Monocyte-macrophage dialogue with adipocytes : implication in human obesity-related type 2 diabetes

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adipocytes: Implication in human obesity-
related type 2 diabetes.**

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SCHOOL OF BIOLOGICAL SCIENCES
2015

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Abbreviations

ACAT1	Acetyl-CoA acetyltransferase
Adipo	Human Adipocytes
ATMs	Adipose tissue macrophages
BMI	Body mass index
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR-2	C-C chemokine receptor type 2
CD	Cluster of Differentiation
CLS	Crown like structures
CM	Conditioned media
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL9	Chemokine ligand 9
CXCL10	Chemokine ligand 10
DCs	Dendritic cells
DIO	Diet induced obesity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FFA	Free fatty acids
FGF	Fibroblast growth factor
HFD	High fat diet
HOMA-IR	Homeostatic model assessment of Insulin Resistance
ICAM-1	Intercellular Adhesion Molecule 1
IFN γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IS	Insulin Sensitivity
LIPE	Hormone Sensitive Lipase

LPA	Lipid A
LPS	Lipopolysaccharide
Mo	Human Monocytes
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MCP-1	Monocyte chemoattractant protein 1
NC	Normal Chow
NEAA	Nonessential amino acids
NKT	Natural Killer T cells
OB-DB	Obese-Diabetic
OB	Obese
OM	Omental Adipose Tissue
OW	Overweight
PPAR- γ	Peroxisome proliferator-activated receptor gamma
p-STAT6	Phosphorylated Signal Transducer and Activator of Transcription 6
RIPA	Radioimmunoprecipitation assay buffer
SC	Subcutaneous Adipose Tissue
SCD	Stearoyl-CoA desaturase
SVC	Stromal Vascular cells
SOCS	Suppressor of cytokine signalling proteins
STAT6	Signal Transducer and Activator of Transcription 6
T2D	Type two diabetes
TLR4	Toll-like receptor 4
TNF- α	Tumor necrosis factor alpha
Treg	Regulatory T cells
WHO	World health organisation
Ym1	Chitinase like 3

Abstract

Mice studies in obesity-induced metabolic disease indicate a major role for monocytes/macrophages, whereas their contribution in regulating this disease in humans is not clear. To this end, we investigated the crosstalk between human monocytes and adipocytes in human obesity and Type 2 Diabetes (T2D). Co-culturing human monocytes with subcutaneous/omental adipocytes increased the expression of measured genes at the basal level. When polarized with IFN γ +LPA or IL-4, the co-cultured monocytes showed impaired expression of inflammatory genes (e.g. *IL6*, *IL1B*, *IL12p40*), but upregulation of non-inflammatory genes (*CCL18*, *CCL17*). This was true for co-cultures with adipocytes from obese-T2D, but not overweight subjects. In contrast, irrespective of their origin, adipocytes co-cultured with monocytes upregulated inflammatory genes expression. An increased concentration of CCL18 and CCL17 was also detected in the culture supernatant from monocytes that were co-cultured with adipocyte conditioned media from obese-diabetic subjects, confirming our gene expression data. Interestingly, *in vivo* plasma of obese-diabetic subjects showed an approximately two-fold increase in CCL18 levels and CCL18 expression correlated with fasting plasma glucose, suggesting its possible role in obesity-T2D. Collectively, we demonstrate that human monocytes upon interaction with adipocytes displayed impaired response to inflammatory or microbial stimuli (IFN γ +LPA) and polarized to an M2-like phenotype, characterized by increased CCL18 expression that was detectable *in vivo* in adipose tissue and plasma. Interestingly, this is contrary to what is known in the mouse system.

1 Introduction

1.1 Obesity

1.1.1 What is obesity?

The World Health Organisation (WHO) characterised obesity as an atypical or disproportionate fat accumulation that can present a potential risk to health (WHO, 2015b). Obesity is considered as a chronic inflammatory disease. The most common causes are bad eating habits, sedentary life style as well as genetic predisposition. Although obesity is a multi-factorial disease (or syndrome), it is mainly caused by the greater nutritional intake than the energy expenditure.

Obesity leads to health impairment and risks of developing pathological associated conditions like insulin resistance (IR), Type 2 Diabetes (T2D) and atherosclerosis, collectively referred to as Metabolic Syndrome.

One of the factors promoting obesity is economic growth and the urban life style. Modernization, energy saving equipment (e.g. cars, internet) as well as broad availability of cheap but energy rich food and drinks contribute to continuous increase of obesity worldwide (Egger, 2009). However, the idea that obesity is a modern disease which is present mainly in modern and economically strong countries is being more and more challenged, as obesity is on a high rise in developing countries or low-income families (Kachi et al., 2015; Marty et al., 2015).

This phenomenon can be explained as low-income families in order to stretch their budget purchase low cost food which is very dense in calories but low in nutrients. Therefore, socioeconomic status of the individual can be considered as one of the contributing factors towards development of obesity.

1.1.2 Facts about obesity globally

The World Health Organization (WHO) has repeatedly reported an increase in worldwide obesity which has more than doubled since 1980. WHO estimated in 2014 that there are currently around 600 million obese individuals and about 1.9 billion overweight adults (WHO, 2015b). This would mean that about 13% of the world's adult population is currently obese and 39% overweight (WHO, 2015b).

Obesity is mostly measured and defined by Body mass index (BMI). The definition of BMI by WHO is stated as the individual's weight in kilograms divided by the square of his height using meters (kg/m^2) (WHO, 2015b). Generally a person with a BMI which is 25 and higher is overweight while those with a BMI which is 30 and more is obese (WHO, 2015b). However, for the Asian population the cut offs of BMI are lower. Overweight starts at 23.5–27.4, while obese starts at 27.5 (Gan and Pang, 2012).

Although BMI is by far the most popular measure of obesity, it may not be the best indicator of obesity as individuals with identical BMI might significantly differ in a content of body fat also termed as Y-Y paradox (Yajnik and Yudkin, 2004).

1.1.3 Obesity as epidemic in Asia

Asia, a continent containing one of the most inhabited regions and countries in the world such as China (over 1.3 billion) and India (more than 1.2 billion). In past few decades, Asia underwent rapid urbanization and economic growth beside marked demographic and epidemiologic changes. While some countries like Singapore or Japan experienced very rapid development, others are still in the process of development, e.g. Indonesia, Philippines. In fact, many Asian countries show striking socio-economic differences between rich and poor. However, it is surprising that obesity

and with its associated pathologies have been underestimated for a long time in this part of the world. This was probably the main reason why until recently most of the Asian countries did not plan for prevention and management of obesity.

Although environmental factors such as socioeconomic status or urbanization are two factors which relate to development of obesity, genetic susceptibility of the Asian population plays also an important role. Several studies reported a higher amount of whole body fat and a higher abdominal adiposity when compared to Caucasians with the same BMI (He et al., 2002; Park et al., 2001). In some Asian populations a direct link for inheritance of central obesity was even shown (Davey et al., 2000; Li et al., 2006).

In Singapore the number of people with obesity has raised in the past few years and surveys show at least one in nine Singapore residents are obese (Gan and Pang, 2012). By ethnic group the highest number of obese is among Malays (24%) following by Indians (16.9%) and then, Chinese (7.9%) (Gan and Pang, 2012).

1.2 Type 2 diabetes mellitus (T2D)

Progressive and long-term obesity is associated with certain type of pathologies. Obese individuals are more prone to insulin resistance (IR), T2D, cardiovascular diseases, stroke, osteoarthritis and some types of cancers (Noto et al., 2013; WHO, 2015b).

It is now a well-accepted fact that obesity is one of the biggest risk factors for T2D, although not all obese patients develop T2D (Kloting et al., 2010). T2D is also termed as a non-insulin dependent or adult originating diabetes (WHO, 2015a).

T2D is the major type of diabetes and represents about 90% of the diabetic cases around the world (WHO, 2015a). In 2014, the International Diabetes Federation (IDF) reported 387 million diabetic people around the world with prediction that this number will increase to 592 million in 2035 (IDF, 2014).

In general, it is recognized that the onset of T2D can occur 5 to 10 years before the first clinical symptoms and in general, this period can be divided into three stages: normal glucose tolerance, impaired glucose tolerance and T2D (Chen et al., 2012). However, T2D can be caused by various conditions of which the most important are β -cell dysfunction and IR.

IR is characterized by a state in which insulin target tissues like muscles or adipose tissue have no longer the ability to respond to normal insulin levels and this is caused by activation of different serine/threonine protein kinases which phosphorylate insulin receptor substrate (IRS) proteins leading to impaired insulin signalling pathway (Kahn et al., 2006). In addition, worsened signalling through serine/threonine protein kinase Akt/PKB plays role in development of IR in the adipose tissue (Mackenzie and Elliott, 2014).

Chronic inflammation is among one of the important causes for the development of insulin resistance, linking the importance of inflammatory cells like monocytes/macrophages in development of this metabolic disease.

1.3 White adipose tissue (WAT)

Adipose tissue was previously considered as a storage tissue for excess calories to be stored in a form of lipids. However, after the discovery and the cloning of the first adipocyte originating hormone Leptin, it was broadly accepted that adipose tissue also functions as an endocrine organ (Zhang et al., 1994). Since then, a growing number of substances produced by the adipose tissue and generally termed as adipokines have been reported. Adipokines can be cytokines, chemokines or hormones having effect on chemotaxis, growth, blood pressure, inflammation and vascularisation (Ouchi et al., 2011).

WAT has certain specific characteristics. It is white to very dark yellow in colour. It plays primary role in energy storage (Fruhbeck et al., 2009; Zeve et al., 2009). WAT stores excessive energy which body does not use in the form of triglycerides (Lazar, 2008; Zeve et al., 2009). However, when body needs energy (e.g. during starvation) stored excessive energy in adipocytes is released in form of fatty acids (FA) (Fruhbeck et al., 2009).

Based on its body location WAT can be divided into: subcutaneous (SC) and visceral or omental (OM) WAT. Depots of white OM adipose tissue are more associated with metabolic difficulties such as increased risk of hyperlipidemia, cardiovascular disease and diabetes in comparison to SC adipose tissue which is suggested to be protective against these metabolic complications (Gesta et al., 2007; Shi and Clegg, 2009; Wajchenberg, 2000). In line with this fact, the ratio of SC/OM adipose tissue mainly correlates with glucose tolerance as well as plasma insulin

levels (Despres et al., 1989; Fujioka et al., 1987; Pouliot et al., 1992). However, some studies did not confirm OM adipose tissue to be more associated with obesity and T2D (Klimcakova et al., 2011).

Some studies report that adipocytes make up about 50 - 70% of the cellular constituents of the WAT and 70% of the proteins from WAT originate from tissue matrix, while the remaining 30% from adipocytes and stromal vascular cells (SVC) (Fain et al., 2004). Indeed, in addition to adipocytes, non-fat cells such as pre-adipocytes, endothelial cells, fibroblasts and various immune cells constitute the cellular constituents of WAT. In a lean WAT, about 5 - 10% of the immune infiltrates are the adipose tissue macrophages or ATMs (Chawla et al., 2011).

It is now well accepted that the non-fat SVC (stromal vascular cells) for example immune cells are one of the main producers of adipokines (90%) inside the human WAT (Fain et al., 2004). In fact, due to this reason immune cells have an important role in regulating the homeostasis or propagating pathogenesis in WATs (Chawla et al., 2011; Huh et al., 2014). Therefore, in the next section, we explain the relation and function of various immune cells present in WAT as well as their role in obesity and subsequent development of insulin resistance.

1.4 Role of immune cells in Obesity and T2D

1.4.1 Adaptive Immune system

1.4.1.1 T-cells

T-cells can be generally divided into CD8⁺ and CD4⁺ subtypes. T-cells modulate immuno-inflammatory response upon interaction with antigen presenting cells (e.g. dendritic cells, monocytes/macrophages and B-cells) which triggers their proliferation and differentiation into different effector cells (e.g. cytotoxic CD8⁺T-cells, CD4⁺Th1, Th2, Th17 or Treg) depending on the microenvironmental stimuli. T-cells in turn by secreting various cytokines (IFN γ or IL-4) play a role not only in the activation and polarization of ATMs but also in either inducing chronic inflammation or checking it and maintaining tissue homeostasis (Liston and Gray, 2014). The number of T-cells increases with obesity both in humans and in mice and correlates with BMI (Rocha et al., 2008; Wu et al., 2007).

1.4.1.2 CD4⁺ T-cells

CD4⁺ T-cells are subdivided into two major categories: T helper cells and Regulatory T-cells (Treg). T helper cells can differentiate into other subtypes depending on the cytokine milieu. These subtypes are Th1, Th2 and Th17 T cells. Th1 produce mostly IFN γ , while Th2 produce IL-4 or IL-13 and Th17 produce IL-17.

Studies in mice with diet induced obesity (DIO) suggested that CD4⁺ T cells present in the visceral adipose tissue are responsible for insulin resistance and glucose homeostasis (Winer et al., 2009). Increased number of inflammatory Th1 cells has been associated with IR (Winer et al., 2009) (**Figure 1.1**). Similarly, Th17 cells are also implicated in obesity-related IR, although their exact role remains questionable. For example, the number of Th17 cells increased in mice on a high fat diet (HFD) and IL-

17 inhibited adipogenesis, but mice lacking IL-17 became more obese independently of their diet (Zuniga et al., 2010).

In lean mice, the number of Treg cells is high while in the insulin resistant mice their number is significantly decreased (Feuerer et al., 2009). In the same study it was shown that expansion of Treg cells *in situ* influenced adipose tissue inflammation, IR and improved glucose tolerance although slightly. In a more recent study, peroxisome proliferator-activated receptor (PPAR- γ) was reported as the main transcription factor involved in Treg accumulation in adipose tissue and necessary for restoration of insulin sensitivity (IS) (Cipolletta et al., 2012). Not only is the PPAR- γ necessary for Treg cell function, it is also a target of anti-diabetic drugs such as pioglitazone (Cipolletta et al., 2012). In human, decreased number of Treg cells in the peripheral blood closely correlates with an increased adiposity, BMI, body weight and leptin levels (Wagner et al., 2013).

1.4.1.3 CD8⁺ T-cells

CD8⁺ T-cells are called cytotoxic T-cells due to their high production of cytolytic proteins (e.g. granzyme). These cells release the inflammatory cytokine, IFN γ . CD8⁺ T-cells increasingly infiltrate adipose tissue of obese mice in comparison to their lean counterparts (Rausch et al., 2008). In another study, increased number of CD8⁺ T-cells and diminished number of CD4⁺ T helper cells and Tregs was reported in HFD fed mice (Nishimura et al., 2009). In the same study, it was observed that infiltration of CD8⁺ T-cells appears before the accumulation of macrophages and genetic deletion CD8⁺ T-cells can decrease macrophage infiltration into the obese adipose tissue (Nishimura et al., 2009).

Although previous studies show the importance of CD8⁺ T-cell in obesity and IR, a recent study shows that these cells can not alone account for improvement of whole body IR (Montes et al., 2013). In this study, antibodies against CD-40L and CTLA-4 were used in order to fully

block the T-cell activation. Mice treated with anti-CD40L antibody showed reduced weight and improved IS while the mice treated with CTLA-4Ig showed no change in IR (Montes et al., 2013). However, in another recent study, no difference in the development and maintenance of CD8⁺ T-cells in lean or obese mice was observed (Khan et al., 2014). Therefore, the role of CD8⁺ T-cells in obesity and development of IR remains unclear and further studies are necessary.

1.4.1.4 B cells

Several studies reported involvement of B-cells in obesity and IR. Some researchers reported very early increase in the accumulation of adipose tissue B cells, which was followed by adipose tissue T-cells and at last ATMs (Duffaut et al., 2009). Another study indicated a fundamental role for B cells in DIO in mice since mice lacking B cells in spite of gaining weight were protected from IR (Winer et al., 2011). Recently, a study done in mice and T2D human subjects reported adipose tissue B cells as critical regulators of pro-inflammatory T-cell functions (DeFuria et al., 2013).

1.4.2 Innate immune system

1.4.2.1 NKT cells

Despite the fact that NKT cells belong to the T-cell family they also show innate-like functions. In HFD fed mice, NKT cells were implicated in the development of adipose tissue inflammation and glucose intolerance (Ohmura et al., 2010). Contrary to this observation, another study showed that mice lacking NKT cells had minimal protection against obesity induced metabolic conditions like IR, glucose intolerance, adipose tissue inflammation and liver steatosis (Mantell et al., 2011). Surprisingly, in mice lacking B cells an accumulation of NKT cell could be observed in adipose tissue together with ATMs (Duffaut et al., 2009). A more recent study

showed increased infiltration of type 1 and type 2 NKT cells following IL-25 treatment in mice and its involvement in improved glucose tolerance and lowered weight gain (Hams et al., 2013). Further studies are necessary in order to clarify the function of NKT cells in obesity and the development of IR.

1.4.2.2 Neutrophils

Neutrophils are the first phagocytic cells to be recruited to the site of inflammation during an innate immune response. Over the past decade, several studies have reported a higher number of circulating neutrophils in obese individuals (Herishanu et al., 2006; Zaldivar et al., 2006).

Neutrophils infiltrate intra-abdominal adipose tissue as early as 3-7 days after the onset of high fat feeding even before the appearance of ATMs (Elgazar-Carmon et al., 2008). These cells produce elastase, different chemokines and myeloperoxidase which are necessary for the recruitment of other immune cells. Indeed, deletion of elastase in neutrophils leads to lessen tissue inflammation and lowered number of ATMs in mice fed HFD (Talukdar et al., 2012). It is also associated with an improved glucose tolerance and IS (Talukdar et al., 2012). Interestingly, it was reported that neutrophils can bind to adipocytes through intercellular adhesion molecule 1 (ICAM-1) and a strong crosstalk between these two types of cells has been described (Elgazar-Carmon et al., 2008).

1.4.2.3 Mast cells

Mast cells play a role in inflammatory and fibrotic diseases. These cells are known for the release of histamine in the body and recognition of pathogens (Abraham and St John, 2010). In the obese humans or mice, the number of adipose tissue mast cells is increased in comparison to their lean counterparts (Liu et al., 2009). In mice lacking mast cells was observed greater glucose homeostasis, less weight gain as well as decrease of inflammatory cytokines and chemokines in plasma or adipose tissue (Liu et al., 2009). In humans, activated adipose tissue mast cells are present in the fibrotic areas, whereas in the mice presence of mast cells is observed to co-localize with ATMs in crown-like structures (CLS) (Divoux et al., 2012; Liu et al., 2009). More recent study indicates mast cells maturation during the process of increased adiposity and a direct role of adipose tissue mast cells in adipose tissue fibrosis (Hirai et al., 2014).

1.4.2.4 Eosinophils

Eosinophils are best known for their role in immune response to helminths, allergy and polarization of immunity towards a Th2 anti-inflammatory phenotype. In the adipose tissue, eosinophils are the main cells producing IL-4 (**Figure 1.1**) and the main contributors in maintenance of anti-inflammatory ATMs during lean conditions (Wu et al., 2011). In the absence of eosinophils, mice fed with HFD develop worsened glucose tolerance, IR and increased adiposity (Wu et al., 2011). A recent study also identified role of eosinophils in development of beige fat which has an important role in thermoregulation (Qiu et al., 2014).

1.4.2.5 Basophils

Despite the fact that basophils are one of the most uncommon cells present in the blood they are becoming more and more recognized for their crucial function in allergic diseases. Surprisingly, to date very little is known on their function in obesity, development of IR or T2D. Human basophils express the leptin receptor and leptin can affect some of the basophils functions such as migration or survival (Suzukawa et al., 2011).

1.4.2.6 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells, which are instrumental in activating T-cells and thus triggering an adaptive immune response. A recent study indicated DCs to regulate macrophage infiltration into obese adipose tissue (Stefanovic-Racic et al., 2012). In this study, a positive correlation between increased numbers of mouse DCs and increased adiposity was observed. In line with this finding, mice lacking DCs showed lower number of ATMs and were resistant to weight gain whereas delivery of bone marrow-derived DCs into lean mice increased number of ATMs (Stefanovic-Racic et al., 2012).

Several studies showed a crucial role of pro-inflammatory CD11c⁺ ATMs (Li et al., 2010; Lumeng et al., 2007a; Nguyen et al., 2007; Wentworth et al., 2010). However, DCs and macrophages share expression of some surface markers and some of the CD11c⁺ cells present in the adipose tissue could also include DCs (Stefanovic-Racic et al., 2012). The same should be taken into account in studies where depletion of CD11c⁺ cells was performed as DCs will be also depleted in addition to inflammatory ATMs (Patsouris et al., 2008).

Figure 1.1: Interaction of different subsets of adaptive and innate Immune cells with adipocytes in lean and obese conditions. Left panel: In lean individuals. Right panel: In obese individuals. M1 and M2 refer to inflammatory and anti-inflammatory ATMs, detailed further in the Macrophage section. Figure adapted from Hruskova and Biswas, 2013.

1.5. Monocytes/Macrophages in Obesity and T2D

Monocytes/macrophages are an integral part of the innate immune system and act as the first line of defence. Thus, they are equipped to respond to infection by recognizing and killing pathogens through phagocytosis and microbicidal activity and the triggering a robust immuno-inflammatory response that ultimately clears the infection.

Once the infection is cleared, these cells also play an important role in tuning down inflammation, in wound healing and tissue repairing responses, which re-instate tissue homeostasis. Thus, monocyte/macrophage show functional diversity, i.e. the ability to tailor their response to diverse microenvironmental signals by displaying equally diverse functions like triggering inflammation, activating T-cells, anti-microbial and anti-cancer response, phagocytosis, and tissue remodelling and repair. Moreover, these cells also have the ability to switch from displaying one function to the other, depending on the dynamic changes in their microenvironment, a characteristic also known as functional plasticity. Thus, the functions of these cells are tightly regulated in time and space and any dysregulation in this response is often associated with a variety of pathologies (Murray et al., 2014; Wynn et al., 2013).

Monocytes and macrophages were among the first immune cells to be associated with chronic inflammation related to obesity and the development of metabolic syndrome (Odegaard and Chawla, 2008). In the following sections we will provide a more extensive description of these cells as this project is focused on the role of monocytes and macrophages in obesity and T2D.

1.5.1 Monocyte/Macrophage: origin and ontogeny

According to the old dogma from the late 60s, it was believed that bone marrow derived monocytes contribute to tissue resident

macrophages (van Furth and Cohn, 1968). Subsequent to their origin in the bone marrow, monocytes circulate in the blood and after a few days home to various peripheral tissues where they differentiate into macrophages or dendritic cells, according to the tissue microenvironmental signal they encounter (Gordon and Taylor, 2005; Mosser and Edwards, 2008). However, seminal studies using fate-mapping approach have challenged this view (Geissmann et al., 2010; Guillemin et al., 2014). These studies show that under homeostatic conditions, most tissue resident macrophages (except in the gut) are of embryonic origin and self-maintain through slow proliferation throughout the lifespan (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Hashimoto et al., 2013; Jenkins et al., 2011). However, under inflammatory conditions as well as in cancer settings, blood monocytes upon reaching the affected tissue differentiate into macrophages (Chittezhath et al., 2014; Ingersoll et al., 2011; Movahedi et al., 2010). However, an exception to this case is during helminth infection, where anti-inflammatory macrophages were shown to exhibit local proliferation (Jenkins et al 2011).

1.5.2 Polarization of Monocytes/Macrophages – “Chameleon” of the body

Phenotypic and functional plasticity are main characteristics of cells of the mononuclear phagocytes lineage (Biswas and Mantovani, 2010). Depending on the microenvironmental stimuli they encounter, monocytes/macrophages display different phenotypes ranging from inflammatory, microbicidal phenotype to an anti-inflammatory, tissue repairing and protumoral phenotype. Historically, two main functional phenotypes have been recognized for macrophages, the classically activated and the alternatively activated states (Stein et al., 1992).

Microbial stimuli like lipopolysaccharides (LPS) and Th1 cytokines like interferon gamma (IFN γ) polarize monocytes/macrophages to a classically activated state. These cells express higher levels of pro-

inflammatory, oxygen and nitrogen radicals, and promote Th1 response (Shiloh et al., 1999). Such classically activated cells are responsible for protection against various types of bacteria, viruses and have anti-tumoral activity (**Figure 1.2A**).

Th2 cytokines like interleukin 4 (IL-4) and interleukin 13 (IL-13) polarize monocytes/macrophages to an alternatively activated state (Stein et al., 1992). These cells were characterized by an increased expression of scavenging receptors and scavenging activity, a reduced pro-inflammatory cytokine expression and a preferential metabolism of arginine to ornithine via arginase 1 expression (**Figure 1.2A**). These cells are mainly related to the clearance of parasite (helminth) infection (Loke et al., 2002). Subsequently, Mills and colleagues demonstrated that macrophages from different mice strains induced differential nitrogen metabolism, with those from Th1 mice strains (e.g. C57/BL6) producing nitric oxide (NO), while those from Th2 strain (e.g. Balb/c) producing ornithine from arginine, in response to LPS or IFN γ (Mills et al., 2000). They also showed that macrophages from Th1 and Th2 strains could drive a Th1 and Th2 cytokine response, respectively. Based on these observations, they proposed that macrophages can be polarized to two distinct states, M-1 and M-2, corresponding to the Th1 and Th2 phenotype, respectively.

Mantovani and colleagues (Mantovani et al., 2002) extended this concept into a general scheme for monocyte/macrophage polarization, wherein M1 state was synonymous to classically activated state, while M2 state included the alternatively activated state. However, variations within the M2 theme have emerged depending on whether the cells were exposed to IL-4, IL-10, dexamethasone, LPS plus immune complex, leading these authors to propose a subdivision of the M2 phenotype (Mantovani et al., 2004). However, others have suggested to subdivide macrophages based on functions (rather than phenotypes) such as host defence (classically activated macrophages), tissue repair (wound healing macrophages) and immunoregulation (regulatory macrophages), reviewed by (Mosser and Edwards, 2008). In fact, these three subsets were

demonstrated to be biochemically and functionally different (Edwards et al., 2006). In particular, wound healing macrophages (which arise in response to IL-4 and synonymous to alternatively activated) express high levels of arginase which helps to convert arginine into ornithine and therefore, stimulate extracellular matrix production (Hesse et al., 2001).

Regulatory macrophages were first reported to arise upon stimulation of macrophages with TLR ligand (LPS) and immuno complexes and defined by the high expression of IL-10 and low expression of IL-12 (Gerber and Mosser, 2001). Other stimuli that induce this phenotype include IL-10, prostaglandins and apoptotic cells (Martinez et al., 2006; Strassmann et al., 1994). Recently, Biswas & Mantovani have proposed a revision of the monocyte/macrophage polarization scheme recognizing three polarization states, namely, M1 (=classical activation), M2 (=alternative activation) and M2-like (to incorporate all other variations of M2 state, for example, regulatory macrophages) (Biswas and Mantovani, 2010).

Transcriptional profiling of monocytes and their differentiation to distinct macrophage phenotypes has contributed greatly in extending our understanding of these polarization states. These studies revealed that M1 macrophages are characterized by expressing inflammatory chemokines (e.g. CXCL9, CXCL10), whereas M2 macrophages express non-inflammatory chemokines (e.g. CCL17, CCL18, CCL22, CCL24) (Martinez et al., 2006) (**Figure 1.2A**).

Similarly, M1 cells in respond to inflammatory stimuli produce high levels of inflammatory cytokines like TNF- α , IL-1 β , IL-12, IL-6, while M2 cells are poor in such a response and instead produce anti-inflammatory cytokines like IL-10 (**Figure 1.2A**) (Gerber and Mosser, 2001). As reviewed recently, M1 and M2 states also show distinct metabolic features relating to glucose, amino acid, lipid and iron metabolism (Biswas and Mantovani, 2012) (**Figure 1.2A**).

Although M1 and M2 phenotypes have been noted in *in vitro* transcriptomic studies as well as pathological settings such as certain tumors, obesity and helminth infections (Ghassabeh et al., 2006; Odegaard et al., 2007), it is increasingly evident that the *in vivo* situation may be more complex.

In vivo, due to the multiplicity of signals and dynamic changes in the microenvironment (e.g. during myocardial infarction, allergic skin and skeletal muscle injury) a purely M1 or M2 state may not be always visible, and instead, a mixture of these phenotype to varying degrees may be expected (**Figure 1.2B**) (Arnold et al., 2007; Egawa et al., 2013; Helm et al., 2014; Nahrendorf and Swirski, 2013; Stables et al., 2011). These observations have given rise to the view that M1 and M2 represent the extremes of spectrum of diverse macrophage phenotypes which occur in response to diverse microenvironmental stimuli (**Figure 1.2B**).

Supporting this concept, recent data on the transcriptome analysis of human macrophages stimulated with 28 or 33 different stimuli now demonstrate macrophages to trigger stimuli-specific transcriptional program resulting in a spectrum of activation states, each corresponding to a particular stimuli (Sudan et al., 2015; Xue et al., 2014). These developments have led to an effort in having a common language to describe macrophage activation and polarization. In this effort Murray et al proposed designating macrophages according to the stimuli they are activated with, for example, M(LPS), M(IL4) and so on (Murray et al., 2014).

A

B

Figure 1.2: Monocyte/macrophage activation and polarization states. (A) Some key characteristic features of M1 and M2 macrophages. Adapted from Biswas and Mantovani, 2012. (B) M1 and M2 as two extremes of a spectrum of macrophage activation states. Grey double arrows indicate the plasticity of these macrophage states, i.e. their ability to switch from one state to the other. Adapted from Xue et al., 2014.

Monocyte/macrophage response needs to be tightly controlled to maintain proper host defence and reinstate homeostasis. For example, a shift in the polarization state of monocytes/macrophages from an inflammatory (M1) to an immunosuppressive (M2-like) state is an important part of many pathophysiological processes such as inflammation and resolution (Auffray et al., 2007; Lawrence and Natoli, 2011). However any defect in this transition or a deregulated polarization of these cells can trigger an aberrant phenotype and function, leading to chronic inflammation or immunosuppression related disease conditions.

Abnormal monocytes/macrophages response has been demonstrated in many human diseases such as sepsis, cancer and atherosclerosis (Biswas et al., 2006; Martin-Fuentes et al., 2007; Swann et al., 2008). Therefore, investigating the aberrant phenotype and function of these cells provides an insight into the immunological and molecular basis of the disease itself.

1.5.3 Polarization of Monocytes/Macrophages in Obesity and T2D

Almost two decades ago, the first link between obesity, inflammation and IR was reported. These initial findings showed increased expression of tumor necrosis factor (TNF) and other pro-inflammatory cytokines in the adipose tissue of obese mice (Hotamisligil et al., 1993). Primary findings reported significant infiltration of macrophages but not neutrophils and lymphocytes into obese adipose tissue in mice (Xu et al., 2003). Simultaneously, other initial study in mice showed positive correlation between number of macrophages present in adipose tissue and adipocyte size as well as body mass (Weisberg et al., 2003). Thus, macrophages are an important source of pro-inflammatory cytokines and key mediator of chronic inflammation in adipose tissue associated with obesity and T2D (Weisberg et al., 2003; Xu et al., 2003).

Increased infiltration of monocyte/macrophage was possibly mediated by the increased expression of monocyte chemoattractant protein-1 (MCP-1/CCL2), as reported in adipose tissue of obese mice (Kamei et al., 2006; Weisberg et al., 2006). In line with this, mice lacking C-C motif chemokine receptor-2 (CCR2) had reduced macrophage content in their adipose tissue, increased adiponectin expression levels, enhanced glucose homeostasis and IR (Weisberg et al., 2006).

The exact mechanism on how obesity-associated metabolic signals translate into an inflammatory response is still not well understood. Analysis of the CD11c⁺ ATMs from obese mice showed a pro-inflammatory (M1) phenotype whereas those from lean animals had an M2-like phenotype characterized by expression of M2 markers like Ym1, Arginase 1 or IL-10 (Lumeng et al., 2007a) (**Figure 1.3**). In line with this observation, ablation of M1-like CD11c⁺ macrophages results in the normalization of IS and decreased expression of inflammatory markers (Patsouris et al., 2008). Other approaches involving macrophage depletion by using clodronate liposomes improved glucose tolerance IS and increased levels of adiponectin in circulation (Feng et al., 2011).

At a molecular level, the phenotypic switch of ATMs from lean to obesity was shown to involve Peroxisome Proliferator Activated Receptor- γ (PPAR- γ) (Hevener et al., 2007; Odegaard et al., 2007). In fact, inactivation of PPAR- γ in lean mice resulted in glucose intolerance and IR in both muscle and liver (Hevener et al., 2007; Odegaard et al., 2007). This phenotype was strongly associated with expression of inflammatory markers (Hevener et al., 2007). This corroborated the fact that a specific deletion of PPAR- γ in macrophage inhibited its polarization to an alternatively activated or M2 phenotype (Odegaard et al., 2007).

Further study revealed a localized recruitment of pro-inflammatory macrophages into adipose tissue of obese mice rather than a switch in the resident macrophage phenotype from M2 to M1 (Lumeng et al., 2008). In the same study, in lean mice, macrophages expressing galactose *N*-acetyl-

galactosamine specific lectin 1 (MGL1) were observed in the interstitial spaces in between adipocytes (independent of CCR2), whereas in the obese mice, heightened recruitment of MGL1⁺CCR2⁺ ATMs were observed in close proximity with necrotic adipocytes. This phenomenon was abolished in *Ccr2*^{-/-} mice (Lumeng et al., 2008).

Figure 1.3: Polarization of macrophages in diet induced obesity in mice. Macrophages in lean mice produce anti-inflammatory cytokines and are M2-like whereas in obese mice macrophages produce pro-inflammatory cytokines and are more M1-like. CLS – crown like structures; FFA – free fatty acids; DIO – diet induced obesity; NO – nitric oxide. Figure adapted from Lumeng et al., 2007a.

The above study along with several other studies in mice suggest the general view that an M1 macrophage phenotype is associated with obesity and one of the reasons for the development of IR (Odegaard and Chawla, 2008; Olefsky and Glass, 2010).

Several subsequent studies reported other phenotypic subtypes of ATMs in obese mice (Fujisaka et al., 2009; Shaul et al., 2010). Three different populations of macrophages were observed in HFD-fed mice at 8

weeks: a) interstitial macrophages MGL1⁺/CD11c⁻; b) Crown like structure(CLS)-associated macrophages which could be either MGL1⁻/CD11c⁺ or MGL1^{med}/CD11c⁺ (Shaul et al., 2010). Contrary to previous studies where M1-like CD11c⁺ macrophages were the predominant population producing inflammatory cytokines in obese mice, in this study, majority of the ATMs (65%) showed a mixed phenotype (MGL1^{med}/CD11c⁺) and expressed both M1 and M2 genes. However, with time, at 12 weeks of HFD, an increase of M2 genes and decrease of M1 genes was observed (Shaul et al., 2010).

Similarly, others reported increased infiltration of M2-like ATMs associated with increased production of IL-10 in obese mice fed HFD (Fujisaka et al., 2009). Later study showed that although CD11c⁺ ATMs were present in adipose tissue of HFD-fed mice, their number did not change once mice were fed normal chow and became IS (Li et al., 2010). This would mean that the initial hypothesis where M1-like ATMs were considered to be predominantly present in obese adipose tissue may need to be reconsidered.

While monocytes and macrophages have been found to infiltrate adipose tissues of obese humans (Cancello et al., 2006; Curat et al., 2004), the exact role of these cells in the pathogenesis of human metabolic syndrome such as T2D is still not well-characterized.

Polarization of ATMs in obese humans also remains very confusing. Some reported that ATMs in obese humans express M2-like surface markers but are also capable of expressing inflammatory cytokines (e.g TNF- α , IL-6 CCL2) both at basal levels and upon treatment, which can contribute to progress of IR (Zeyda et al., 2007). Flow cytometry study of human ATMs revealed two distinct populations comprised of: a) CD11c⁻CD206⁺ present at the adipocyte junctions; b) CD11c⁺CD206⁺ present in CLS (Wentworth et al., 2010). The CD11c⁻CD206⁺ ATMs expressed high levels of TNF- α , IL-6, IL-8, IL-1 β as well as IL-10, whereas the CD11c⁺

CD206⁺ ATMs expressed genes involved in lipid metabolism and T-cell chemoattractants (Wentworth et al., 2010).

More recently, it was shown that human ATMs in progressive obesity to be more M2-like rather than M1-like and the expression of some of the M2-like genes (e.g. CD163, IL-10) was found to correlate with the IR indicator, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (Fjeldborg et al., 2014). Moreover, the number of ATMs in human OM adipose tissue was also found to be a strong indicator of liver steatosis or fibro-inflammation (Tordjman et al., 2009). In other recent study, it was shown that increased number of ATMs in obese adipose tissue can also involve local proliferation of macrophages in these tissues in response to CCL2 (Amano et al., 2014; Haase et al., 2014). This increased proliferation of ATMs was more observed in the CLS and these macrophages expressed M2 markers like CD206 (Haase et al., 2014). Collectively, these studies call for re-consideration the widely held view that ATMs in obese animal or human are mostly pro-inflammatory/M1-like and perform a careful characterization of monocytes and macrophages in human obesity.

Contrary to all the studies showing inflammation in adipose tissue (mediated by M1-like macrophages) is detrimental, more recent study discussed the importance of adipose tissue inflammation for healthy adipose tissue expansion (Wernstedt Asterholm et al., 2014). Inhibition of pro-inflammatory response in adipose tissue rose ectopic lipid accumulation and glucose intolerance (Wernstedt Asterholm et al., 2014). This would mean that local adipose tissue inflammation can be rather adaptive response allowing storage of excess nutrients.

1.6 Other triggers involved in development of IR and T2D

1.6.1 Adipocyte death

One of the possible events attracting macrophages into obese adipose tissue is adipocyte death. Taking into consideration the phagocytic capacity of macrophages which is essential for the removal of death cells and cell debris, it was shown that about 90% of ATMs in obese adipose tissue was present around dying adipocytes (Cinti et al., 2005). In mice on HFD, around week 16 80% of adipocytes are death and correlate with increased amounts of IL-6, TNF- α as well as increased number of CD11c⁺ cells (Strissel et al., 2007). However, in adult humans it was showed that only 10% of adipocytes are renewed every year, regardless of their metabolic status (Spalding et al., 2008). Therefore, adipocyte death might play rather smaller role in development of T2D.

1.6.2 Free fatty acids (FFA)

In addition to adipokines, adipocytes release FFA. As primary cells storing fat, they gather FFA throughout triglyceride lipolysis and under IR state lipolysis fails to stop which in turn rises the levels of FFA (Guilherme et al., 2008).

It was reported that some of the FFA such as palmitate can activate inflammatory pathways for example Toll-like receptor-4 (TLR4) in macrophages (Shi et al., 2006). Contrary to that, some studies did not observe direct activation of TLR4 with FFA (Erridge and Samani, 2009). Saturated fatty acids, palmitate and stearate can induce production of inflammatory cytokines TNF- α , IL-8 and IL-1 β in human macrophages (Haversen et al., 2009). Similarly, several studies showed that mice lacking TLR4 and fed HFD are protected from development of IR (Davis et al., 2008; Kim et al., 2007).

1.6.3 ER stress

ER functions in a cell as an organelle responsible for protein production. If the ER homeostasis is disturbed, also named ER stress, the cell starts unfolded protein response in order to restore function of ER.

In obese humans and animals, it was reported increased ER stress (Boden and Merali, 2011; Ozcan et al., 2006). In fact, treatment of obese and diabetic mice with chemical chaperones could resolve hyperglycaemia, improve IS as well as insulin action in liver (Ozcan et al., 2006). Accordingly it was showed that ER stress can act as one of the factors involved in alternative macrophage differentiation (Oh et al., 2012).

1.6.4 Hypoxia

Due to storage of excess nutrients in obese humans and animals adipose tissue expands. Subsequently, insufficient adaptation of adipose tissue vasculature causes a lesser oxygen dispersion in the tissue and increases hypoxia. Indeed, studies in obese mice showed hypoxia in the adipose tissue associate with increased infiltration of macrophages and T-cells (Rausch et al., 2008). Other study showed hypoxia to be involved in decreased amounts of insulin sensitising hormone adiponectin (Ye et al., 2007). At the same time, inhibition of hypoxia-inducible factor 1 in adipocytes improves IS and production of adiponectin (Jiang et al., 2011). However, recent study used venous catheterisation in order to measure hypoxia in a human SC adipose tissue (Hodson et al., 2013). This study concluded that even though adipose tissue of obese individuals is less oxygenated, it is not hypoxic. Therefore, further investigation into the role of hypoxia in obesity and development of IR is necessary.

1.6.5 Endotoxemia

In recent years, increasing number of studies showed importance of gut microbiota in obesity and development of IR (Cani et al., 2007; Parks et al., 2013). It is indeed due to these gut microbiota that an increased plasma level of bacterial lipopolysaccharide (LPS) was observed (Cani et al., 2007). Study in human showed increased production of inflammatory cytokines such as TNF- α or IL-6 in adipocytes treated with LPS (Creely et al., 2007). At the same time, LPS can induce TLR4 signalling in obese animals (Cani et al., 2007).

1.7 Summary and Outstanding questions

In summary, current evidence in the field of obesity and T2D support the involvement of immune cells in adipose tissue inflammation and IR. In particular, monocytes/macrophages are key players in this process. While most studies from mice have indicated recruitment of these cells into adipose tissues where they mediate chronic inflammation, the scenario is more complex.

Neither a consistent phenotype for ATMs are evident across different mice studies available nor has been done a systematic analysis of macrophage polarization phenotype in obesity. Moreover, the plasticity of these cells may complicate assessing these cells phenotype. Interestingly, another confounding issue is the divergence between mice and humans. For example a 3 month HFD in mice can induce a more 'acute' inflammation than a more chronic and gradual induction of inflammation that occurs during a more gradual development of obesity, (e.g. taking couple of years) in humans. In fact, anti-inflammatory therapies have been successful in attenuating IR in mice, while such attempts have failed in clinical trials (Ofei et al., 1996; van Asseldonk et al., 2011).

In fact, there is very little information on the phenotypic and functional characterization of monocytes/macrophages in the human system during obesity and T2D. The lack of this information together with very little attempts to test the implication of the existing mice ATMs data (e.g. the M2 to M1 shift in macrophage phenotype) to human obesity and T2D has left many questions to be answered. For example: What is the contribution of monocytes/macrophages in human obesity and T2DM? Do human adipocytes from these settings crosstalk with monocyte/macrophages to modulate their polarization phenotype? Does this crosstalk result in an aberrant phenotype? Does this phenotype relate to a defective immune response and T2D pathogenesis?

The general hypothesis of this project is that an aberrant response of monocyte/macrophage plays a key role in the pathogenesis of obesity-related T2D in humans. In particular, we hypothesize that the interaction between monocytes/macrophages and adipocytes during this disease, results in polarizing monocytes/macrophages to an abnormal phenotype which favours pathogenesis.

1.8 Present Study: Rationale and objectives

In view of the unresolved issues and pending questions discussed above, we undertook the present study to investigate the role of monocytes/macrophages in human obesity and T2D. This is especially important since most of the data in this area come from mice and results from mice studies failed to validate in clinical trials, urging the need to study humans. This is one of the primary reasons why the present study was performed using the human system.

In particular, we investigate in this study the crosstalk between human monocytes and adipocytes in an obesity and obesity-related T2D setting.

The present study aimed to:

1. Investigate whether human adipocytes influenced the phenotype of human monocytes (and *vice versa*).
2. Investigate the polarization status of such monocytes and whether this relates to an aberrant or impaired immune phenotype.
3. Investigate whether the monocyte phenotype changes depending on the type of adipocytes (degree of adiposity): i.e. whether they are from overweight versus obese versus obesity-related T2D individuals.

Answers to these issues would provide valuable insights into the role of monocyte/macrophage in obesity and obesity-related T2D in humans.

2 Materials and Methods

2.1 Criteria for selection of patients

Study population consisted of subjects from four different cohorts (**Table 2.1**). Cohort 1 included lean subjects with a body mass index (BMI) of 20 to 23 kg/m². Cohort 2 subjects were overweight with a BMI of 23 to 27.5 kg/m². These subjects were all healthy male, aged 21 to 40 years old, non-smoking with no previous medical history or use of any medication 6 months prior to recruitment. Cohorts 3 and 4 were obese subjects recruited from both the National University Hospital and Khoo Teck Puat Hospital, aged 21 to 65 years old with a BMI of ≥ 35 kg/m² who opted for bariatric surgery. T2D was defined as having glucose intolerance with a fasting glucose of >6.0 mmol/L. A detailed medical history was collected from each subject and this included physical examination, presence of other cardiovascular disease risk factors (e.g., hypertension, dyslipidemia), presence of albuminuria, and all existing medications prescribed. Adipose tissue specimens were obtained from these patients from both the OM and SC abdominal area at the time of bariatric surgery without any additional surgical procedure. Subjects in this study were excluded if they had a history of type 1 diabetes, malabsorption syndrome, Crohn's disease, ulcerative colitis, pancreatitis, and a history of steroid use (e.g., corticosteroids). Plasma samples were available from all the four cohorts. However, adipose tissue for pre-adipocyte isolation was available only from a limited number of patients undergoing bariatric surgery: n=7 for Obese-Diabetic (OB-DB), n=5 for Obese (OB) and n=3 for healthy, overweight (OW) subjects. All subjects provided a written informed consent. Ethics approval was obtained from the National Healthcare Group Domain Specific Review Board (Singapore).

Table 2.1: Baseline characteristics of subjects.

Cohorts	(1) Lean (n= 9)	(2) Overweigh t (n= 32)	(3) Obese (n=39)	(4) Obese Diabetic (n=20)
Age (y)	23.33 ± 0.71	30.22 ± 6.35	37.62 ± 10.54	38.90 ± 9.67
BMI (kg/m ²)	22.03 ± 0.51	24.99 ± 1.83	38.19 ± 7.75	39.8 ± 4.85
HbA1c (%) ¹	ND	5.37 ± 0.25	5.68 ± 0.49	ND
Fasting Plasma Glucose (mmol/L)	4.33 ± 0.17	4.43 ± 0.28	5.21 ± 0.69	9.90 ± 3.42
Insulin (mU/L) ²	4.31 ± 1.55	9.05 ± 3.99	19.68 ± 13.37	10.68 ± 10.29
HOMA-IR ²	0.83 ± 0.31	1.79 ± 0.82	4.56 ± 3.54	3.9 ± 4.11
Triglycerides (mmol/L) ³	0.62 ± 0.20	0.99 ± 0.49	1.75 ± 0.88	2.36 ± 3.05
Total cholesterol (mmol/L) ³	5.09 ± 0.81	4.64 ± 0.86	5.18 ± 1.03	4.79 ± 0.85
HDL cholesterol (mmol/L) ³	1.71 ± 0.26	1.31 ± 0.32	1.22 ± 0.29	1.19 ± 0.22
LDL cholesterol (mmol/L) ⁴	3.09 ± 0.87	2.88 ± 0.80	3.27 ± 0.92	2.80 ± 0.70

Values are expressed as mean ± standard deviation. ¹For HbA1c, n = 31 for overweight subjects, n = 11 for obese subjects, ND = not determined. ²For Insulin and HOMA-IR, n = 29 for obese subjects, and n= 10 for obese diabetic subjects. ³For Triglycerides, Total cholesterol and HDL cholesterol, n = 33 for obese subjects, and n = 18 for obese diabetic subjects. ⁴For LDL cholesterol, n = 18 for obese subjects, and n = 17 for obese diabetic subjects.

2.2 Isolation and culture of human peripheral blood monocytes

Human blood was obtained from the Blood Bank of Singapore Health Science Authority. PBMCs were then isolated from apheresis blood by Ficoll-Paque PLUS (GE Healthcare Life Sciences AB, Upsala, Sweden) density gradient centrifugation method. Monocytes were then isolated from PBMC using human CD14 microbeads and LS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell number and

viability was assessed by using haemocytometer and Trypan blue (Invitrogen™, Life Technologies, Carlsbad, USA). Subsequently, isolated monocytes were plated into the 12-well plate (Corning® Costar®, Sigma-Aldrich, St. Louis, USA) for experiment with adipocyte conditioned media or for co-culture experiment into the 12-well plate inserts (Transwell®, Corning Incorporated Life Sciences, Tewksbury, USA) at 1.5×10^5 cells/well in 1 ml/well incomplete Iscove's Modified Dulbecco's Medium (IMDM) media (HyClone Modified IMDM containing HEPES and 4 mM L-glutamine without serum) for 1 h in tissue culture incubator at 37 °C and 5% CO₂. Afterwards, the incomplete IMDM was removed and replaced with 1ml/well of complete IMDM containing 5 % human serum (Gemini Bio Products, West Sacramento, USA) and Pen Strep (Invitrogen, Gibco Life Technologies, Carlsbad, USA). Cells were then rested for 1 h and treated as described in the section about co-culture of human blood monocytes with human SC and OM adipocytes.

2.3 Primary human SC and OM adipocytes

Freshly isolated adipose tissue was processed as described previously with some modifications (McGillicuddy et al., 2009). Briefly, adipose tissue was minced and digested with 1 mg/ml collagenase (Sigma-Aldrich) and 2% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h in a 37°C incubator shaker. Digested tissue was filtered through 100µm cell strainer and centrifuged at 400 g for 5 min. The pellet formed (stromal vascular cells) was subsequently subjected to 3 rounds of washing each in 10 ml Hank's balanced salt solution (HBSS) (GIBCO). The pellet, containing adipose tissue macrophages (ATMs), was then re-suspended in 10 ml Dulbecco's modified Eagle medium (DMEM) (GIBCO) containing 15% foetal bovine serum (Thermo Scientific), 1% non-essential amino acids (NEAA) (GIBCO), 1 µg/ml human recombinant fibroblast growth factor (FGF) (GIBCO) and 1% penicillin/streptomycin (GIBCO). Cell suspension was then incubated in a 10 cm Petri dish at 37°C for 1 h after which the non-adherent cells, containing adipose-derived stromal cell

population, were transferred to a T75 culture flask. Human pre-adipocytes were ready to be differentiated when they displayed fibroblastic morphology. Pre-adipocytes were then plated at a density of 0.05×10^6 cells/well in a 12-well plate. Mature SC/OM adipocytes at day 16 were then used for co-culture or conditioned media experiments with monocytes. The original adipocytes maintenance media was removed before co-culture experiment due to insulin content, which could have a potential influence on monocytes. Original media was replaced with fresh complete IMDM media (1 ml/well) and left overnight. On the following day, overnight medium was collected for experiments with conditioned media, stored at -80°C and new complete IMDM medium was added into the wells containing adipocytes. Adipocytes were then rested for 1 h and used for co-culture experiment.

2.4 Co-culture of human blood monocytes with human SC and OM adipocytes

Inserts with monocytes were placed into the wells with attached adipocytes and left co-culture overnight. Next day, media were removed and both types of cells were lysed using 1ml/well TRIzol[®] Reagent (Invitrogen[™], Life Technologies, Carlsbad, USA) and stored at -20°C for further analysis.

2.5 Culture of human blood monocytes with SC and OM adipocytes conditioned media (CM)

Monocytes were pre-incubated with IMDM or SC/OM conditioned media (CM) containing 5% human serum and left overnight. Next day the media was removed and replaced with fresh complete IMDM and treated with 100 ng/ml LPA (Gomez-Flores et al., 1997) and 20 ng/ml IFN γ (Pelegrin and Surprenant, 2009) or 10 ng/ml IL-4 (Bonder et al., 1999; Satoh et al., 2010) and left to incubate overnight. After incubation, cell-free

supernatants were collected and stored at -80 °C, while monocytes lysed using TRIzol[®] Reagent and stored at -20 °C for further analysis.

2.6 Real-time or quantitative PCR (Q-PCR)

Adipocytes and monocytes were lysed in TRIzol[®] Reagent and total RNA was prepared and DNase treated using Nucleo Spin II kit (Macherey Nagel, Dueren, Germany) according to the manufacturer's instructions. The purity of RNA was assessed by measuring the ratio of A260/280 nm, using a spectrophotometer, NanoDrop 1000 (Thermo Scientific, Wilmington, USA). RNA samples showing a 260/280 ratio of 1.8–2.0 was considered to be of good quality. Subsequently, total RNA (0.27 µg) was reverse transcribed using TaqMan Reverse Transcription kit (Invitrogen[™], Applied Biosystems, Life Technologies, Carlsbad, USA) as described in manufacturer's instructions on a 2720 Thermal Cycler (Invitrogen[™], Applied Biosystems, Life Technologies, Carlsbad, USA). Two µl of cDNA was then used to carry out Q-PCR using human primers and iQ[™] SYBR Green Supermix (Bio-Rad, Hercules, USA) on a CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, USA). Data were processed and analysed by Bio Rad CFX Manager 2.1 software (Bio-Rad, Hercules, USA) and normalized to housekeeping gene β -actin gene expression levels. All gene expression data were expressed as a fold change in mRNA expression as comparison to the control cells.

2.7 ELISA

Cell-free overnight supernatants from experiment performed with SC/OM adipocyte CM were analysed using ELISA against different cytokines. Human PARC/CCL18 ELISA kit and human TARC/CCL17 ELISA kit was purchased from (Sigma-Aldrich, St. Louis, MO, USA). The assay was performed according to manufacturer's instructions. All cell supernatants were used directly while human plasma was diluted 300 times in order to measure the concentration of CCL18. Colour intensity of

samples was measured at 450 nm on TECAN Infinite® 200 PRO System (Tecan Group AG, Männedorf, Switzerland), using TECAN-icontrol version 3.4.2.0 software. Results were expressed in pg/ml.

2.8 Human ATMs sorting

The adipose tissue was processed as described in section 2.3 Primary human SC and OM adipocytes. Whole cell suspension containing adipocytes and SVC were pelleted and washed with FACS buffer [DPBS, 0.5% BSA (Sigma-Aldrich, St. Louis, MO, USA), 2mM EDTA (Ambion, Life Technologies, Eugene, OR, USA), 0.1% sodium azide (BDH, 103692K)]. Cells were then stained against CD14, CD45, CD11c and CD206 as previously published (Wentworth et al., 2010).

Table 2.2: List of antibodies used for ATMs isolation.

Antibody	Specification	Company
CD14	FITC	MACS, Miltenyi Biotec, Bergisch Gladbach, Germany
CD45	PerCP-Cy 5.5	eBioscience, San Diego, CA, USA
CD11c	PE	eBioscience, San Diego, CA, USA
CD206	APC	eBioscience, San Diego, CA, USA

For list of antibodies used refer to the **Table 2.2**. After 30 min incubation cell were washed twice with FACS buffer and resuspended to desired volume. Subsequently cells were separated by using BD FACS Aria™ II sorter (BD Bioscience, San Jose, CA, USA) and appropriate software BD FACS Diva version 8.0.1. ATMs were then collected and lysed by using 500 µl of 1ml/well TRIzol® Reagent.

2.9 Immunofluorescence

Immunofluorescence was performed on paraffin embedded SC and OM adipose tissue from obese subjects. Paraffin embedded tissue sections were first de-paraffinised as per standard protocol. De-paraffinised slides were microwaved in Dako targeted retrieval solution (Dako, Glostrup, Denmark) for 15 min and subsequently permeabilized with 0.2% Triton X-100 (Merck, Darmstadt, Germany) for 45 min at room temperature. Slides were blocked with normal goat serum (Dako, Glostrup, Denmark) for 1h at room temperature in the dark humidified chamber. Primary and secondary antibodies were diluted as per manufacturer recommendation in antibody diluent (Dako, Glostrup, Denmark). For the list of antibodies used, please refer to **Table 2.3**. Sections were incubated with required primary antibodies overnight in the dark humidified chamber at 4 °C and with secondary antibodies for 1h at room temperature. Lastly the sections were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 15 min in the dark at room temperature. Slides were mounted with fluorescent mounting media (Dako, Glostrup, Denmark) and visualized by using Olympus IX81 (Olympus, Tokyo, Japan).

Table 2.3: List of antibodies used for adipose tissue immunofluorescence.

Antibody	Specification	Company
CD68	Primary antibody – Mouse anti-human	Biolegend, San Diego, CA, USA
CCL18/PARC	Primary antibody – Goat anti-human	R&D Systems, Minneapolis, MN, USA
Goat IgG control	Isotype	R&D Systems, Minneapolis, MN, USA
Alexa Fluor 488	Secondary antibody – Donkey anti-goat	Life Technologies, Eugene, OR, USA
Alexa Fluor 568	Secondary antibody – Donkey anti-mouse	Invitrogen, Molecular Probes, Eugene, OR, USA

2.10 Statistical analysis

Statistical analysis was performed using the paired t-test on a GraphPad Prism software. All statistical tests for qPCR were performed on the gene expression values normalized to that of the house keeping gene, β actin. $p < 0.05$ was considered statistically significant. Statistical analysis of CCL18 concentration in plasma was performed using One-way ANOVA followed by Dunn's Multiple Comparison Test. Correlation studies were performed using Pearson and Spearman test based on the normality test.

3 Results

3.1 Monocytes show modulation in their basal expression of inflammatory and non-inflammatory genes upon interaction with adipocytes from obese-diabetic, obese or overweight individuals.

Studies in mice have shown that monocytes and macrophages infiltrate adipose tissue during obesity and contribute to the pathogenesis of obesity-related metabolic disease (Lumeng et al., 2007a; Xu et al., 2003). However, in humans, the interaction of monocytes with adipocytes and how this may skew the phenotype of these cells to support pathogenesis of obesity-related metabolic disease is poorly understood.

As a first step towards understanding the interaction between monocytes and adipocytes in humans, we used a co-culture approach wherein blood monocytes from healthy donors were cultured alone or in the presence of SC or OM adipocytes from obese (OB) or obese-diabetic (OB-DB) individuals in a transwell co-culture system. Thereafter, the monocytes were screened for the expression of a panel of inflammatory (M1) (e.g. *IL6*, *IL1B*, *IL12p40*, *CXCL10* and *CCL5*) and non-inflammatory (M2) genes (e.g. *CCL18*, *CCL17*) to assess their polarization state upon interaction with the adipocytes. In addition, other non-inflammatory M2 genes (*CCL22*, *IL10*) and some metabolic genes (e.g. *CD36*, *SCD*) were initially measured. However, these genes did not show any consistent and/or significant modulation through our experiments and were excluded from further studies (**Appendix Figure 1.1A-B**).

As compared to monocytes that were cultured alone (Mo), monocytes co-cultured with SC adipocytes from obese-diabetic individuals [Mo+Adipo SC (OB-DB)] showed an increased basal expression for all the five M1 cytokines/chemokines genes i.e. *IL6*, *IL1B*, *IL12p40* (from now on

labelled as IL12) *CXCL10* and *CCL5* (**Figure 3.1A**). However, significant change in expression was observed for *IL6*, *IL1B*, *IL12* and *CCL5*. The M2 genes *CCL18* and *CCL17* also showed a significantly increased basal expression in the adipocyte co-cultured monocytes as compared to Mo (**Figure 3.1A**).

Next, we checked the effect of monocytes co-cultured with SC adipocytes from obese individuals (OB). As evident from (**Figure 3.1B**), monocytes co-cultured with SC (OB) adipocytes displayed a similar effect on the expression of M1-M2 genes with only *IL1B*, *CXCL10*, *CCL5*, *CCL18* and *CCL17* showing significant increase compared to Mo.

To cover the entire spectrum of adiposity, ranging from overweight to obese to obesity-induced insulin resistance, we decided to also include SC adipocytes from healthy, overweight individuals (OW), to serve as a 'non-obese' internal control for the OB and OB-DB adipocytes. Surprisingly, monocytes when co-cultured with SC (OW) adipocytes showed a marked and significant increase in basal gene expression level of all the M1 and M2 genes studied when compared to Mo (**Figure 3.1C**). Interestingly, the upregulation of these M1 and M2 genes was much higher to those seen for monocytes co-cultured with OB or OB-DB adipocytes (compared with **Figure 3.1A, B**).

A similar trend of increased basal expression of the M1 and M2 genes was observed in Mo co-cultured with OM (OB-DB) adipocytes with minor deviations (**Figure 3.2A**). Although, the M1 and M2 gene expression was increased in monocytes co-cultured with SC (OB) adipocytes this could not be clearly observed in monocytes co-cultured with OM (OB) adipocytes though *IL6* and *CXCL10* did show a trend (**Figure 3.2B**).

Collectively, the above results demonstrated that co-culturing monocyte with adipocytes induced an increased gene expression of both M1-M2 cytokines and chemokines in these cells. However, the effect on monocyte inflammatory gene expression depended on whether OW, OB or

OB-BD adipocytes were used, with the most marked increase being observed upon exposure to SC adipocytes from OW individuals, while those exposed to SC adipocytes from OB or OB-DB individuals, showing clearly a lesser increase.

Figure 3.1: Modulation of inflammatory and non-inflammatory genes in monocytes – SC adipocytes co-culture. Basal gene expression level of inflammatory or non-inflammatory genes in obese/diabetic (A), obese (B) or overweight individuals (C). Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic, n=5 donors for obese and n=3 donors for overweight. Statistically significant was considered * $p < 0.05$.

A

B

Figure 3.2: Modulation of inflammatory and non-inflammatory genes in monocytes – OM adipocytes co-culture. Basal gene expression level of pro-inflammatory or non-inflammatory genes in OB-DB (A), OB (B). Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic and n=4 donors for obese. Statistically significant was considered * $p < 0.05$.

3.2 Adipocytes upregulate their basal expression of inflammatory genes upon interaction with monocytes

Having determined the effect of adipocyte-monocyte co-culture on the basal gene expression of monocytes, we investigated next the effect of this co-culture on adipocyte basal gene expression.

SC adipocytes from OB-DB individuals upon co-culturing with healthy donor monocytes [Adipo SC (OB-DB)+Mo, **Figure 3.3A**] showed trend of increased expression of inflammatory genes *IL6*, *IL1B* and *CCL5*, as compared to the adipocytes that were cultured alone [Adipo(OB-DB), **Figure 3.3A**]. Among these upregulated genes, none of the genes showed statistical significance (**Figure 3.3A**).

The expression of non-inflammatory M2 genes *CCL17* and *CCL18* in the co-cultured adipocytes was minimal (**Figure 3.3A**, right panel) and did not show any modulation as these are human monocyte-macrophage lineage specific genes (Mantovani et al., 2002). Therefore, further assessment of these genes in the adipocytes was discontinued. The same case was true for a few adipocyte metabolic genes (e.g. *CD36*, *SCD*, *ACAT*, *LIPE*) which were initially included in our assays, but later discontinued due to the lack of any consistent modulation (data not shown).

Study on the basal gene expression in SC adipocytes from OB individuals upon co-culture with normal monocytes also demonstrated a significant increase in the expression of inflammatory genes *IL6* and *CCL5* as compared to SC (OB) adipocytes that were cultured alone (**Figure 3.3B**).

Lastly, when SC adipocytes from OW individuals were co-cultured with monocytes, they showed a marked increase in all the four inflammatory genes with *IL6*, *IL1B* and *CCL5* displaying significant

upregulation as compared to the adipocytes cultured alone (**Figure 3.3C**). Interestingly, increase in inflammatory gene expression in these SC (OW) adipocytes was strikingly higher than those in SC (OB/DB) or SC (OB) adipocytes upon co- culture with monocytes (**Figure 3.3C**).

Similar trend as seen in SC OB-DB co-cultured monocytes was also observable for OM adipocytes from OB-DB individuals upon co-culturing with monocytes, wherein they significantly upregulated *IL6* but not other genes studied (**Figure 3.4A**). Recapitulating a similar trend as in SC (OB) also OM (OB) adipocytes co-cultured with monocytes showed an increase in *IL6* and *CCL5* expression with both showing statistical significance (**Figure 3.4B**).

Collectively, the above results indicated a general trend of upregulated basal expression of inflammatory cytokine/chemokine genes in SC/OM adipocytes upon co-culturing with normal monocytes. However, the degree of upregulating these inflammatory genes varied depending on whether the adipocytes came from OB-DB, OB or OW individuals.

Figure 3.3: Basal gene expression level of inflammatory or anti-inflammatory genes in SC adipocytes. Co-culture in obese/diabetic (A), obese (B) or overweight patients (C). Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic, n=5 donors for obese and n=3 donors for overweight. Statistically significant was considered * $p < 0.05$.

A **B**

Figure 3.4: Basal gene expression level of inflammatory or anti-inflammatory genes in OM adipocytes. Co-culture in obese/diabetic (A), obese (B). Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic and n=5 donors for obese. Statistically significant was considered * $p < 0.05$.

3.3 Monocytes exposed to conditioned media from OB-DB and OW adipocytes show differential modulation of gene expression under basal and polarizing conditions.

Our previous results indicated that co-culturing monocytes with adipocytes modulated the basal expression for specific M1-M2 genes in these monocytes. As a second approach to confirm these data and to ascertain whether soluble factors released by adipocytes were responsible for inducing these changes in the monocytes, we treated normal monocytes with the conditioned medium from OW, OB and OB-DB adipocytes and study their basal gene expression.

Cell-free conditioned medium (CM) were collected from overnight cultures of SC and OM adipocytes from OW, OB and OB-DB individuals. Thereafter, monocytes from healthy donors were cultured overnight in normal media or conditioned media from adipocytes and expression of the same panel of M1 and M2 as described earlier was studied.

Figure 3.5A shows that monocytes cultured with CM of OB-DB adipocytes displayed a general increase in the basal expression of all the indicated M1 genes with *IL1B* and *CXCL10* showing a significant upregulation, as compared to monocytes cultured in normal medium (**Figure 3.5A**). From M2 genes only *CCL18* was significantly upregulated in these CM-treated monocytes (**Figure 3.5A**).

In line with these observations, monocytes cultured with CM of OB adipocytes also showed a similar trend with significant upregulation of *IL6*, *IL1B* and *CXCL10* with respect to monocytes cultured in normal medium (**Figure 3.5B**). Both *CCL17* and *CCL18* showed significant upregulation in these CM-cultured monocytes (**Figure 3.5B**).

In contrast to above data, when monocytes were cultured with CM from OW adipocytes, they exhibited a marked and significant upregulation

of M1 genes *IL6*, *IL1B* and *CCL5* gene expression (**Figure 3.5C**). In fact, the degree of upregulation of these genes was much higher to that induced in the monocytes treated with OB-DB or OB adipocyte CM. M2 genes *CCL17* and *CCL18* also showed a consistent and significant increase in the OW adipocyte CM treated monocytes (**Figure 3.5C**).

Monocytes cultured with CM from OM OB-DB and OB adipocytes also showed a similar trend in upregulation of several M1 and M2 genes as compared to monocytes cultured with normal medium, although this varied depending on OB or OB-OB individuals (**Figure 3.6A-B**).

Figure 3.5: Basal gene expression in monocytes cultured with SC adipocyte conditioned media. Monocytes were cultured overnight in normal medium (Mo) or CM from SC adipocytes of obese/diabetic (A), obese (B) and overweight individuals (C). Expression was measured by real-time PCR with actin being as the housekeeping gene. The basal gene expression data are presented as mean $\pm$ SEM, from $n=7$ donors for obese/diabetic, $n=5$ donors for obese and $n=3$ donors for overweight. Statistically significant was considered * $p<0.05$.

A

B

Figure 3.6: Basal gene expression in monocytes cultured with OM adipocyte conditioned media. Monocytes were cultured overnight in normal medium (Mo) or CM from OM adipocytes of obese/diabetic (A), and obese (B) individuals. Expression was measured by real-time PCR with actin being as the housekeeping gene. The basal gene expression data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic, and n=5 donors for obese. Statistically significant was considered * $p < 0.05$.

Having demonstrated that exposure to adipocyte CM differentially modulated the basal expression of M1 and M2 genes in monocytes, we next asked whether exposure to adipocytes CM also impacted the response of monocytes to defined M1 or M2 polarizing stimuli and their target gene expression. To study this, monocytes were first cultured in normal medium or with CM from OB-DB, OB or OW adipocytes overnight. Thereafter, cells were put in normal medium and challenged with the prototypical M1 stimuli, LPA+IFN γ or M2 stimuli, IL-4. Cells were then assessed for their ability to upregulate the expression of the M1 or M2 target genes.

Upon IFN γ +LPA treatment, monocytes cultured in normal medium hugely upregulated the expression of M1 inflammatory genes *IL6*, *IL1B*, *IL12*, *CXCL10* and *CCL5*, as expected. In comparison to these monocytes, monocytes cultured with CM from OB-DB adipocytes exhibited a markedly downregulated expression (i.e. lesser induction) of all the above M1 genes. However, the trend was opposite for IL-4-treated monocytes and M2 gene induction (**Figure 3.7A**). Monocytes cultured with CM from SC OB-DB adipocytes, showed a significantly increased expression of *CCL17* and *CCL18* as compared to monocytes cultured in normal medium, upon IL-4 stimulation. These results indicate that CM from OB-DB adipocytes inhibited M1 polarization of monocytes in response to IFN γ +LPA, while augmenting its M2 polarization in response to IL-4.

Next, we repeated the same experiment with CM from OB adipocytes. Upon IFN γ +LPA stimulation, monocytes cultured in normal medium upregulated all M1 genes. In contrast, monocytes cultured in CM from SC OB adipocytes demonstrated a consistent lower response for *IL6*, *IL1B*, *IL12* whereas *CXCL10* showing a significantly higher expression and *CCL5* showing similar levels of expression as compared to IFN γ +LPA treated monocytes that were cultured in normal medium (**Figure 3.7B**). However, upon IL-4 treatment, monocytes cultured with CM of OB adipocytes as compared to those cultured in normal medium showed a

marked increase in *CCL17* and *CCL18* expression with *CCL18* showing significance (**Figure 3.7B**).

Lastly, we compared the response of monocytes cultured in OW adipocyte CM to that of monocytes cultured in normal medium, upon IFN γ +LPA stimulation. Monocytes cultured with OW adipocyte CM showed a significant and massive increase in the expression of inflammatory genes *IL6*, *IL1B* and *IL12* when compared to monocyte cultured in normal medium (**Figure 3.7C**). In fact, the increased expression of these inflammatory genes was in contrast to their inhibition in monocytes cultured with OB-DB or OB adipocyte CM. However, the IFN γ +LPA induced *CXCL10* and *CCL5* expression in the OW adipocyte CM cultured monocytes showed no change (*CXCL10*) or decreased expression (*CCL5*) with respected to their normal medium control (**Figure 3.7C**). In case of IL-4 treatment, monocytes cultured with CM from OW adipocytes showed significantly increased *CCL17* but not *CCL18* expression in comparison to monocytes cultured in normal medium (**Figure 3.7C**). This was in contrast to the IL-4 response in monocytes exposed to OB-DB adipocyte CM which showed a huge upregulation of *CCL18* but not *CCL17* (**Figure 3.7A**).

A decreased expression of M1 genes like *IL6*, *IL1B* and *IL12* could also be seen for the monocytes cultured overnight with CM from OM adipocytes of OB-DB or OB individuals, upon IFN γ +LPA treatment (**Figure 3.8A**, compare with IFN γ +LPA treated monocytes cultured in normal medium) (**Figure 3.8B**). However, the expression of *CXCL10* somehow show opposing trends and failed to meet statistical significance in response to OB vs. OB-DB adipocyte CM. However, both M2 genes *CCL18* and *CCL17* were significantly increased upon IL-4 stimulation in these samples (**Figure 3.8B**).

Figure 3.7: Gene expression in monocytes cultured with SC adipocyte conditioned media, following treatment with M1 and M2 stimuli. Monocytes cultured with CM from obese/diabetic (A), obese (B) and overweight (C) adipocytes. Expression was measured by real-time PCR with actin being as the housekeeping gene. The gene expression data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic, n=4 donors for obese and n=3 for overweight. Statistically significant was considered * p<0.05.

Figure 3.8: Gene expression in monocytes cultured with OM adipocyte conditioned media, following treatment with M1 and M2 stimuli. Monocytes cultured with CM from obese/diabetic (A), or obese (B) adipocytes. Expression was measured by real-time PCR with actin being as the housekeeping gene. The gene expression data are presented as mean $\pm$ SEM, from n=7 donors for obese/diabetic, and n=4 donors for obese. Statistically significant was considered * $p < 0.05$.

In conclusion, exposing monocytes to adipocyte CM skewed their response to M1- or M2-polarizing stimuli. For example, IFN γ +LPA induced an massive increase in the expression of inflammatory cytokine genes *IL6*, *IL1B* and *IL12* in monocytes cultured with OW adipocyte CM, whereas those cultured with OB-DB adipocyte CM showed a marked inhibition of these genes, in response IFN γ +LPA . Concomitantly, OW adipocyte CM induced a modest increase in *CCL18* expression, whereas OB-DB adipocyte CM induced it's markedly increase in monocytes, upon IL-4 treatment. These suggest OW adipocytes to preferentially induce an inflammatory (M1) phenotype in monocytes, while OB-DB adipocytes to induce a M2-like, immunosuppressive phenotype in monocytes.

3.4 Monocytes exposed to OB-DB adipocyte conditioned medium show increased production of chemokines CCL17 and CCL18.

Since monocytes cultured with CM from OB-DB adipocytes displayed a consistent M2-like phenotype characterized by the enhanced expression of M2 genes *CCL17* and *CCL18* under basal and IL-4 stimulated condition, we decided to further validate this observation at a protein level. For this purpose, the production of chemokine CCL17 and CCL18 was measured in the supernatants of monocytes that were cultured in normal medium or OB-DB SC/OM adipocyte CM for overnight and subsequently challenged with the M2-stimuli, IL-4.

Monocytes exposed to the OB-DB adipocyte CM displayed a significant increase in CCL18 and CCL17 as compared to monocytes cultured in normal medium (Mo), upon IL-4 treatment (**Figure 3.9A-B**). Moreover, a similar trend of enhanced CCL17 and CCL18 production could also be recapitulated in IL-4-treated monocytes that were cultured in CM from OM (OB-DB) adipocytes versus normal media (**Figure 3.9C-D**).

The above results corroborate the gene expression data confirming that indeed exposure to OB-DB adipocyte or its conditioned media skewed macrophages to an M2-like phenotype characterized by upregulated expression the chemokines CCL17 and CCL18 both at a gene and protein levels. Notable, among these two chemokines, CCL18 showed a more consistent and significant increase both under basal (untreated) and IL-4 treated conditions in the adipocyte CM exposed monocytes and therefore followed-up in subsequent experiments.

A **B** **C** **D**

Figure 3.9: Increased production of CCL18 and CCL17 in monocytes cultured with SC or OM adipocyte conditioned media. Increased production of CCL18 (A-C) and CCL17 (B-D) can be measured in monocytes cultured with adipocytes CM from OB/DB adipocytes and treated with IL-4. Concentration of CCL18 and CCL17 was measured by sandwich ELISA. The data are presented as mean $\pm$ SEM, from n=5 donors for CCL18 and n=3 donors for CCL17, SC and OM indicate different sources of adipocytes. ND stands for the not detectable concentration of protein. Statistically significant was considered * $p < 0.05$.

3.5 Adipose tissue macrophages from OB individuals show increased CCL18 expression in vivo

Having demonstrated that monocytes upregulated the expression of the M2 chemokine CCL18 upon interaction with OB-DB or OB adipocytes CM *in vitro*, we wanted to find out next if this observation was also true *in vivo*. For this purpose, we isolated SC and OM adipose tissue macrophages (ATMs) from OB individuals and investigated the expression of CCL18 in these cells.

ATMs were FACS sorted from adipose tissue of OB individuals based on CD14, CD45, CD11c and CD206 expression as described before (Wentworth et al., 2010) (**Figure 3.10A-left and right**). qPCR analysis of these ATMs showed increased expression of CCL18 as compared to control macrophages (i.e. monocyte-derived macrophages, MDMs) from healthy donors (**Figure 3.10B**). The same increased expression was confirmed in ATMs from OM adipose tissue (**Figure 3.10C**)

Immunofluorescence staining also confirmed CCL18 expression by OB ATMs in adipose tissue (**Figure 3.11A-B**). Together, the above data confirm the increased expression of CCL18 in ATMs from obese individuals *in vivo*, corroborating our findings from the *in vitro* studies.

Figure 3.10: Increased expression of CCL18 by SC and OM ATMs from OB patients. ATMs from SC or OM adipose tissue depots were isolated from lymphocytes and preadipocytes based on expression of CD14, CD45 (A-left panel) and based on expression of macrophage markers CD206 and CD11c (A-right panel). Increased expression of CCL18 was measured by qPCR in ATMs from SC (B) and OM (C) depots of obese individuals. Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, for ATMs from obese $n=3$ donors, with two replicates. Statistically significant was considered * $p<0.05$.

Figure 3.11: Expression of CCL18 by SC ATMs from OB patients. Immunofluorescence staining was done on SC adipose tissue from OB subject using (A) anti-human CD68 (red) and anti-human CCL18 (green) as well as with appropriate isotype control where staining as expected did not occur (panel B).

3.6 Elevated amount of CCL18 in the plasma of OB-DB and OB patients.

Since both our experiments showed monocytes interaction with adipocytes (or their CM) *in vitro* as well as ATMs *in vivo* from OB individuals correlated with increased expression of CCL18, we wonder whether elevated levels of this chemokine could also be detected in the circulation of these individuals.

Figure 3.12A demonstrates the concentration of CCL18 present in the plasma of healthy lean, OW, OB and OB-DB individuals, as measured by ELISA. As evident, plasma from OB and OB-DB subjects showed significantly higher concentration of CCL18 as compared to that of healthy lean or OW individuals. In addition, a trend showing a gradation in CCL18 from OB-DB→OB→OW could be also detected (**Figure 3.12A**). Since OB and OB-DB subjects showed increased concentration of CCL18, we investigated a possible relationship between concentration of CCL18 in plasma and the clinical parameters related to obesity and T2D (i.e. plasma glucose, Hb1c, triglycerides). Analysis of the present cohort of patients showed that concentration of CCL18 correlated significantly with plasma glucose and triglyceride levels (**Figure 3.12B-C**).

In conclusion these data not only show a significantly increased concentration of CCL18 in plasma in OB and OB-DB subjects but also its relationship to some of the important clinical parameters associated with obesity and T2D.

Figure 3.12: Elevated amounts of CCL18 in the plasma of OB-DB and OB individuals and correlation between CCL18 plasma levels and some clinical parameters of insulin resistance/obesity. (A) ELISA measurement of CCL18 plasma levels in lean (n=13), overweight (OW n=25), obese (OB n=37), obese-diabetic (OB-DB n=20) individuals. Correlation analysis of CCL18 plasma levels (lean, OW, OB and OB-DB) with (B) levels of fasting plasma glucose (n=89), (C) amount of triglycerides (n=79). Statistically significant was considered * $p < 0.05$.

4 Discussion

It is believed that during obesity, infiltrating blood monocytes/macrophages contributes to low-grade inflammation in the adipose tissues which promotes the pathogenesis of metabolic diseases. Most of these observations come from mice studies which indicate macrophages to possess an inflammatory M1 phenotype expressing TNF- α (Hotamisligil et al., 1993), and other pro-inflammatory cytokines (Weisberg et al., 2003; Xu et al., 2003) during obesity. These pro-inflammatory cytokines in turn amplify local inflammation and induce insulin resistance in adipocytes. In spite of these studies, the crosstalk between monocytes/macrophages and adipocytes, how this interaction polarizes monocytes/macrophages and how it impacts the pathogenesis of insulin resistance and obesity-related metabolic disease is still not well understood. Importantly, the situation is even lesser known in humans.

In the present study, we attempted to characterise the interaction of monocytes with adipocytes and how this interaction might influence the polarization and response of monocytes, in the context of obesity-related T2D in humans. To this end, we co-cultured human monocytes with SC or OM adipocytes from OW, OB and OB-DB patients in order to investigate the effect of adipocytes on monocytes polarization and response.

Our results revealed an increased expression of several inflammatory (M1) cytokine/chemokine genes in monocytes co-cultured with SC/OM adipocytes or CM from these adipocytes (**Figures 3.1-3.2 and 3.5-3.6**). This observation correlates with previous findings where direct co-culture of 3T3-L1 adipocytes and murine macrophage cell line RAW264.7, led to an upregulation of inflammatory genes (Suganami et al., 2005; Yamashita et al., 2007). While we did observe significant increase in *IL6* as reported in the above study, co-cultured RAW cells showed increased TNF- α expression, which we failed to observe in our results. This may be

due to the use of different systems: primary cells versus cell lines and murine versus human cells.

When comparing the response of monocytes co-cultured with SC versus OM adipocytes (or their CM) from OB-DB or OB subjects, the upregulation of inflammatory genes in the monocytes (as compared to those cultured alone) was observed to a lesser extent for co-cultures involving OM adipocytes than SC adipocytes or their respective CMs (**Figures 3.1A-B vs 3.2B and 3.5A-B; vs 3.6B**). This could arise due to the use of adipocytes from different tissue compartments (OM vs SC) which are known to have different characteristics (Hellmer et al., 1992; Jove et al., 2014). The fact that SC adipocytes induce more inflammatory genes is in contrast with the well-accepted fact that subcutaneous adipose tissue plays a less important role in obesity and in the onset of T2D (Despres et al., 1989; Fujioka et al., 1987; McLaughlin et al., 2011).

An important aspect of our study was to study how monocytes were affected by adipocytes from OW, OB and OB/DB subjects, which represents different stages of adiposity and the development of obesity followed by metabolic disease. While monocytes co-cultured with OB or OB-DB adipocytes or their CM showed a similar trend of increased inflammatory gene expression (**Figures 3.1A-B, 3.5A-B**), those cultured with OW adipocytes (or their CM) showed an immense increase in inflammatory gene induction (**Figure 3.1C, 3.5C**). In these monocytes, the expression of *IL6* and *IL1B* was the highest and clearly higher than in monocytes co-cultured with OB or OB-DB adipocyte or their CM media. These observations may suggest a possible shift in monocytes during the course of adiposity, with those in early stage of adiposity (i.e. OW) showing increased inflammatory capacity as compared to those in the later stage of adiposity (i.e. OB-DB). Although such a concept needs validation by future *ex vivo* human studies, an increased inflammatory capacity of monocytes under OW settings is in line with the widely reported inflammatory phenotype of monocyte/macrophages in obesity (Chawla et al., 2011; Lumeng et al., 2007b).

Importantly our results add a new twist to the scenario by suggesting a skewing of monocyte response from an inflammatory to an immunosuppressive phenotype upon progression from OW to OB-DB condition. Such a skewing of monocytes/macrophages has also been reported during sepsis, cancer progression and cardiac infarction (Biswas and Mantovani, 2010; Shalova et al., 2015). Unfortunately, the same experiments could not be performed with the OM OW adipocytes as the unavailability of the sample limited such an analysis. Side-by-side comparison of the ability of OW SC and OM adipocytes to induce such an immense increase in IL6 and IL1B expression in monocytes would be interesting.

Our results also documented the expression of the non-inflammatory chemokine CCL17 and CCL18, which represent M2 genes that are specific to human macrophages (Biswas and Mantovani, 2010). A consistent trend showing upregulation of *CCL18* and *CCL17* could be observed in monocytes co-cultured with adipocytes, irrespective of the patient groups, under basal conditions (**Figures 3.1A-C**). This was also reproduced for monocytes cultured with the adipocyte CM (**Figures 3.5A-C and 3.6A-B**). Thus, under basal conditions, while monocytes co-cultured with adipocytes from OB, OB-DB or OW (or their CM) showed upregulated expression of M2 genes *CCL17* and *CCL18*, the markedly increased induction of inflammatory M1 genes (e.g. *IL6*, *IL1B*, *IL12*, *CCL5*) in monocytes co-cultured with OW adipocytes as compared to OB-DB adipocytes (or their CM), suggested a preferential skewing to an immunosuppressive phenotype for the latter case, as discussed above.

Having studied the basal phenotype of monocytes co-cultured with adipocytes or their CM, we further investigated the potential of these cells to respond to M1 (i.e. IFN γ +LPA) and M2 (i.e. IL-4) stimuli. Monocytes exposed to CM from OW adipocytes showed markedly increased expression of several inflammatory (M1) genes (e.g. *IL6*, *IL1B*, *IL12*) as compared to monocytes cultured alone, upon IFN γ +LPA challenge. These

results indicate that adipocytes from OW subjects promote an inflammatory phenotype in monocytes. This is in line with previous mice studies on an M1 phenotype of macrophages during obesity (Hotamisligil et al., 1993; Lumeng et al., 2007a).

In contrast to the above, when monocytes were cultured with OB-DB adipocyte CM, the expression of inflammatory genes (*IL6*, *IL1B*, *IL12*) were significantly decreased as compared to monocytes alone, upon IFN γ +LPA challenge. Concomitant with this observation, monocytes cultured with OB-DB monocytes showed a clearly heightened induction of *CCL18* (than monocytes alone) upon IL-4 treatment, as compared to their counterparts treated with OW adipocyte CMs. These data once again confirmed the skewing of monocytes conditioned with OB-DB adipocyte media to an M2-like phenotype.

The impaired expression of inflammatory genes upon LPA challenge is suggestive of an immunosuppressive phenotype and possibly, an impaired response to infection. This is in line with the fact that OB-DB subjects have an increased susceptibility to infection and sepsis (Falagas and Kompoti, 2006; Huttunen and Syrjanen, 2013). Although, the immunological basis of this clinical observation has not been understood, our present results provide an insight to this by demonstrating the skewing of monocytes to an immunosuppressive phenotype. A similar situation is also seen in sepsis and endotoxin tolerance, where a chronic and basal upregulation of low-grade inflammation initiates a negative feedback loop which impaired response to further inflammatory stimuli, but upregulation of anti-inflammatory and M2 genes (Pena et al., 2011; Porta et al., 2009; Shalova et al., 2015). In fact, adipocytes are known to constitutively produce large amount of inflammatory cytokines/chemokines which could provide a basis for the low-grade inflammatory condition (Zoccali et al., 2003). In addition, the basal expression of genes like *IL6* and *CCL5* in adipocyte-conditioned monocytes may also contribute to this low-grade inflammation.

While many of the mouse studies have shown an M1 inflammatory phenotype of macrophages related to obesity and insulin resistance, our studies show a different result. This can be due to a number of reasons.

Firstly, the studies that assign an M1 phenotype to obesity-related macrophages have often based their conclusions on just two-three M1 vs M2 markers, and not an expanded panel of polarization markers as suggested very recently in the new nomenclature of macrophage activation (Murray et al., 2014). For example, a study assessing effect of weight loss in humans on M1 vs M2 status of macrophages used CD40 as an M1 marker and CD206 and CD163 as M2 marker (Aron-Wisnewsky et al., 2009). Similarly in a mice study, ATMs were assessed for polarization by using TNF, IL6 for M1 and IL10, Arginase I for M2 polarization (Lumeng et al., 2007c). Instead, we utilized a panel of multiple markers to screen for polarization (M1: *IL6*, *IL1B*, *IL12p40*, *CXCL10* and *CCL5* and M2: *CCL17*, *CCL18*, *CCL22* and *IL10*) in our study.

Second, most studies focussing on the M1 polarization of ATMs in obesity are in mice and very little has been done in humans. It is important to mention here that the inflammatory conditions induced in the adipose tissue of mice in response to HFD (which is often a couple of months) may differ from that associated with obesity gained in humans, typically over a number of years. The latter may represent a “smouldering” of inflammation which often triggers immunosuppressive characteristics, as seen in cancer (Mantovani et al., 2008).

Third, previous study have shown considerable divergence of polarization markers between human and mouse macrophage (Biswas and Mantovani, 2010). For example, Arg1, Fizz1, Ym1 are mouse M2 macrophage markers and are not expressed in human M2 macrophages, that contrary to mouse exclusively express chemokines CCL17 and CCL18 (Beyer et al., 2012; Martinez et al., 2006). Thus, to assess monocyte-macrophage polarization in human settings, the markers need to be carefully chosen. The M1 and M2 markers used in our study are also

documented by high-resolution profiling in human macrophages (Beyer et al., 2012).

Several studies have shown ATMs to show mixed phenotype with co-expression of inflammatory M1 (TNF, IL6, IL1, IL23) as well as M2 features (IL10, IL1RA, TGF β , CD163) (Bourlier et al., 2008; Zeyda et al., 2007). Indeed, profiling human ATMs during fat mass enlargement showed them to exhibit several features of M2 tissue remodelling phenotype with downregulation of pro-inflammatory genes (IL8 and COX2) (Bourlier et al., 2008). In mice, as well such mixed phenotypes for CD11c⁻CD206⁺ ATMs have been noted with co-expression of inflammatory (TNF α , IL-6, IL-8, IL-1 β) and anti-inflammatory (IL-10) cytokines (Wentworth et al., 2010).

More recently, the *in situ* proliferation of ATMs with M2-like features was reported during obesity (Haase et al., 2014). Another study showed human ATMs in progressive obesity to be more M2-like rather than M1-like and expression of some of the M2-like genes correlated with Homeostatic model assessment of Insulin Resistance (HOMA-IR) (Fjeldborg et al., 2014). In agreement with this observation, during obesity in mice, ATMs were shown to increase M2-like and decrease M1-like genes at 12 weeks of HFD (Shaul et al., 2010). Taken together, these observations support our results on the mixed phenotype of OB-DB adipocyte-conditioned monocytes at basal level and their immunosuppressive and M2-like phenotypes, upon stimulation. Functionally as well such an immunosuppressive phenotype is in line with the clinically observed impaired immunity in T2D subjects. However, further study of ATMs in different adiposity condition in humans will clarify our findings as well as whether this phenotype is restricted to the expansion of a particular subset of these cells *in vivo*.

Besides investigating the effect of adipocytes on the gene expression of monocytes, we also measured the changes in gene expression in the SC/OM adipocytes itself in our co-culture experiment. However, the gene expression study in adipocytes was limited to the

inflammatory genes, since M2 genes like *CCL17* and *CCL18* were specific to monocyte/macrophage lineage and not found to be expressed in adipocytes. Our measurements revealed monocyte-co-cultured adipocytes to display a general trend of upregulating inflammatory genes (as compared to adipocytes cultured alone), although this effect varied depending on the particular gene studied, type of adipocytes (SC vs OM) and source of the adipocyte (OW showing more gene expression than OB-DB) (**Figures 3.3A-C and 3.4A-B**). *IL6* and *CCL5* were two inflammatory genes which showed the most clear upregulation in the adipocytes (**Figure 3.3A-C, Figure 3.4A-B**). In line with this observation, co-culture of 3T3-L1 adipocyte with macrophage cell line was previously shown to upregulate IL-6 and CCL5 expression (Yamashita et al., 2007). In fact, adipocytes are a source of various cytokines (Zoccali et al., 2003). In particular, IL6 expression by adipocytes has been considered to be one of the key inflammatory cytokines that promote insulin resistance (Kern et al., 2001; Rotter et al., 2003; Senn et al., 2002). In addition, CCL5 and its receptor (CCR5) expression have been reported in WAT of obese mice and visceral fats in humans (Huber et al., 2008; Kitade et al., 2012; Wu et al., 2007). In mice, genetic deletion of CCR5 showed its relevance in recruitment macrophages, adipose tissue inflammation and insulin resistance (Kitade et al., 2012).

Taken together, our present finding that monocytes promote an inflammatory phenotype in adipocytes is suggestive of their role in supporting insulin resistance in adipocytes. Moreover, since we showed increased IL6 expression by both the cell types, it is tempting to speculate that the presence of monocytes/macrophages could potentially support maturation and growth of adipocytes. This is in agreement with previous reports that IL-6 is gradually increased in process of adipocytes differentiation (Vicennati et al., 2002).

One of the most consistent features of monocytes co-cultured with adipocytes or their CM was the upregulation of the chemokine gene *CCL18*. Indeed, *CCL18* was upregulated in monocytes cultured with OB-

DB adipocytes CM under basal as well as upon IL-4 stimulation, as compared to monocytes cultured alone (**Figure 3.7**). An interesting trend showing a preferentially higher induction of *CCL18* as compared to *CCL17* was seen for monocytes treated with CM of OB-DB adipocytes while the reverse was seen for monocytes treated with CM of OW adipocytes (**Figure 3.7A, C: compare CCL18 vs CCL17 within each panel**). The *CCL18* expressing phenotype of these OB-DB CM-treated monocytes was also validated at the protein level by ELISA (**Figure 3.9**).

Increased expression of *CCL18* was reported in association with several diseases such as arthritis (Schutyser et al., 2001), ovarian carcinoma (Schutyser et al., 2002), hypersensitivity pneumonitis (Pardo et al., 2001), lymphoblastic leukaemia (Struyf et al., 2003) or in genetic susceptibility to tuberculosis (Jamieson et al., 2004). However, to date no report on *CCL18* expression in myelomonocytic cells in obesity related T2D exists.

Chemokine *CCL18* was for the first time independently described by several groups as a gene specific for humans (Adema et al., 1997; Hieshima et al., 1997; Kodelja et al., 1998). *CCL18* was described as a chemokine which is most closely related to macrophage-inflammatory protein-1 α (MIP-1 α) whose generation occurred due to gene duplication of two MIP-1 α genes (Hieshima et al., 1997; Tasaki et al., 1999). As this chemokine is specific for humans or primates, the information on its *in vivo* function is still very limited due to the inability to use mice models. *CCL18* have been reported to show chemotactic activity on T-cells as well as induce the recruitment and induction of Treg (Chenivesse et al., 2012; Guan et al., 1999; Melief et al., 2013). *CCL18* induces collagen production in lung fibroblasts and contributes to generation of fibrotic tissue (Atamas et al., 2003; Luzina et al., 2006a; Luzina et al., 2006b). More recently, tumor macrophage-derived *CCL18* was shown to promote EMT, and metastasis of breast cancers (Su et al., 2014).

To date, production of CCL18 under basal conditions has been reported in eosinophil, monocytes, macrophages and dendritic cells (Schraufstatter et al., 2004; Vissers et al., 2001; Vulcano et al., 2003). Increased expression of CCL18 was reported in macrophages in response to M2 stimuli such as IL-4, IL-13 and IL-10 (Kodelja et al., 1998) as well as in decidual, lung and tumor associated macrophages (Chenivesse et al., 2012; Su et al., 2014; Svensson-Arvelund et al., 2015).

In our study we confirmed the expression of CCL18 in ATMs *in vivo*. Indeed, flow-sorted ATMs isolated from both SC and OM adipose tissue depots of obese individuals showed increased CCL18 gene expression (**Figure 3.10**). This was also validated by immunohistochemistry (**Figure 3.11**). These findings confirm our *in vitro* results and suggest CCL18 expression as a possible characteristic feature of monocyte/macrophages related to obesity. Unfortunately, due to limited availability of bariatric surgery-derived adipose tissue, we could not perform the same assays on adipose tissues from different patient groups such as lean or overweight. It would be of great interest to procure such rare materials for a comparative study of CCL18 in the future.

The human specificity of CCL18 expression by monocytes/macrophages and the little information on its cognate receptors severely limits means to assess its function *in vivo*. As mentioned above, there are some reports which suggest CCL18 expression by human macrophages to recruit Tregs and maintain immune tolerance in lung (Chenivesse et al., 2012; Melief et al., 2013). It is possible that monocytes/macrophages in OB-DB subjects by CCL18 expression induce recruitment of Treg cells, thereby promoting immunosuppression. Furthermore, given the role of CCL18 in fibrosis and EMT (Atamas et al., 2003; Luzina et al., 2006a; Luzina et al., 2006b; Su et al., 2014), CCL18 expression by monocytes/macrophages could also stimulate fibrosis in adipose tissue (Furukawa et al., 2015).

In fact, collagen deposition and fibrosis have a relevant role in the adipose tissue dysfunction and metabolic dysregulation (Khan et al., 2009; Sun et al., 2013). Although these functional attributes are in line with the clinico-pathological symptoms seen in obese/T2D settings and the M2 phenotype of monocytes/macrophages, further *ex vivo* functional studies would confirm these facts. Moreover, the molecular mechanism(s) by which CCL18 expression and the immunosuppressive phenotype of adipocyte 'conditioned' macrophages is induced remains to be characterized. So is the identification of the adipocyte-derived soluble factor(s) that induce this phenotype in the monocytes. These are some of the areas of future investigations. At present, the difficulties in adequate amount of bariatric surgery tissues from different subject groups, isolation of tiny number of ATMs and the difficulty in using them in *ex vivo* functional assays, the purification and identification of adipocyte-derived soluble fraction that induce the monocyte phenotype and the human specificity of key molecules like CCL18 pose some of the challenges for such studies.

Elevated levels of CCL18 in plasma or serum have been reported in patients with different pathological conditions such as systemic sclerosis, lymphoblastic leukaemia or Gaucher disease (Boot et al., 2004; Kodera et al., 2005; Struyf et al., 2003) but not in obesity and T2D. Considering that CCL18 was a characteristic feature of adipocyte-conditioned monocytes and ATMs in obesity, we wondered if increased levels of this chemokine could also be reflected in the plasma. We tested the levels of CCL18 in the plasma of CCL18 in lean, OW, OB and OB-DB patients. Surprisingly, plasma of OB-DB and OB subjects showed significantly more CCL18 than overweight or lean subject (**Figure 3.12**). Importantly, this is suggestive of CCL18 levels to be related with the degree of adiposity. Further correlation studies indicated correlation between CCL18 levels to some parameters of insulin resistance like fasting plasma glucose, glycated haemoglobin and obesity parameters such as triglycerides (**Figure 3.12**). This is an interesting observation since high levels of CCL18 were reported in plasma of Gaucher disease individuals (Boot et al., 2004; Boot et al., 2006) and Niemann-Pick disease (Brinkman et al., 2005) and both of these genetic

diseases are characteristic for improper metabolism of lipids. Therefore, it would be imperative to hypothesize that higher concentration of lipids present in obesity and T2D could drive increased production of CCL18. However, it remains to be known whether triglycerides directly act on the ATMs to induce this phenotype.

Nevertheless, our correlation results clearly point towards a link between CCL18 and clinical parameters of insulin resistance and obesity. However, future studies need to clarify whether CCL18 itself plays a pathological role in insulin resistance and T2D or is an attribute associated with this disease. If CCL18 does play a role in the pathogenesis of obesity related T2D itself, does it directly affect adipocyte insulin signalling or does it influence other cells and factors in the obese adipose tissue which indirectly affects insulin resistance? These questions open a new channel of investigation into the role of CCL18 in adipocyte biology.

5 Conclusion

In summary, our study reports new findings on the interaction of monocytes and adipocytes in obesity and T2D settings in humans. Using an *in vitro* co-culture model and adipocyte CM, we demonstrated OW adipocytes to show marked induction of inflammatory genes (e.g. *IL6*, *IL1B*, *IL12*) in monocytes, while those from OB-DB subjects showed comparatively lesser induction of these inflammatory genes and more expression of the M2 gene *CCL18*.

Interestingly, this trend was also reflected in their capacity to respond to polarization stimuli with OB-DB adipocyte CM-treated monocytes showing significantly impaired inflammatory gene expression in response to LPA+IFN γ , but heightened *CCL18* expression to IL-4 stimulation (compared to their only monocyte counterparts). In contrast, OW adipocyte CM-treated monocytes showed unimpaired and even markedly heightened induction of inflammatory genes in response to LPA+IFN γ , but a modest induction *CCL18* expression in response to IL-4, compared to their only monocyte counterparts. These results suggest a skewing of monocytes from an inflammatory phenotype in OW conditions to an immunosuppressive, M2-like phenotype in OB-DB conditions (**Figure 5.1**). Co-culturing with monocytes also affected adipocytes (irrespective of their source, e.g. or OB-DB) to express heightened levels of inflammatory genes which is suggestive of amplifying inflammation in these cells and thereby promoting their insulin resistance (**Figure 5.1**).

Thus, our results indicate for the first time that monocytes and adipocytes interact by affecting each other in a distinct manner: promoting insulin resistance on one hand and immunosuppression on the other hand, the two clinical hallmarks associated with OB-DB subjects (**Figure 5.1**). Finally, we also identify the chemokine *CCL18* as a consistent characteristic of monocytes/macrophages in obesity/T2D, which not only correlated with increased plasma levels in these subjects, but also with

selected clinical parameters of insulin resistance and obesity (e.g. fasting glucose levels, HbA1c, triglycerides).

These results open future research directions into investigating the dynamic changes in monocyte/macrophage phenotypes during the progression of obesity and T2D and the relevance of their CCL18 expression in the pathogenesis of these conditions. This would require isolation of monocytes/macrophages (ATMs) from obese and obese-T2D subjects and their deep pheno-profiling, which would need considerable standardization experiments to resolve the issue of limiting cell number obtainable from such clinical samples.

In addition, investigations into the role of CCL18 in pathogenesis of obesity-related T2D would require studying the *in vitro* and *in vivo* effects of this chemokine on adipose tissue biology, specifically, insulin signalling in adipocytes, angiogenesis and tissue remodelling of adipose tissue which has direct implications on adipose tissue expansion and insulin resistance.

Figure 5.1: A simplified scheme showing the interaction of monocytes with adipocytes from overweight (OW) or Obese-T2D (OB-DB) subjects. Modulated genes from our results are indicated for each cell. The boxes with red outline indicate possible health outcomes. The figure suggests a skewing of monocytes from an inflammatory to an immunosuppressive phenotype from OW to OB-DB. The impaired response of such immunosuppressive (CCL18 expressing) M2-like cells to respond to microbial stimuli (such as LPS) would mean increased susceptibility to infection, a well-known condition in T2D subjects. In contrast, adipocytes upon interaction with monocytes assume an inflammatory status (characterized by expression of inflammatory cytokines, e.g. IL1, IL6) which is known to support insulin resistance in these cells. Thus, the adipocyte-monocyte crosstalk would propagate two well-known clinical hallmarks of T2D pathogenesis: promoting insulin resistance and impaired immune response to infection”.

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7 Appendix

Figure 1.1: Modulation of metabolic genes in monocytes – SC adipocytes co-culture. (A) Basal gene expression level of metabolic genes in obese/diabetic and (B) expression of metabolic genes in polarized monocytes. Expression was measured by real time PCR with actin being as the housekeeping gene. The data are presented as mean $\pm$ SEM, from n=3.

8 Author's Publications, Posters, Awards, Invited talks

Publications

Hruskova Z and Biswas SK. A New “Immunological” Role for Adipocytes in Obesity. *Cell Metab.* 2013; 17(3):315-317.

Conferences Attended

2012	7th ENII EFIS/EJI Spring School on Advanced Immunology,
Alghero, Italy
2013	6th International Singapore Symposium of Immunology,
Singapore, Singapore
Poster Presentation.
2013	Immunometabolism: From Mechanism to Therapy, Toronto,
Canada
Poster Presentation.
2013	6th HKU-Pasteur Immunology Course, Hong-Kong
Presented short talk.
2015	The Crossroads of Lipid metabolism and Diabetes.
Copenhagen, Denmark
Poster Presentation.
2015	RIKEN IMS-JSI International Symposium on Immunology,
Yokohama, Japan
Poster Presentation and short talk.