Establishment and Validation of *In vitro* Model of Adipose Tissue Macrophages in Obesity and Diabetes

Synopsis Submitted to

The Maharaja Sayajirao University of Baroda

For the completion of degree

Doctor of Philosophy in Biochemistry

By

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Abstract

Obesity associated low grade chronic inflammation is involved in the development of insulin resistance. It is believed that during obesity, there is an unbalanced phenotypic shift from alternatively activated macrophages (M2), being anti-inflammatory towards classically activated macrophages (M1), which are pro-inflammatory. The anti-inflammatory macrophages preserve the insulin sensitivity by secreting IL10 and IL13. On the other hand, inflammatory cytokines like TNFα produced by the M1 macrophages in the obese adipose tissues, contribute to the development of insulin resistance. However, within the Adipose Tissue Macrophages (ATMs), a new subpopulation of macrophages have been described. These are the Metabolically activated macrophages – MMe and are different from M1 and primarily found in the adipose tissues of obese human. We developed an in-vitro model of MMe and characterized their phenotype, surface markers and metabolic signatures in comparison to M1 and M2. We elaborate on the subtle differences between MMe from M1 with respect to their role in adipose tissues.

Introduction

Obesity is a global epidemic and enhances the risk of several clinical problems. Its prevalence has increased drastically in last 30 years[1]. Nutrient excess and adiposity activate several metabolic pathways including inflammatory signaling, lipotoxicity, aberrant adipokine secretion, adipose tissue hypoxia, fibrosis, endoplasmic reticulum(ER) stress, and mitochondrial dysfunction and development of insulin resistance [2]. Inflammation arising within the adipose tissue has been identified as a major source of systemic inflammation which further impairs insulin signaling pathway [3,4].

Adipose Tissues (AT) plays a pivotal role in energy homeostasis. In addition, AT is also an important endocrine organ, secreting various adipokines. Other than adipocytes, residing immune cells and endothelial cells are also major source of several adipokines [5]. Obesity causes increase in the number of adipocytes (hyperplasia) well as volume

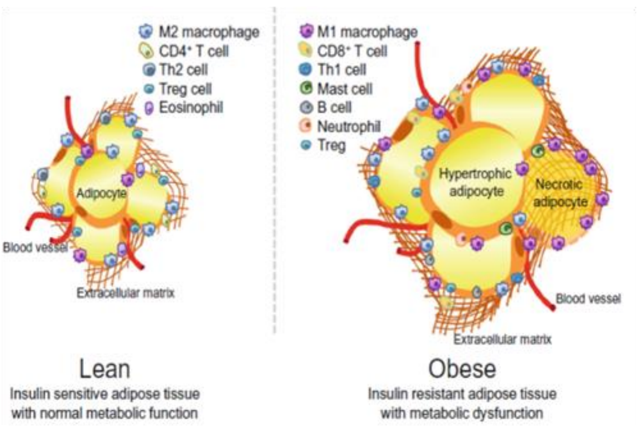

Fig 1: Adipose Tissue Remodeling during obesity [6]

(hypertrophy) leading to increase in adipose mass [7]. It also brings on alterations in the immune cells composition in AT. It includes increase in macrophages, neutrophils CD4^{+}T cells, B cells and mast cells while reduced eosinophils and T_{reg} cells. Macrophages are the most prominent immune cells during obesity constituting up to 40-50% of all AT cells in obese mice compared to 10% in lean mice [8]. Cinti et al., demonstrated that hypertrophic adipocytes death encourages the macrophage accumulation in the AT forming crown like structures [9].

Macrophages are important cells of the immune system that are formed in response to an infection or accumulating damaged or dead cells. Based on the inflammatory state, macrophages are classified as **classically activated**, M1, known to be pro-inflammatory or **alternatively activated**, M2 that are anti-inflammatory. Along with the well-characterized inflammatory properties. these macrophage sub-types have signature metabolic characteristics. Activation of macrophages by pro-inflammatory stimuli causes them to undergo a metabolic change towards glycolysis and away from oxidative phosphorylation (OXPHOS). On the other hand M2, shows significant upregulation of aerobic glucose metabolism and fatty acid oxidation [10]. The key drivers of metabolic characters of ATMs are hypoxia, lipid rich environment and adipocyte cell death. Hypoxia increases the expression of *Hif1α* which promotes secretion of inflammatory cytokines and upregulates GLUT1 for better glucose uptake required for augmented glycolysis [11].

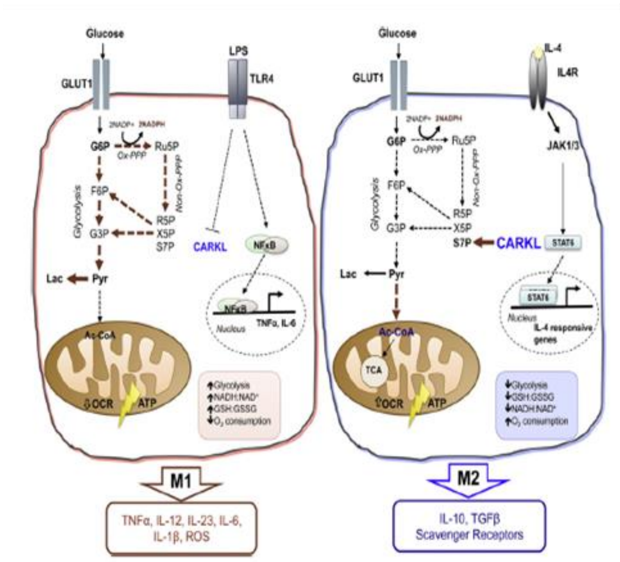

Fig 2: Metabolic Reprogramming in Macrophages[12]

Diet induced obesity involves a phenotypic shift in ATMs from anti-inflammatory phenotype (M2) to pro-inflammatory state (M1) [13]. In addition, HFD is found to aggravate pro-inflammatory cytokine secretion from M1 macrophages through activation of TLR4, which is marker for M1macrophages [14]. During obesity, similar to LPS, saturated FFAs, such as palmitic acid, are reported to cause IKK activation and IκBα phosphorylation through the activation of TLR4 in different cell types including adipocytes, vascular cells, and macrophages followed by the NF-κB and subsequent inflammatory signaling. Therefore, cytokines released from the adipose tissue and ATMs due to pro-inflammatory signals (FFA interacting with TLR4) during obesity, such as TNFα, are key drivers of insulin resistance [15] while the anti inflammatory macrophages preserve the insulin sensitivity [16].

However, various studies have demonstrated that the Majority of macrophages in AT, although pro-inflammatory, are distinct from M1.These display altered lysosomal activity [17] express distinguishing surface markers [18] and have unique metabolic activation [19]. This sub-type known as Metabolically activated Macrophages – MMe, promotes AT dysfunction. For example, secreted factors from activated macrophages affect the adipocyte mitochondrial bioenergetics [20].

It is therefore, important to study different aspects of MMe function and differentiation with respect to their role in adipose tissue. The mechanistic details like what molecular pathways control the differentiation and function of such cells will be instrumental in uncovering new drug targets for obesity and insulin resistance. An in vitro model will be very helpful in this regard. So far, the reports in this field have used animal models or human primary cells.

We hypothesize that macrophages involved in metabolic dysfunction in adipose tissues implicated in obesity can be recreated in an *In-vitro* system.

We have developed such a model and studied characterized various aspect of their phenotype and function such as surface markers, metabolic signature, cytokine profile and their effect on adipose tissues.

With this information we planned our objectives as below:

Major Objectives

1. Establishing a cell based model of Adipose Tissue Macrophages(ATMs)
2. Effect of ATMs on Adipocytes
3. Mass Spectrometric analysis of Macrophages subtypes

Objective1:Establishing a cell based model of Adipose Tissue Macrophages

We used THP-1, human monocyte cell line for our studies. Different stimulants were used to get in-vitro macrophage subtypes.

A) **In-vitro differentiation of M1, M2 and MMe**

THP-1 cells are maintained in RPMI media supplemented with 10% FBS and 1% PS at 37°C under5% CO_2. StimulatingTHP-1 with PMA gives resting macrophages(M0). Then, these were further stimulated with LPS & IFNγ to mimic M1 macrophages, while IL4 was used to differentiate M2 macrophages. To imitate MMe, M0 cells were stimulated with palmitate, insulin and glucose.

Inflammatory Characters: Both M1 macrophages and MMeshows upregulated levels proinflammatory cytokines (TNFα, IL1β, IL6) expressions although at different levels while M2 macrophages do not produce them.

Surface Markers:We found CD319 and CD274, M1 markersare negligible in MMe and M2. Moreover, MMedisplay differential markers CD36, ABCA1 and PLIN2. These markers are known be involved in lipid metabolism and also induced in M2 macrophages as well, but to a different extent. However, CD209 is upregulated in M2 while suppressed in MMe and M1 macrophages.

Metabolic features:Here in our in-vitro model, we also observed LPS & IFNγ stimulation upregulated glycolytic genes PKM2, PFKFB3 and PDK1. As reported in various studies we also find increased GLUT1 expression for glucose uptake and FAS (for prostaglandin synthesis) which is needed to maintain inflammatory state. On the other hand, MMe exhibits increased transcript levels of PPARγ, Cpt1α and PGC1α indicating high lipid metabolism.M2 is skewed towards lipid metabolism. Also oil-red-O(ORO) staining showed lipid accumulation in MMe.

B) Effect of disrupting metabolism on Macrophages phenotype:

Since, changes in energy metabolism is a hallmark of macrophage differentiation.

To study the role of metabolism on polarization, we used metabolic inhibitors.

Glycolysis inhibition by 2DG: Inhibiting the glycolysis was affecting the cytokine expression in M1 but not in MMe.Also FAS was downregulated.

Fatty Acid oxidation inhibition by Etomoxir: Etomoxir is a Cpt1α inhibitor. Treatment with etomoxir affected the cytokine expression in M1 and MMe. The extent of reduction was less in MMe in comparison to M1. Although there were no changes in FAS. On contrary, it increased CD36 and PLIN2 in all subtypes.

C) Autophagy pathway is altered in MMe

We checked the mRNA levels of autophagy genes. Atg5 and Atg7 upregulation suggests autophagy induction in MMe. LC3-II and p62protein accumulation is also observed in MMe using western blot. Additionally, increased expression of LAMP2 and LIPA also indicate lysosomal dependent lipid metabolism. Flux needs to be confirmed by changes in LC3 when inhibiting autophagy using hydroxychloroquine.

D) ER Stress

Nutritional stress induces ER stress in MMe. We found increased levels of XBPs and ATF4 transcripts and upregulated GADD34 and CHOP. On the other hand, M1 shows higher expression of ATF3,GADD34 and CHOP incomparison to XBPs.

E) Mass Spectometery

Mass spectometery data shows 359, 281 and 439 exclusive proteins in M1, M2 and MMe and these were used for pathway enrichment. M1 are enriched in pathways involved in Interferon signaling, that is important for cytokine production.M2 are involved CREB phosphorylation and glycosphingolipid mechanism. MMe showed to be involved in wnt ligand biogenesis, beta oxidation of VLCFA and PPP.

Objective2:Effect of ATMs on adipocytes

Firstly the protocol for adipocyte differentiation is standardized. Formation of lipid droplets was confirmed using ORO staining and qRT-PCR of differentiation markers. Next, conditioned media is used to observe the effect on adiponectin expression from adipocytes. M1 and MMe conditioned media (CM) both affects the adiponectin production but at different extent. But at the same time MMe CM did not alter the expression of other adipogenesis markers in comparison to M1, indicating its finer role in adipocyte homeostasis. We are also looking at its effect on insulin sensitivity. We observed MMe CM is better in preserving insulin sensitivity than that of M1.

Conclusion

We are able to establish a cell based model for Metabolically Activated Macrophages very similar to those in the adipose tissue of obese individuals. Key cytokine markers, IL1β, IL6 and TNFα were found to be upregulated in both M1 and MMe. Different cell surface markers were also checked where CD319 and CD274 was upregulated in M1 while CD36 and ABCA1 upregulated in MMe indicating the increased lipid uptake. M1 favors glycolysis whereas M2 and MMe are more inclined to fatty acid metabolism. Yet,MMe displayed high lipid accumulation in comparison to M2 while it was negligible in M1. This nutritional stress leads to ER stress and altered lysosomal activity and autophagy. Since, metabolic reprogramming plays a key role in macrophage phenotype. Modulating these energy pathways was affecting the inflammatory functions as well as surface molecules. Inhibiting the glycolysis decreased expression of IL1b and TNFa. Disrupting fatty acid uptake also upregulated the PPARγ which in turn caused upregulation of CD36 and PLIN2. Even though these macrophages are involved in AT inflammation which is the key in IR, but are also beneficial in maintaining the adipocyte homeostasis. We observed that MMe CM reduced the adiponectin levels like M1. But MMe did not affected the adipogenesis. MMe was better in preserving the insulin sensitivity than M1 while M2 CM did not affect pAkt. This indicates that MMe are involved in inflammation in adipose tissues. But having distinct phenotypes, it tries to preserve the adipocyte function. Thus MMe plays a dynamic role in adipose tissue homeostasis.

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Poster Presentations and Conferences Attended During Ph.D.:

1. Poster presented **"Mass Spectrometric analysis of In-vitro Macrophages subtypes"** at International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran**"**, 3-4 Mar,2023, organised by Department of Biochemistry, M. S. University of Baroda,Vadodara ,Gujarat.
2. Abstract **"Evaluation of Major Anti-diabetics agents on Macrophages"** at International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran**"**, 3-4 Mar,2023, organised by Department of Biochemistry, M. S. University of Baroda, Vadodara ,Gujarat.
3. Abstract **"*Sida cordifolia* is effective in reducing cellular aggregation"** at International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran**"**, 3-4 Mar,2023, organised by Department of Biochemistry, M. S. University of Baroda,Vadodara ,Gujarat.
4. Participated in **"Science Conclave 2020"**, 28 Feb,2020, organised by Faculty of Science, M. S. University of Baroda,Vadodara,Gujarat.
5. **National Symposium** on **"Trends in Biochemistry and Inaugration of Prof. L.J. Parekh Mmorial Lectures Series"** organized by Department of Biochemistry, M. S. University of Baroda in 2019.
6. **Hands-on Training Workshop, "**Basic Cell Culture Technology" at NCCS,Pune, May 14^{th} – 17^{th}, 2018

7. **Workshop** on "Hands-on basic flow cytometry" at NCBS-TIFR, Bangalore, May 11^{th} – 13^{th}, 2022
8. **Virtual Workshop** on "Computational Genomics" conducted by NCBS-TIFR, Bangalore, and CPMBB, TNAU, Coimbatore.June 8^{th} – 12^{th}, 2022
9. **Short Term Course** on "Biostatistics: A User's Perspective" conducted by IISER, Pune,Dec 10^{th} – 18^{th}, 2022