like monocytes expressing CD14 dim ($^{+}$) in the subjects with dyslipidemia compared to the control group ($p <0.0001$, 3.8% ± 6.67% vs 3.66% ± 2.22%, arithmetic mean and SD; Fig 3C). When

evaluating the $CD14^{++}CD68^{+}CD80^{+}$and $CD14^{+}CD163^{+}CD206^{+}$populations, we found higher percentages of pro-inflammatory monocytes in the dyslipidemia patients (p= 0.0355, 51.65%, 60.80%-26.53% vs 28%, 39.13%-13.63%, median and IQR; Fig 3B). However, no differences between M2-like monocytes from healthy and dyslipidemia groups, were found (p= 0.5383; Fig 3C).

Metabolic stimuli promote differentiation to metabolic, classical, and alternative macrophages.

We aimed to investigate the effects of high concentrations of molecules such as, glucose, insulin, and palmitate, in the polarization of macrophages into different phenotypes. Once the monocytes were purified from healthy and dyslipidemia subjects, cells were incubated with the metabolic stimuli mentioned above, dyed, and analysed by flow cytometry (Fig 4A). We obtained different macrophages subsets such as M1 ($CD14^{+}CD68^{+}CD80^{+}$), M2 ($CD14^{+}CD163^{+}CD206^{+}$) and MMe ($CD14^{+}ABCA1^{+}CD36^{+}PLIN2^{+}$), being M1 the most abundant subset among the polarized cells (p= 0.0004; Fig 4B).

Higher differentiation of circulating monocytes into metabolically activated macrophages (MMe) in dyslipidemia.

Since the population of MMe has been recently described, there is of interest to consider different subpopulation levels based on the expression of one, two or three metabolic markers. When we compared MMe obtained from culture from dyslipidemia and from healthy subjects, we found that the percentage of differentiated MMe from the group with dyslipidemia is higher than the percentage from the control group (Fig 5A) when macrophages were evaluated with 2 (p= 0.0249, 2.71%, 2.05%-3.31% vs 3.41%, 2.3%-5.08%, median and IQR) or 3 metabolic markers (p= 0.0340, 2.08%±1.14% vs 2.88%±1.35%, arithmetic mean and SD). Moreover, in dyslipidemia subjects, we observed a positive correlation between the BMI and the percentage of MMe (p= 0.0401, r= 0.4217; Fig 5B). Nevertheless, there was no relationship between the monocyte derived-MMe percentage and blood lipid levels either in dyslipidemia patients (NS; Fig 5C) or control subjects. Despite an expected relationship between ABCA1 median fluorescence intensity (MFI) and HDLc levels (Fig 5D), no significant correlation was observed between these parameters in dyslipidemia subjects (p= 0.9133). Interestingly, there was a positive correlation between ABCA1 MFI and LDLc concentrations (p= 0.0019, r= 0.5897; Fig 5D). Regarding macrophages M1 and M2 monocytes differentiated in presence of GM-CSF, LPS and IFN-γ or M-CSF and IL-4, respectively, we found no difference in the percentages of neither M1 nor M2 macrophages, between controls and patients with dyslipidemia (NS; Fig 5E).

Metabolically activated macrophages from patients and controls produce equal amounts of cytokines.

To evaluate the MMe function, we recollected supernatants from the macrophage cultures treated with metabolic stimuli in order to determine concentration of different cytokines. First, we compared the cytokines levels produced by pro-inflammatory macrophages M1 and MMe. In the control group, we found higher levels of IL-12 (p= 0.0156, 0.97%, 0.33%-1.67% vs 2.07%, 1.71%-2.15%, median and IQR; Fig 6A) in the MMe supernatants, while IL-1β, IL-6, IL-8, IL-10 and TNFα were equally produced by MMe and M1 macrophages (data not shown). Interestingly, in dyslipidemia patients (Fig 6B), we found a higher concentration of IL-10 in the MMe supernatants (p= 0.0391, 4.21%, 2.4%-4.61% vs 56.33%, 2.98%-104%, median and IQR) and higher IL-6 levels in M1 supernatants (p= 0.0156, 390.8%, 1726%-55.84% vs 273.5%, 570.8%-31.3%, median and IQR). Since it is known that when culturing monocytes with metabolic stimuli there is also differentiation not only towards MMe, but also towards M1 and M2 macrophages, we evaluated the intracellular expression of IL-10 by flow cytometry in MMe to corroborate that these cells, in fact, produce IL-10 (85.8%, 82.5%-86.3%, median and IQR; Fig 6B). When we evaluated cytokine concentrations, produced by MMe from healthy and dyslipidemia subjects, we did not observe differences between groups (NS, Fig 6C). Additionally, we did not find differences among the cytokine concentrations in serum samples from healthy and dyslipidemia subjects, with the exception of IL-12 (p= 0.0003, 0.43%, 0.52%-0.12% vs 3.12%, 4.05%-2.63%, median and IQR, Fig 6D), that was higher in the group with dyslipidemia.

Discussion

Dyslipidemia is an important public health problem in industrialized countries, great importance due to its contribution to the development of cardiovascular diseases . Dyslipidemia can have different presentations, whether there is a high level of triglycerides, a high level of total cholesterol, HDL cholesterol, LDL cholesterol, or any combination of high serum lipid levels . Systemic metabolism, in addition to storing and providing energy to the body, influences cellular metabolism. Immune cells, after stimulation by some antigens, suffer metabolic changes that directly influence their functional spectrum. In innate immune cells, the metabolic changes after activation lead to epigenetic changes that condition cells to respond differently to a second stimuli, either enhancing or attenuating their functional phenotype, a physiological condition known as immune training .

In metabolic diseases such as dyslipidemia, monocyte phenotype may be shaped by high blood lipid levels. We hypothesized that elevated serum levels of cholesterol and triglycerides observed in patients with dyslipidemia, could induce the expression of metabolic markers in circulating monocytes, providing them with a phenotype similar to the recently described metabolic activated macrophages onocytes are cells with a short half-life in circulation of around 1 day; however, studies suggest reprogramming can take place at the level of progenitor cells in the bone marrow trained monocytes have been observed 3 months after priming .

It has been reported that the phenotype of metabolic monocytes was $CD36^{+}$ $ABCA1^{+}$; however, in that study the phenotype was missing PLIN2 and CD14 markers, and the metabolic markers were evaluated individually, with possible overlap with other subpopulations such as M2. This is the first study reporting circulating monocytes with the complete metabolic phenotype (MoMe) in healthy subjects. This population correspond to approximately 3.4% of total monocytes, with the majority of these cells showing high CD14 expression. This could be related to its pro-inflammatory functional spectrum because its similarities, at least in terms of CD14 expression, to the intermediate monocyte phenotype (CD14high CD16low), to which proinflammatory properties are attributed .

When evaluating the percentages of the different populations of circulating monocytes, we found that healthy subjects showed similar levels of MoMe and M2-like monocytes, which are predominantly $CD14^{+}$ (dim). Regarding the M1-like monocytes, predominantly $CD14^{++}$ (high), higher frequencies were found in dyslipidemia subjects. This is in accordance with the literature, as hypercholesterolemia induces loss of hematopoietic stem and progenitor cells quiescence and enhances the propensity to generate pro-inflammatory monocytes . We consider that the levels of MoMe may not be influenced by high levels of lipids since the effect of this metabolic alteration has an impact on epigenetic mark and not necessarily in the expression of metabolic receptors. In innate immune cells that are subjected to a stimulus, whether infectious or sterile, undergo long-term metabolic, transcriptional, and epigenetic reprogramming . It is when these cells are subjected to a secondary stimulus, equal to or different from the first stimulus, when they present a greater response capacity, for example, increased proliferation, receptors expression and cytokines production . We need further studies to evaluate the possible epigenetic modifications, including levels of histone acetylation and methylation, in order to clarify this hypothesis.

It is well known that patients with complicated metabolic diseases such as diabetes mellitus 2 or metabolic syndrome, are characterized by a pro-inflammatory state where molecules as glucose, insulin, and palmitate, are elevated, regulating inflammation and with M1 macrophages playing a key role preserving the inflammatory environment . However, it has been proposed that these metabolites also promote the presence of metabolically activated macrophages (MMe), which display a different phenotype from the classically activated (M1) .

Since monocytes from dyslipidemia subjects are exposed to high triglycerides and cholesterol levels, we hypothesize that such condition serves as a priming stimuli driving macrophages stimulated in vitro with high concentrations of glucose, insulin, and palmitate to a stronger polarization into the MMe phenotype.

In dyslipidemia patients and healthy subjects, metabolic stimuli promote polarization into pro-inflammatory MMe, pro-inflammatory M1 and anti-inflammatory M2 macrophages, being M1 the most abundant polarized subset. This was expected free fatty acids (FFA) such as palmitat (the most prevalent dietary FFA, induce

metabolic inflammation via TLR4 through NF-kB, MAPK and PI3K, and induce proinflammatory cytokines production such as IL-6, TNF-α, IL-8, and IL-1β .

While no difference was observed between the percentages of MoMe comparing control and dyslipidemic subjects, *in vitro*differentiated monocyte-derived MMe percentages are higher in dyslipidemia than in the control group. This finding suggests, accordingly with our hypothesis, that elevated triglycerides and cholesterol levels serve as the first stimulus inducing immune training in monocytes, which after *in vitro* stimulation with glucose, insulin and palmitate in high concentrations, respond differently to those not exposed to elevated lipids in circulation.

Furthermore, in dyslipidemic conditions, the higher the body mass index (BMI) the higher the MMe percentage. It has been previously reported that macrophages from adipose tissue, show a positive correlation between CD36 expression and BMI . Nevertheless, this is the first work reporting the correlation between monocyte-derived MMe (full phenotype) frequencies and BMI.

ABCA1, which is present in the plasma membranes of cells such as monocytes/ macrophages, participates in cholesterol efflux donating it to ApoA1 to form HDL, inducing anti-inflammatory signaling . In , we did not find a relationship between serum HDL concentrations and ABCA1 expression, but we found a direct association between LDL concentrations and ABCA1 expression. This finding suggests that the differentiation into MMe may be a result of the metabolic impairment and are not associated in a direct manner to the metabolism of lipids. However, we observed that high levels of LDLc are related to a greater expression of ABCA1 in MMe, suggesting a high cholesterol efflux and HDL formation. The above, could indicate a beneficial role of this cell population in late stages of dyslipidemia. It would be interesting to evaluate molecules such as SIRT6, involved in the expression of ABCA1 .

Regarding the MMe function, we confirm that although they produce pro-inflammatory cytokines such as IL-6 , they do so to a lesser extent than classic M1 macrophages in subjects with dyslipidemia. Interestingly, we also found that these MMe produce IL-10, a cytokine with potent anti-inflammatory and regulatory activities , and that their production is higher in MMe compared to M1. Regarding the polarization to classic M1 and alternative M2 macrophages, we found no differences between healthy people and those with dyslipidemia; in this sense, Baardman and colleagues have shown that hypercholesterolemia alters macrophage metabolism and phenotype, attenuating the inflammatory phenotype in M1 macrophages.

Our results support the idea that pre-existing metabolic alterations such as elevated serum lipid levels, condition monocytes to differentiate into a specific phenotype after a second stimulus. This is supported by our results from our *in vitro* model where the second stimulus corresponds to the combination of high concentrations of glucose, insulin and palmitate. We observed a preferent polarization of monocytes into MMe in dyslipidemia group compared to healthy subjects. Interestingly, we observed that MMe from dyslipidemia patients produce higher levels of the regulatory cytokine IL-10. It has been previously described that MMe have a dynamic role in obesity (18). MMe have an important role in controlling metabolic alterations of high-fat diet fed mice at early stages, however, even when maintaining their metabolic markers expression, their function becomes detrimental at advanced stages of obesity. It is clear that our experimental approach focuses in monocyte derived macrophages and not in adipose tissue associated macrophages. However, the production of IL-10 as a regulatory mechanism by monocyte derived MMe results of great relevance. In this regard, it is important to point out that our study was carried out in young people with dyslipidemia, who did not present other comorbidities. For this reason, we cannot predict whether metabolically activated monocytes/ macrophages would have a different behavior pattern in subjects with a longer time of evolution of the disease, different metabolic alterations or in the presence of other comorbidities. To elucidate if the chronic exposure of circulating metabolic monocytes to high concentrations of metabolites (such as glucose or free fatty acids) leads to a different functional profile, further follow-up studies should be carried out.

In conclusion, this is the first study in which monocytes with metabolic phenotype (expressing CD36, ABCA1 and PLIN2) in systemic circulation are described by multiparametric analysis. Our study highlights the dual role of metabolically activated macrophages, which regulate the pro-inflammatory milieu characteristic of

dyslipidemia through the production of IL-6 and IL-10.

Acknowledgements

We thank the University Health Center from the Universidad Autónoma de San Luis Potosí. This work was supported by CONACyT.

Disclosure

The authors declare that they have no disclosures related to this manuscript.

Author contributions

All authors were involved in drafting or revising the article, and all authors approved the final version to be published.

Data sharing and data accessibility

All data pertinent to this article are included herein.

References

FIGURE LEGENDS

Fig 1. Circulating monocytes express metabolic markers. Peripheral blood was obtained from healthy and dyslipidemic individuals to analyse metabolic markers in circulating monocytes. (A) Cells were analysed by multi-parametric flow cytometry. (B) Bloodstream monocytes with metabolic phenotype (MoMe) levels according to ABCA1, CD36 (n= 19) and PLIN2 (n= 20) expression in healthy subjects. (C) CD14 expression in metabolic monocytes. Data shown in panels B and C had a non-Gaussian distribution and correspond to the median and Q1 and Q3. Groups in panel C were compared using one-way ANOVA (Friedman test). *p < 0.05, **p < 0.01, ***p < 0.001.

Fig 2. Metabolic monocytes in healthy individuals and in patients with dyslipidemia. Bloodstream MoMe percentages from subjects with elevated lipid levels (triglycerides >150 mg/dl, total cholesterol >200 mg/dl) and healthy subjects. (A) MoMe percentages evaluated with 2 (n= control 19, dyslipidemia 31) and 3 (n= control 20, dyslipidemia 32) metabolic markers. Relative expression of PPAR-γ mRNA in healthy individuals (n= 4) and in patients with dyslipidemia (n= 8). (B) Different MoMe subsets according to their CD14 expression, divided into CD14 negative (n= control 20, dyslipidemia 31), dim (n= control 19, dyslipidemia 31) and high (n= control 19, dyslipidemia 31) expression. (C) Correlations between metabolic monocytes percentages and total cholesterol (n= 29) or triglycerides (n= 29) or body mass index (D, n= 27) in subjects with dyslipidemia. All data shown had a non-Gaussian distribution and correspond to the median and Q1 and Q3. Groups were compared using Mann-Whitney test in panel A and B. Spearman r analysis was performed in panel C. Linear regression was performed. P value represents the likelihood of a nonzero slope.

Fig 3. Evaluation of M1- and M2-like monocytes levels in healthy subjects and in patients with dyslipidemia. (A) Using flow cytometry, starting from monocytes gate defined by FSC and SSC parameters, doble cells were eliminated and then, CD14 expression was evaluated. (B) CD14 expression in M1-like monocytes (n=26) from dyslipidemia subjects and $CD14^{++}CD68^{+}CD80^{+}$comparation between patients (n= 26) and controls (n= 8). (C) CD14 expression in M2-like monocytes from subjects with dyslipidemia (n=26) and $CD14^{+}CD163^{+}CD206^{+}$comparation between patients (n= 26) and controls (n= 8). Data shown in bars graph B had a non-Gaussian distribution and correspond to the median and Q1 and Q3. Data shown in individual values graphs and bars graph C had a normal distribution and correspond to the arithmetic mean and SD. Groups were compared using paired t test, Mann-Whitney test, or unpaired t test. *p < 0.05, ***p < 0.001, ****p < 0.0001

Fig 4. Metabolic stimuli promote differentiation to metabolically activated (MMe), classical (M1), and alternative macrophages (M2). Monocytes were purified from healthy and dyslipidemia

subjects, cells were incubated with metabolic stimuli, dyed, and analysed by flow cytometry for the expression of characteristic polarization markers, M1 ($CD14^+CD68^+CD80^+$), M2 ($CD14^+CD163^+CD206^+$) and MMe ($CD14^+ABCA1^+CD36^+PLIN2^+$). (A) Cell culture conditions and MMe gating strategy. (B) Comparison of MMe, M1 and M2 percentages, obtained from the incubation with metabolic stimuli of monocytes from dyslipidemia patients (n= 9) and control subjects (n= 6). Groups were compared using two-way repeated measures ANOVA. *p < 0.05, ***p < 0.001

Fig 5. Metabolically activated (MMe), classical (M1), and alternative (M2) macrophage differentiation in dyslipidemia and healthy subjects. MMe (glucose, insulin, palmitate), M1 (IFN-γ, LPS) and M2 (IL-4) macrophages obtained from culture from dyslipidemia and healthy subjects. (A) MMe comparison between control and dyslipidemia subjects evaluated with 2 (n= control 21, dyslipidemia 28; median and IQR) and 3 (n= control 21, dyslipidemia 28; arithmetic mean and SD) metabolic markers. (B) Correlation between MMe percentages and body mass index (n= 24). (C) Correlation between MMe percentages and triglycerides (n= 27), total cholesterol (n= 27), HDLc (n= 27) and LDLc (n= 26). (D) ABCA1 median fluorescence intensity compared with HDLc (n= 27) and LDLc (n= 25). (E) Monocyte-derived M1 (n= control 9, dyslipidemia 23) and M2 (n= control 9, dyslipidemia 22) macrophages in control and dyslipidemia subjects. Groups were compared using Mann-Whitney test or unpaired t test. Spearman r analysis was performed. Linear regression was performed. P value represents the likelihood of a nonzero slope. *p < 0.05

Fig 6. Cytokine production by macrophages from dislypidemic patients and healthy controls. Supernatants from polarization cultures with metabolic stimuli or LPS were collected and analysed by flow cytometry using a Cytometric Bead Array kit. (A) IL-12 quantification in M1 and MMe cell culture supernatants from healthy subjects (n= 7). (B) IL-10 (n= 8) and IL-6 (n= 7) quantification in M1 and MMe cell culture supernatants from patients. Representative plot of flow cytometry (n= 3) for $CD14^+CD36^+PLIN2^+$macrophages expressing IL-10 (red) compared with undyed control (blue). (C) Cytokine concentrations produced by MMe from healthy and dyslipidemia subjects. (D) IL-12 quantification in serum from control (n= 7) and dyslipidemia subjects (n= 13). Data shown in A, B and D had a non-Gaussian distribution and correspond to the median and Q1 and Q3. Groups were compared using Wilcoxon matched-pairs signed rank test or Mann-Whitney test. Groups were compared using two-way ANOVA. *p < 0.05, ***p < 0.001

TABLES

Table 1. Demographic and clinical data of dyslipidemia patients and control subjects

	Dyslipidemia	**Control**	**p**
n	32	22	-
Age in years (mean±SD)	19.5, 25.5-18	24, 28-18	NS
Sex (F/M)	M: 23, F: 9	M: 15, F: 7	-
Total cholesterol (mg/dl)	208.5, 223.3-201	158.5, 186.5-141	<0.0001
Triglycerides (mg/dl)	192.5, 240.8-119.8	102, 129.3-70.75	<0.0001
HDLc (mg/dl)	44.3, 62.2-35.43	50.4, 68.98-41.7	0.1628
LDLc (mg/dl)	125.5, 135.7-106.9	80.35, 113.8-64.3	0.0005

Data correspond to median and Q3-Q1. Abbreviations: F, female; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; M, male; mg/dl, milligram/deciliter; NS, not statistically significant.

FIGURES

Figure 1

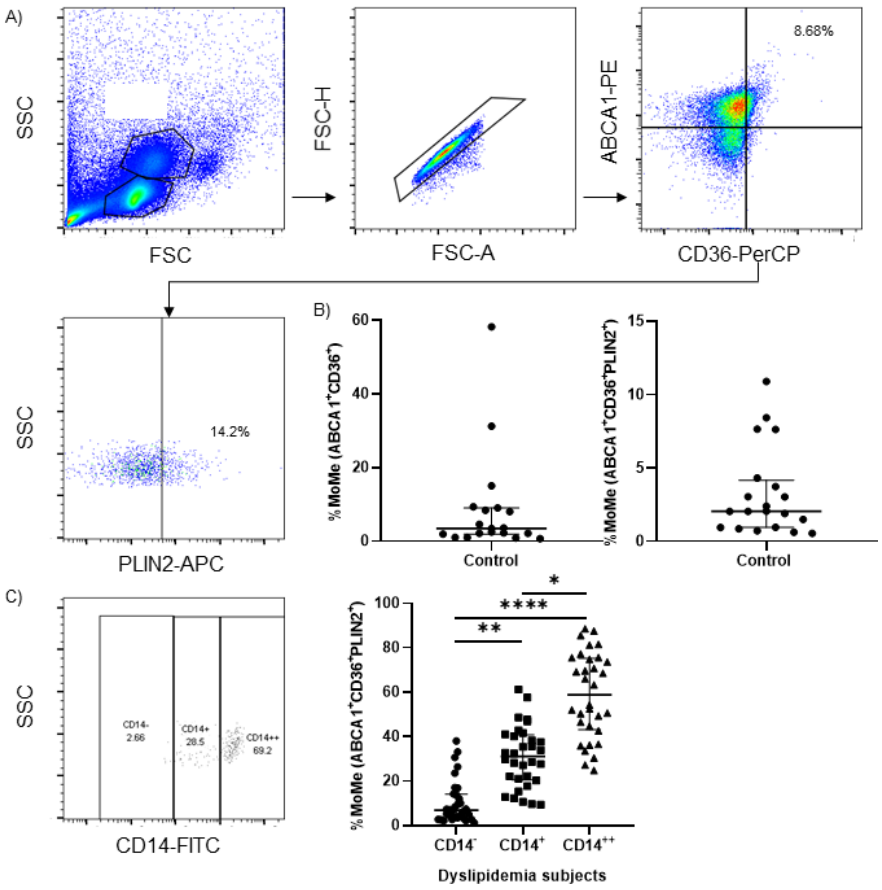

Figure 2

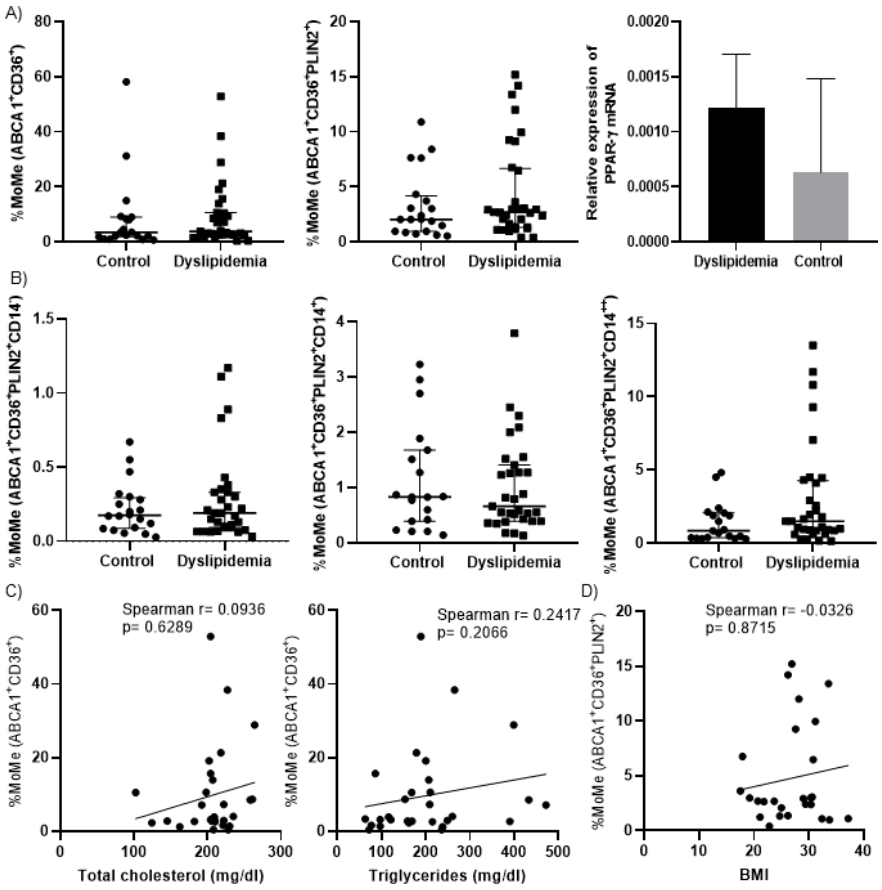

Figure 3

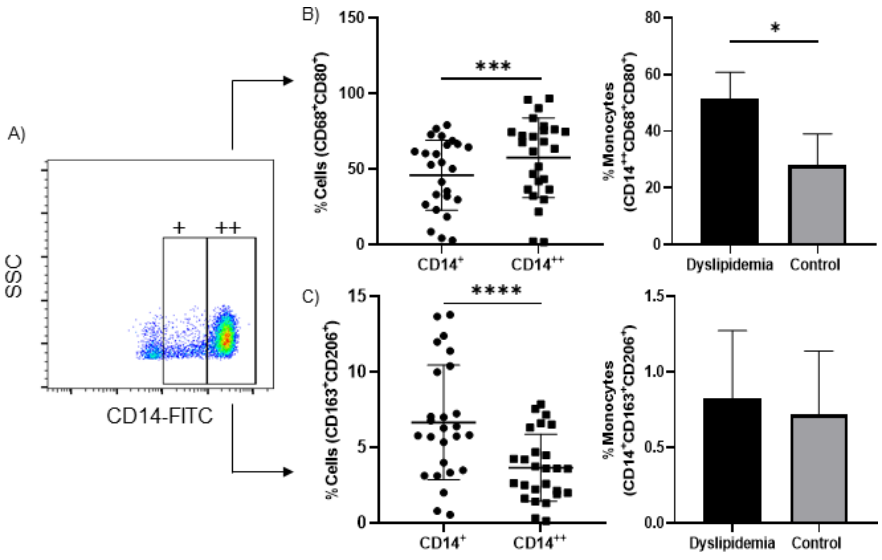

Figure 4

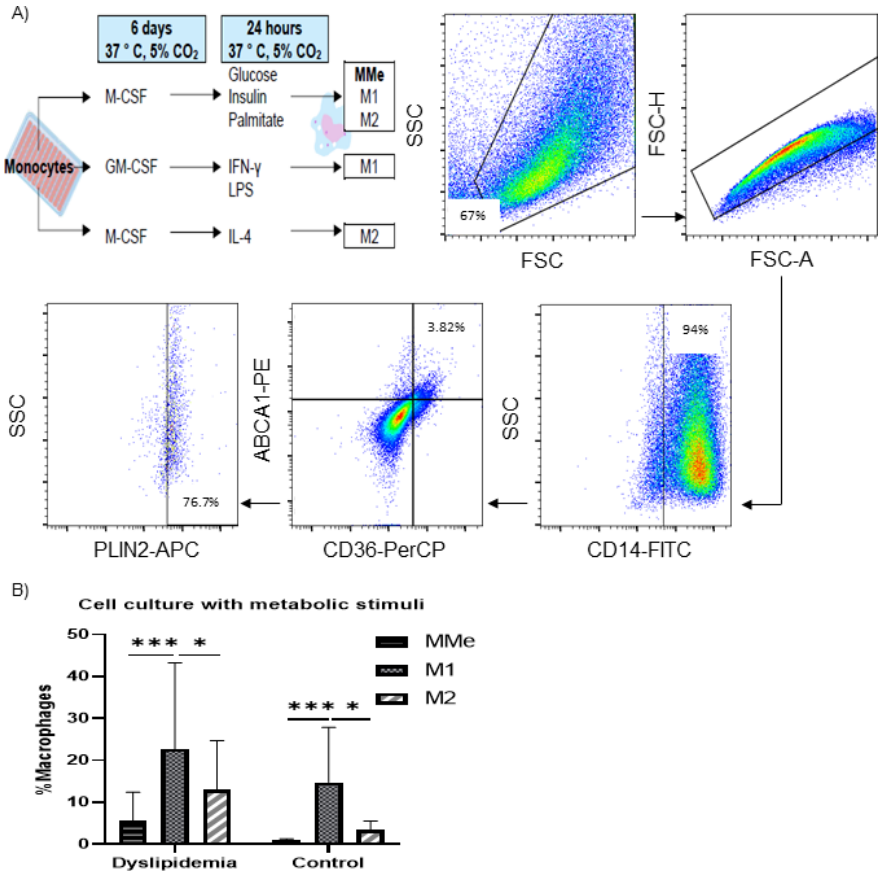

Figure 5

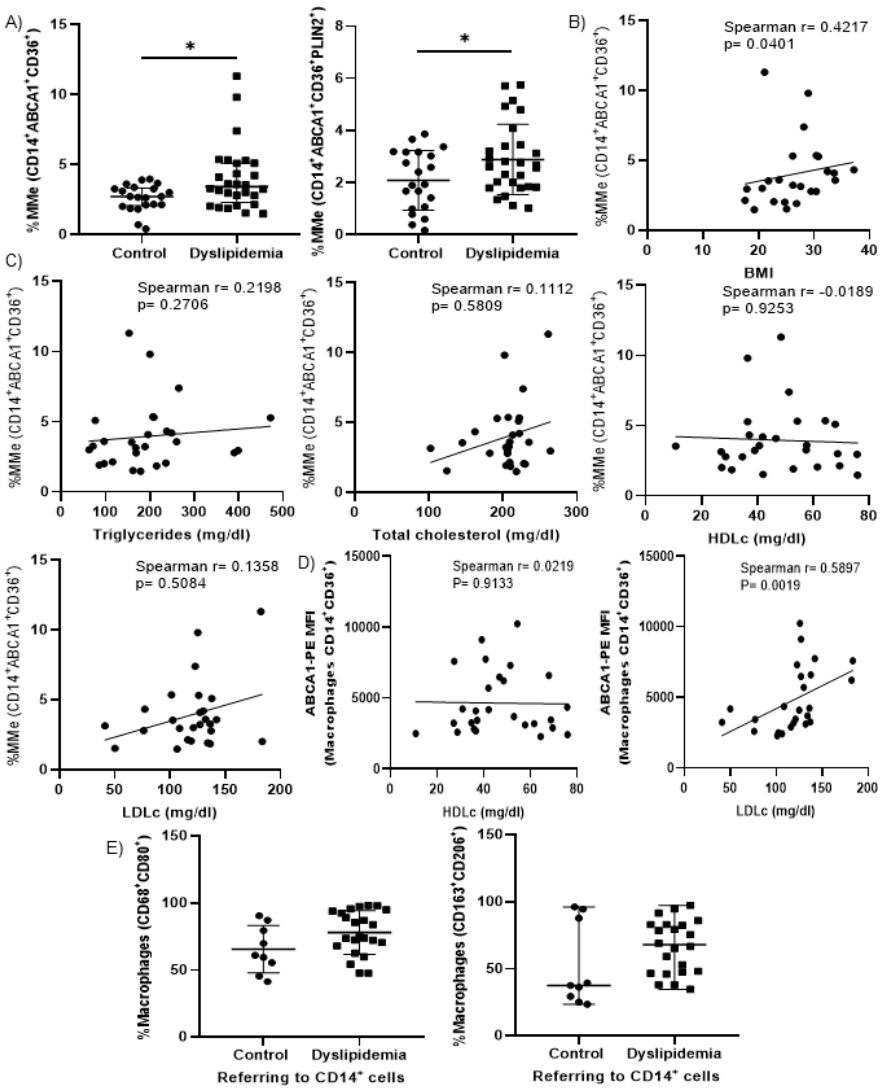

Figure 6