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SELECTIVE TARGETING OF INFLAMMATORY FUNCTIONS OF FAT-ACTIVATED MACROPHAGES FOR THE TREATMENT OF OBESITY-ASSOCIATED TYPE-2 DIABETES

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1. Summary

Inflammatory adipose tissue macrophages play a key role in obesity-associated pathologies, including type 2 diabetes. Previous evidence supports the idea that macrophage functional reprogramming could be used to treat these diseases. However, these potential approaches are currently limited by the lack of treatments that inhibit inflammatory macrophages in adipose tissue without disrupting beneficial homeostatic functions of tissue macrophages in this and other tissues. Our hypothesis is that inflammatory macrophages in fat adipose tissue have specific transcriptional profiles that distinguish them from other inflammatory macrophages, and that these differences can be exploited to design strategies to selectively inhibit or reprogram pathogenic adipose tissue macrophages. The main objective of the project has been the identification of the specific inflammatory gene signature of human macrophages activated by fat, so that this gene signature can be used for the design of protocols that make it possible to specifically inhibit its pathogenic effect in obesity. We also planned to develop a mouse obesity model to analyze in vivo the inflammatory functions and pharmacological sensitivity of human macrophages. Our results in macrophages acutely exposed in vitro to the fatty acid palmitate, characteristically enriched in the environment of obese visceral fat, have identified gene profiles associated with inflammatory and metabolic processes, and sensitive to anti-inflammatory modulators. Likewise, the analysis of macrophage populations resident in healthy and obese visceral adipose tissue in a mouse model has allowed the identification of new gene profiles, not directly associated with inflammation, of macrophages in a context of chronic obesity in vivo. These results could promote a more precise knowledge about the pathogenesis of obesity, and help improve therapeutic strategies for the treatment of associated pathologies.

2. Results

1*. Determination of the transcriptome of blood-derived human macrophages exposed to palmitate in vitro, and comparative analysis of inflammatory responses to palmitate in human and mouse macrophages*.

Data in the literature have established that obese adipose tissue presents high concentrations of the fatty acid palmitate, which induces a state of pro-inflammatory activation in macrophages resident in this tissue. This activation towards a chronic state of low-grade inflammation exacerbates obesity-associated pathologies, including the risk of type 2 diabetes.

Our analysis has identified genes specifically regulated by palmitate in human macrophages in vitro, among them genes induced by XBP1, which promotes the stress response "Unfolded Protein Response" (UPR), and the inhibition of genes, such as IL10, regulated by the MAF factor.

We have observed a similar result of XBP1 activation by palmitate in bone marrow-derived mouse macrophages, as well as in macrophages resident in mouse visceral adipose tissue. In this section we have also identified a common effect of palmitate on human and mouse macrophages in the induction of pro-inflammatory cytokines genes such as *TNF*, *IL6*, *IL1b* and the repression of the anti-inflammatory cytokine *IL10*.

The results of this section lend support to the concept that the inflammatory and stress response to palmitate is essentially conserved between human and mouse macrophages, which suggests the usefulness of developing in vivo approaches in mouse models.

We have also performed RNAseq experiments on human macrophages derived from monocytes and exposed to LPS, CL264 (TLR7 ligand) or palmitate, for 30 minutes, 2 hours, 4 hours or 12 hours. The raw data from these analyses have already been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) with access number GSE156921. We are currently conducting experiments to complete this information with functional data that support the particularities of macrophage activation induced by palmitate, as well as trying to

validate these results by analyzing macrophage samples obtained from inflammatory pathologies.

2. Determination of the transcriptome of human macrophages exposed to serotonin (5-HT) in vitro, and to intravenous immunoglobulin (IVIg) in vivo.
Previous results by our team and others have shown that serotonin (5-HT) and, separately, intravenous immunoglobulin (IVIg) can attenuate inflammatory functions in macrophages, hence one of our objectives included the analysis of the effect of these compounds in the expression of genes relevant to the inflammatory phenotype of fatty acid-activated macrophages.

We have identified a repressive effect of 5-HT on the genes that encode for enzymes involved in the synthesis of cholesterol in macrophages, while, on the other hand, 5-HT increases the expression of genes encoding for proteins responsible for cholesterol efflux in macrophages.

Regarding IVIg, we have obtained the transcriptome of human monocytes exposed to IVIg in vivo and determined the effects of IVIg on the different subpopulations of peripheral blood monocytes in vivo.

The results with 5-HT have been obtained in RNAseq experiments performed on human macrophages derived from monocytes and exposed to BW723C86 (5-HT2B serotonin receptor agonist) or serotonin (5-HT). The raw data from these analyses have already been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) with access number GSE161774. We are currently carrying out functional and metabolomic analyses that allow us to validate the hypothesis that serotonin alters intracellular lipid metabolism by altering the expression / function of the LXR and SREBP factors.

The results obtained with IVIg treatment in humans are derived from RNAseq and flow cytometry experiments performed on cells (monocytes, T lymphocytes, B lymphocytes, NK cells) isolated from individuals before and after intravenous immunoglobulin infusion (IVIg, 6 hours). The transcriptional results have not yet been deposited in the Gene Expression Omnibus repository. All these results form the basis of an article

currently being written, and in which we describe the ability of IVIg to promote the appearance of "Myeloid-Derived Suppressor Cells" (MDSC) in peripheral blood.

3. Identification of intracellular metabolites induced by palmitate in mouse macrophages in vitro, and in populations of macrophages resident in visceral adipose tissue in healthy and obese conditions.

Observations obtained in this project showed that palmitate induced an inflammatory gene profile in both human and mouse macrophages. These results led us to analyze the contribution of relevant metabolic pathways in the pro- and anti-inflammatory activity of macrophages, such as the use of glucose and the mitochondrial oxidation of fatty acids, in the induction of pro-inflammatory genes by palmitate. Our results indicated that macrophages activated with classical inflammatory stimuli (e.g. bacterial products such as LPS), or by palmitate show comparable sensitivity to the inhibition of glycolysis with 2-DG (a non-metabolizable glucose analog). Likewise, they were not affected by a low concentration of etomoxir (which inhibits the transport of long chain fatty acids to the mitochondria) in terms of the expression of genes of cytokines and chemokines characteristic of the inflammatory phenotype associated with obesity: *Il6*, *Tnf*, *Ccl2*. Although we have observed differences in the degree of sensitivity of some genes (*Cpt1a*, *Ccl2*) to 2-DG depending on whether they are induced by LPS or by palmitate, these were moderate. These results suggest that inflammatory responses to pathogens and lipids share activation mechanisms and highlight the opportunity to identify new differentiating elements between both types of response.

Since the experiments with these drugs did not reveal a specific susceptibility between palmitate and LPS to the inhibition of the metabolic pathways tested, we analyzed the profiles of metabolites of the Krebs cycle and various lipid derivatives in macrophages stimulated with LPS or palmitate. We have determined that macrophages stimulated with palmitate have a different lipid profile than macrophages stimulated with inflammatory bacterial products (LPS). This lipid profile is reproduced in macrophages of different origin (bone marrow or adipose tissue) cultured in the presence of palmitate and is also characteristically elevated in vivo in the macrophage population considered more inflammatory and pathogenic (CD11c cells) in obese adipose tissue. These results identify metabolic characteristics that distinguish inflammatory macrophages in a context of obese adipose tissue versus macrophages whose inflammatory activity has been induced by microbial stimuli. We are currently

conducting trials with palmitate processing inhibitors in different types of macrophages to determine whether it is possible to attenuate the inflammatory activation of macrophages by palmitate without suppressing their responsiveness to microbial products.

4. Determination of the transcriptome of different populations of mouse macrophages obtained from healthy and obese visceral adipose tissue in vivo. Adipose tissue macrophages comprise different subpopulations, whose specific role in the homeostasis and pathologies of obese fat tissue is not well characterized. In order to study them in detail, we established an obesity induction protocol in mice with a diet rich in fat and sugar with 60% kcal from fat. This model reproduces the metabolic and inflammatory pathology associated with obesity in humans. Our analyses in different populations (monocytes, CD206 + / CD301 + macrophages and CD11c + macrophages) isolated from adipose tissue of normal and obese mice showed that obesity causes a reversal in the ratio of CD206 + / CD301 + macrophages and CD11c + macrophages. Our data also showed that the CD11c + population already exhibited some pro-inflammatory characteristics (higher expression of *Il6* and *Il1b*) under homeostatic conditions. In these analyses we observed that the expression of inflammatory genes characteristically induced by acute treatment with palmitate in human and mouse macrophages in vitro varied little within the same population of adipose tissue macrophages in vivo (being relatively stable up to 32 weeks of diet rich in fat), but the inflammatory bias of the tissue in obesity was associated with the enrichment of the tissue in inflammatory CD11c + macrophages.

Very recently we have been able to carry out RNA sequencing (RNAseq) of these populations and obtain the transcriptome of monocytes, CD206 + macrophages and CD11c + macrophages from adipose tissue of normal and obese mice. We are currently analyzing these results and the preliminary study confirms that the obese state does not induce a marked profile of inflammatory genes in these populations but mainly alters responses associated with the ability of macrophages to respond to type I and II interferon, as well as processes related to cell migration and extracellular matrix remodeling. We are currently confirming these observations in new experiments. The transcriptomic and metabolic characterization of macrophages from healthy and obese adipose tissue, together with the study of the impact of lipid metabolites derived from palmitate on its pro and anti-inflammatory functions constitute the core of a

manuscript in preparation with which we hope to conclude the most recent and novel results of the project.

3. Relevance and possible future implications

Given the pathological relevance of macrophages in inflammatory conditions associated with obesity, the transcriptomic data that have been generated during the project (and especially those of macrophages exposed to palmitate) have allowed us to identify a whole series of genes whose expression could be linked to this pathology, and that might constitute potential markers of it. The validity of the results generated is supported by the identification of a gene expression program of the "Unfolded Protein Response" among the genes most increased in macrophages after exposure to palmitate. These results are complemented by the identification of in vivo gene profiles of different populations of macrophages resident in adipose tissue of healthy and obese mice, whose preliminary analysis reveals novel gene signatures with respect to those identified with palmitate treatment. The study of the potential usefulness of these genes as biomarkers is currently beginning, through the analysis of macrophages of adipose tissue from obese patients.

In addition, and given the prevalence of diseases whose treatment requires the use of serotonin reuptake inhibitors (SSRIs), the data we have generated show that serotonin downregulates the expression of genes that encode for enzymes that control cholesterol metabolism, while enhancing the expression of genes that encode molecules involved in cholesterol efflux. If these results are confirmed at the protein and metabolic level, our results will provide relevant information regarding the potential consequences of SSRI treatments in patients with clinical depression. On the other hand, the identification of increased lipid metabolites induced by palmitate in inflammatory macrophages of obese adipose tissue in vivo and in vitro opens the opportunity to apply pharmacological modulators of their synthesis to selectively attenuate the inflammatory activation of macrophages from obese fatty tissue without altering beneficial inflammatory responses to, for example, microbial agents.

4. Scientific bibliography generated in the project

Due to the long period from the awarding of the project (2016) to the formalization of the agreement between the CSIC (institution where the subproject 201619-31 is carried out) and the *Fundació La Marató de TV3*, almost 2 years elapsed before starting the specific objectives of this project. This situation, and to a certain extent the circumstances associated with the COVID-19 pandemic in 2020, have delayed the completion of some results that have not yet been consolidated in publications. Thus, we are currently preparing a manuscript describing the immunomodulatory capacity of IVIg in inflammatory macrophages and its possible applicability in obesity; and another describing the transcriptomic and metabolic characterization of macrophages from healthy and obese adipose tissue. Finally, various methodologies used in this project have been set up in various works by the group, the results of which have contributed to this project. The funding support by the *Fundació la Marató de TV3* is acknowledged in these works.

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