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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Ο ΡΟΛΟΣ ΤΟΥ MIR-155 ΣΤΗΝ ΑΝΤΙΣΤΑΣΗ ΣΤΗΝ ΙΝΣΟΥΛΙΝΗ ΚΑΙ Η ΡΥΘΜΙΣΗ ΤΟΥ ΑΠΟ ΤΗΝ ΛΕΠΤΙΝΗ ΣΤΑ ΜΑΚΡΟΦΑΓΑ

ΑΖΝΑΟΥΡΟΒΑ ΜΑΡΙΝΑ

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Ο ρόλος του MiR-155 στην αντίσταση στην ινσουλίνη και η ρύθμιση του από την λεπτίνη στα μακροφάγα.

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ΠΕΡΙΛΗΨΗ

Η παχυσαρκία, που τα τελευταία χρόνια έχει πάρει επιδημικές διαστάσεις, σχετίζεται με χαμηλού βαθμού φλεγμονή και αυξημένο κίνδυνο για λοιμώξεις. Γενικά τα παχύσαρκα άτομα τείνουν να εμφανίζουν διάφορες παθήσεις, όπως είναι ο διαβήτης τύπου ΙΙ, η αθηροσκλήρωση και οι καρδιακές ανεπάρκειες. Ο στόχος της έρευνας αυτής είναι να κατανοήσουμε πως η αντίσταση στην ινσουλίνη , που είναι ένα από τα βασικά χαρακτηριστικά της παχυσαρκίας, επηρεάζει τα μακροφάγα και την δράση τους. Επιπρόσθετα, ελέγχθηκε και η επίδραση της λεπτίνης στα μακροφάγα, που είναι μία ορμόνη που υπερπαράγεται στον λιπώδη ιστό και έχει δειχθεί να έχει προ- φλεγμογόνο δράση.

Τα αποτελέσματα έδειξαν ότι η αντίσταση στην ινσουλίνη, επηρεάζει την παραγωγή προφλεγμονωδών μορίων από τα μακροφάγα. Ποιο συγκεκριμένα τα επίπεδα κιτοκινών όπως της ιντερλευκίνης 6 και του TNFα, και μορίων όπως το micro RNA 155 (miR155) πέφτουν. Η πτώση των επιπέδων παρατηρήθηκε είτε σε μακροφάγα από ποντίκια που έχουν αντίσταση στην ινσουλίνη είτε είναι παχύσαρκα με ή χωρίς μόλυνση με λιποπολισακχαρίτη. Από την άλλη, η επίδραση με λεπτίνη σε μακροφάγα από φυσιολογικά ποντίκια έδειξε αύξηση των κιτοκινών και του MiR-155 με ή χωρίς επιμόλυνση με λιποπολυσακχαρίτη. Επίσης η λεπτίνη προκάλεσε πτώση των επιπέδων των υποδοχέων της ινσουλίνης και του IGF, που αποτελούν τους κύριους υποδοχείς μέσω των οποίων γίνεται η μετάδοση του σήματος από την ινσουλίνη.

Τα αποτελέσματα αυτά δείχνουν ότι η παχυσαρκία που οδηγεί στην δημιουργία αντίστασης στην ινσουλίνη μεταβάλει τα μακροφάγα σε έναν λιγότερο δραστικό φαινότυπο (M2) με μειωμένη προ- φλεγμονώδη ικανότητα που μπορεί να εξηγήσει τα αποτελέσματα που παρατηρούνται στην μονάδα εντατικής θεραπείας, οπού κλινικές μελέτες δείχνουν ότι οι παχύσαρκοι σηπτικοί ασθενείς έχουν χαμηλότερο δείκτη θνησιμότητας

από αυτούς με κανονικό βάρος . Από την άλλη πλευρά, η υπερπαραγωγή της λεπτίνης που παρατηρείται στον λιπώδη ιστό των παχύσαρκων ατόμων ναι μεν οδηγεί στην δημιουργία φλεγμονής τοπικά του ιστού, αλλά επίσης επάγει την δημιουργία αντίστασης στην ινσουλίνη στα μακροφάγα περιφερειακά., μειώνοντας τα επίπεδα των υποδοχέων της.

Συμπερασματικά, η επαγόμενη από την λεπτίνη αντίσταση στην ινσουλίνη στα μακροφάγα των παχύσαρκων ατόμων οδηγεί στην μείωση της φλεγμονής περιφερειακά στον οργανισμό και έχει ως αποτέλεσμα τα άτομα στην μονάδα εντατικής θεραπείας να μπορούν σε μερικές περιπτώσεις να ανταπεξέλθουν καλύτερα στην θεραπεία μετά από διάφορες μόλυνσης.

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MASTER THESIS

ROLE OF MIR-155 IN INSULIN RESISTANCE AND ITS REGULATION BY LEPTIN IN MACROPHAGES

MARINA AZNAOUROVA

ABSTRACT

Obesity, that in the past few years has reached epidemic proportions, is collated with a low grade inflammation and high risk of infections. Generally, obese individuals tent to develop a variety of inflammatory conditions, such as type II diabetes andatherosclerosis. The purpose of this study is to understand how insulin resistance affects macrophages and their function. Furthermore, the effects of leptin, which is hormone overproduced in adipose tissue of obese individuals, on the properties and the function of macrophages were tested.

The results showed that insulin resistance, effects the production of pro- inflammatory molecules in macrophages. More specifically, there is a reduction of the levels of pro- inflammatory cytokines, such as interleukin -6 (IL-6) and TNFa, and molecules, such as micro RNA 155 (miR-155). The reduction of the levels was observed, either in macrophages from insulin resistance mice or from mice fed with high fat diet and were either naïve or after Lipopolysaccharide (LPS) treatment. On the other hand leptin treatment of wild type macrophages showed an increase of cytokines levels (IL-6, TNFa) and MiR-155 with or without LPS stimulation. Also, treatment with leptin reduced the levels of insulin and IGF receptors.

These results indicate that obesity that causes insulin resistance switch macrophages to a less pro- inflammatory phenotype (M2 phenotype) which can explain the results that are observed in ICU, were clinical studies showed that obese individuals with sepsis have lower mortality rates than the normal ones. On the contrary, the overproduction of leptin in the adipose tissue of obese individuals can cause inflammation locally, but can also induce insulin resistance to the macrophages of the periphery, by reducing the levels of its receptors.

To conclude, leptin- induced insulin resistance in macrophages of obese individuals, leads to reduction of the inflammation in the periphery of the organism and that leads patients of the ICU to have better outcomes after treatments against infection.

INDEX

1. Introduction

1.1 Obesity and Inflammation

Obesity is a medical condition that has reached epidemic proportions worldwide and especially in the developed countries (Figure1.1). In the United States obese individuals have reached over 30% of the population and taken together the ones that are overweight the percentage extents to 60%, as defined by having a Body Mass Index (BMI, kilograms/meter^2) of at least 30. Universally, it is estimated that more than five hundred million adults and nearly forty three million children beneath the age of five years are obese. In Greece, obese population was dramatically increased in the past 2 decades, since there is a switch from a Mediterranean lifestyle and diet to the adoption of more 'western life' ways. Certain habits, like 'fast food' consumption and sedentary lifestyle, are the most common reasons for obesity development.

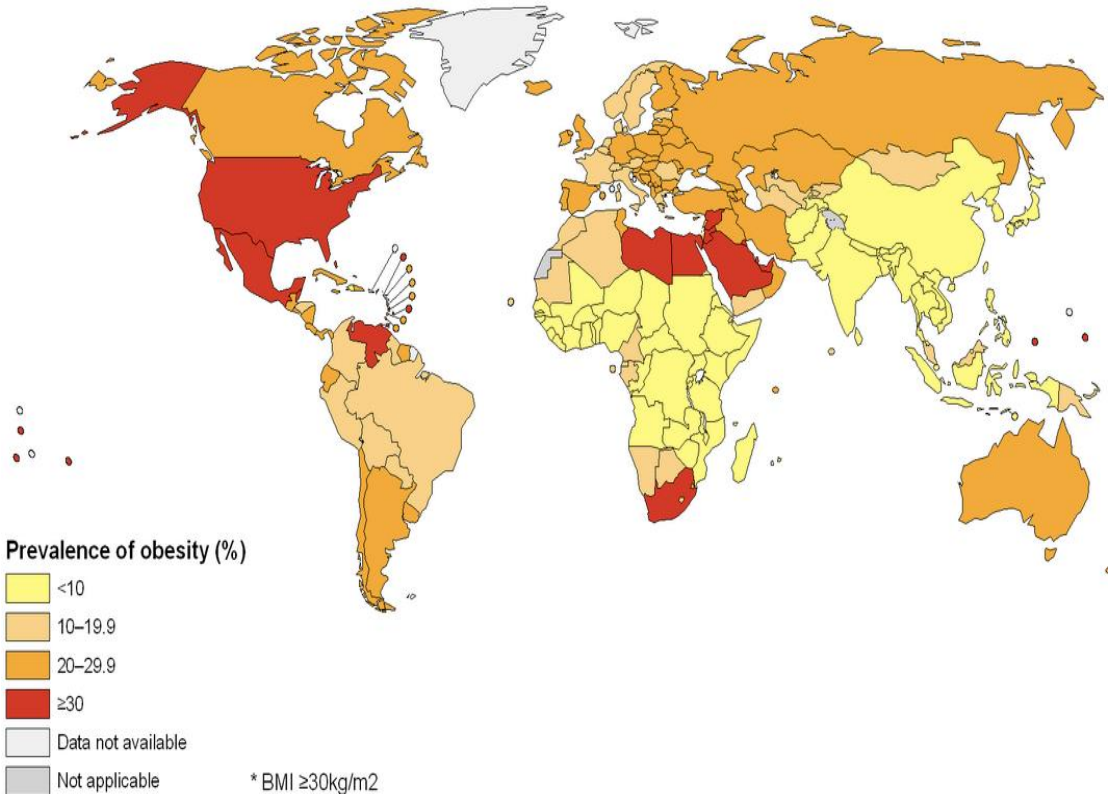


Figure 1.1: Map of obesity distribution over the world.

Obesity is associated with a various types of health disorders that are clinically known as the Metabolic Syndrome. The Metabolic Syndrome is characterized as the presence of three or more of the following features:

obesity, hyperglycemia, hypertension, low HDL cholesterol levels, and/or hypertriglyceridemia. Obese individuals tend to develop several diseases such as type II diabetes, coronary heart disease, osteoarthritis, atherosclerosis, kidney dysfunction etc. (Figure 1.2). There are also other diseases that are multifactorial, such as cancer, in which obesity plays a significant role. However, it is not obesity per se that is responsible for these health disorders but relatively the inflammatory anomalies that are interwoven with this condition. Obese individuals tend to develop chronic inflammatory diseases such as atherosclerosis diabetes and kidney dysfunction (reviewed by Heilbronn and Campbell, 2008). Furthermore, it has been noticed that these individuals have higher risk to develop sepsis and multi-organ failure after systemic infection (reviewed by Vachharajani, 2008, Ciesla et al., 2006).

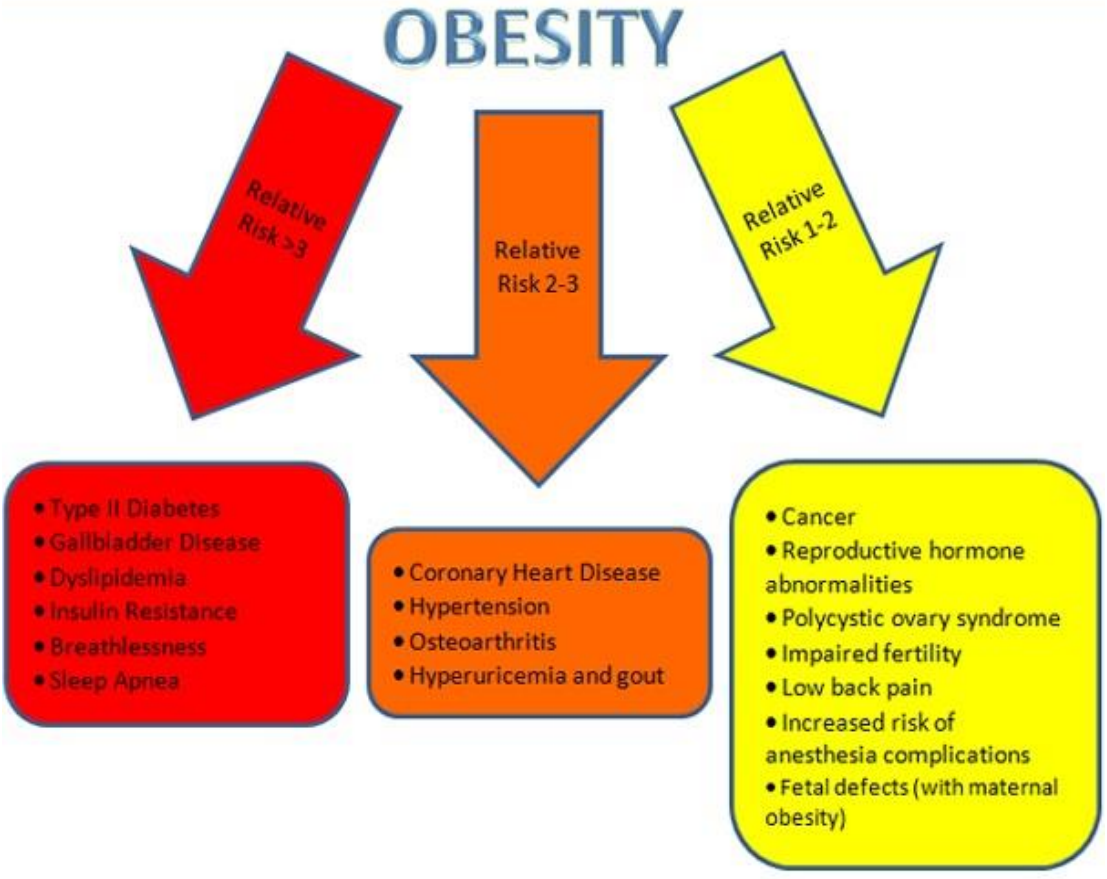


Figure 1.2: Obesity associated health disorders. Relative risk>3 refers to those diseases that are most possible for an obese individual to develop, relative risk 2-3 are the ones that come next and relative risk 1-2 are the health disorders that are associated with obesity but their development is multifactorial.

Obesity is associated with low grade inflammation by increase in circulating levels of pro-inflammatory cytokines such as IL-6, TNF-a and acute phase proteins (CRP and haptoglobin). Studies suggest that diet may reduce the circulating levels of this molecules and other markers of inflammation (Kopp H.P. et al. 2003). Metabolic Syndrome, Cardiovascular disease and diabetes are strongly associated with elevated levels of this pro-inflamatory molecules. CRP is the most important marker for cardiovascular disease (Ridker P. et al. 2002) and IL-6 for diabetes, where IL-6 may interfere with insulin signalling through induction of proteins that bind to the insulin receptor (Senn J. et al.2003). Also obese individuals develop endothelial dusfunction. Clinical and experimental data support a link between systemic inflammation and endothelial dysfunction. Mounting evidence shows that disturbed endothelial function may be an early marker of an ongoing atherosclerotic process. Thus, endothelial dysfunction has increasingly been recognized to play an important role in a number of conditions associated with a high prevalence of atherosclerotic CVD. Inflammatory cytokines are important protagonists in the formation of atherosclerotic plaques, eliciting effects throughout the atherosclerotic vessel. Importantly, the development of atherosclerotic lesions, regardless of risk factors (e.g., diabetes, hypertension, obesity), is characterized by the disruption of the normal function of endothelial cells.

For all these conditions, adipose tissue is the key player. Adipose tissue is progressively recognized not only as a depot for storage of fat but also as a complex endocrine organ. Adipose tissue is now known to secrete a large number of proteins that are called adipokines and can act in an autocrine, paracrine, or endocrine way to control various metabolic functions and immune responses. In obese individuals, this tissue develops an inflammatory milieu which eventually leads to several conditions including insulin resistance. Although many components of the immune system have been found to play a role in either promoting or attenuating adipose tissue inflammation, macrophages are key players. They are the major subpopulation of the white adipose tissue (WAT) which is the majority of adipose tissue in the human body and the location of the energy storage (Fantuzi. et al., 2005). WAT macrophages are bone marrow derived and they infiltrate the adipose tissue from the circulation. The characteristics of these macrophages, and the cytokines that they

produce while infiltrating the adipose tissue, seem to play a major role in the inflammatory phenotype of the obese individuals.

1.2 Macrophage polarization

Macrophages are a heterogeneous population of innate myeloid cells involved in health and disease. They are the most plastic cells of the hematopoietic system, found in all tissues, and their main function is to respond to pathogens and to modulate the adaptive immune response through antigen processing and presentation. Macrophages, derived from monocyte precursors, undergo specific differentiation depending on the local tissue environment (reviewed by Steinman and Idoyaga, 2010). The various macrophage functions are linked to the type of receptor interaction on the macrophage and, crucially, to the cytokine milieu where the macrophages resides in. There are two main distinct subpopulation types of macrophages: the classically activated M1 and the alternative M2 macrophage phenotype (reviewed by Gordon and Taylor, 2005, Mantovani et al., 2002) (Figure 1.3). In past few years there is a further classification of the M2 phenotype. M2 macrophages can be further divided into four subsets: M2a, M2b, M2c and M2d based on gene expression profiles (Mantovani et al., 2004). The M2a subtype is elicited by IL-4 or IL-13 (common IL-4R-alpha, CD124), M2b by IL-1R ligands or exposure to immune complexes plus LPS, the M2c subtype by IL-10, TGF-beta, and glucocorticoid hormones and M2d which is characterized by an IL-10highIL-12low M2 profile with some features of tumor-associated macrophages (TAMs) (reviewed by Hao, 2012). M2d's have phenotypic and functional attributes similar to ovarian TAMs but distinct from M2a-c. Although these subpopulation types are distinct, their functional and expressional differences are not yet well understood and the usually referred as one population: M2.

M1 and M2 macrophages are not only activated differently but also act in a very distinct way. M1 macrophages are generally activated by TLR ligands and IFN-γ stimulation and are characterized by the expression of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, promotion of Th1 response, and strong microbicidal and tumoricidal activity. In contrast, M2 macrophages are alternatively activated by IL-4, IL-13, IL-10 and TGFβ and are considered to be involved in parasite containment and promotion of tissue remodeling and tumor progression and to have immunoregulatory functions (reviewed by Locati and Mantovani, 2013).

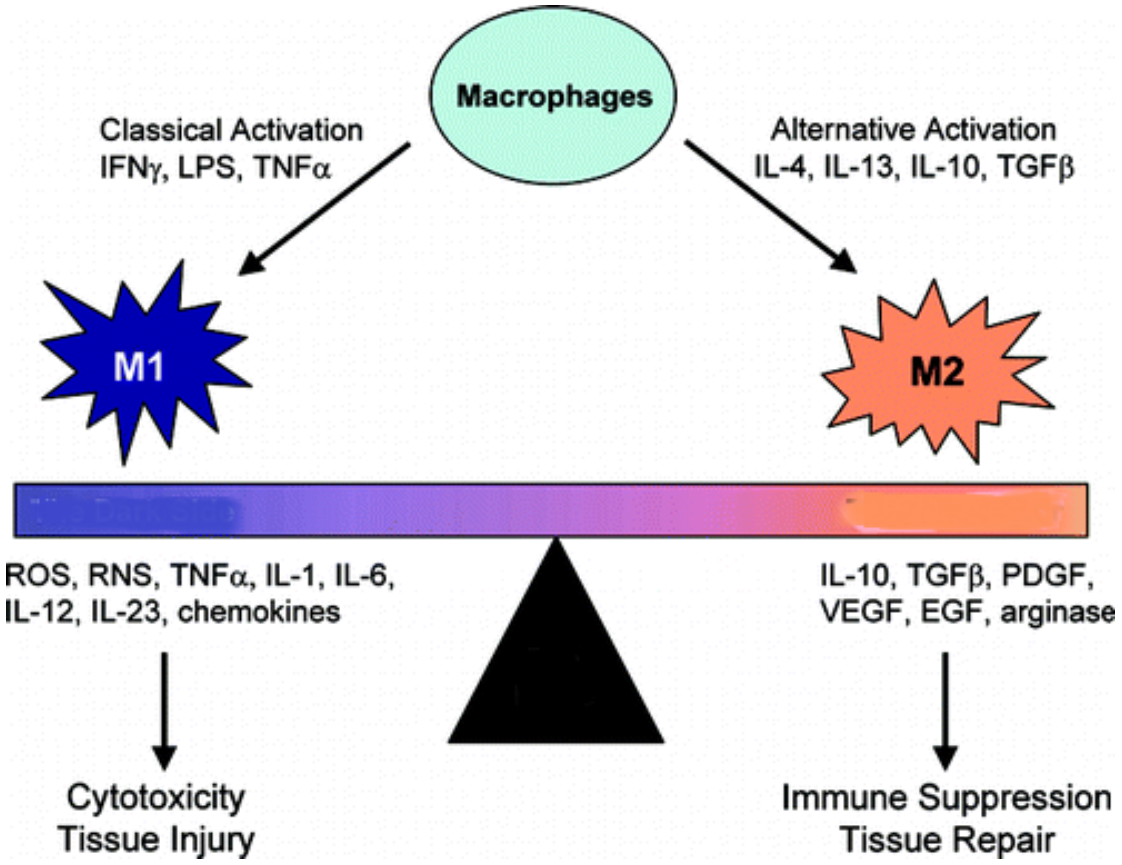


Figure 1.3: Macrophages subpopulations and their characteristics. M1 macrophages are characterized by cytotoxicity and tissue injury and they are mostly activated by INFγ, TNFα and LPS. The cytokines that are produced by those macrophages are mostly IL-6, TNFa, IL-1, IL-12 and IL-23.They also enhance the production of iNOS and chemokines. On the other hand, M2 macrophages are mostly act as immune suppressors and they help in tissue repair. They alternatively activated by IL-4, IL-13, IL-10 and TGFβ and they produce different molecules such us IL-10, VEGF, TGFβ, PDGF and arginase.

1.2.1 Macrophage polarization during infection

When tissues are challenged by pathogens, macrophages are recruited to the infected area and try to keep the homeostatic status together with the resident macrophages of the tissue. The polarization of the recruited macrophages depends on the type of the infection. Generally, in early stage of bacterial infections, macrophages tend to polarized into M1 phenotype. When the pathogen associated molecular patterns (PAMPs) of bacteria are recognized by pathogen recognition receptors (such as Toll-like receptors, TLRs), macrophages are activated and produce a large amount of pro-inflammatory mediators including TNF-α, IL-1, and nitric oxide (NO), which kill the invading organisms and activate the adaptive immunity (Wynn TA. et al.2013). If this inflammatory response of macrophages cannot be control, huge amounts of cytokines are produced and this can lead to sepsis (Stearns-Kurosawa DJ. et al 2011). In order to counteract the excessive inflammatory response, macrophages undergo apoptosis or polarize to an M2 phenotype to protect the host from excessive injury and facilitate wound healing (Murray PJ. et al 2011). Microarray analysis and transcriptional profiling of peripheral blood cells showed that typical M1 genes and M1-related genes were replaced by M2 markers during treatment of patients with typhoid fever (Thompson LJ. et al.2009). LPS, large molecules in the outer membrane of gram-negative bacteria, play a critical pro-inflammatory role in acute infections. As the infection persists, host may present a LPS-tolerant state, and macrophages are polarized to M2 phenotype. A recent study has confirmed that the p50 subunit of NF-κB served as the key regulator of M2-driven LPS-tolerant state in this transformation (Porta C. et al.2009). However, when the excessive injury is reduced, M2 phenotype macrophages also induce an immunosuppressive state, resulting in a more susceptible situation to re-infection.

Macrophage polarization is also involved in virus infection, and M2 phenotype macrophages can suppress inflammation and promote tissue healing. During infections with parasites, both M1 and M2 phenotype macrophages are involved depending on the subtype and duration of infection (Liu Y.. et al.2014).

1.2.2 Macrophage polarization in adipose tissue

Adipose tissue macrophages (ATMs) recruitment to the adipose tissue is an early event of the obesity- induced adipose depot inflammation. ATMs that are in the lean adipose tissue are different from the ones that are in obese. Generally obesity not only promotes infiltration and migration of macrophages, but also induces a shift in macrophage balance towards the M1 phenotype (Figure 1.4).

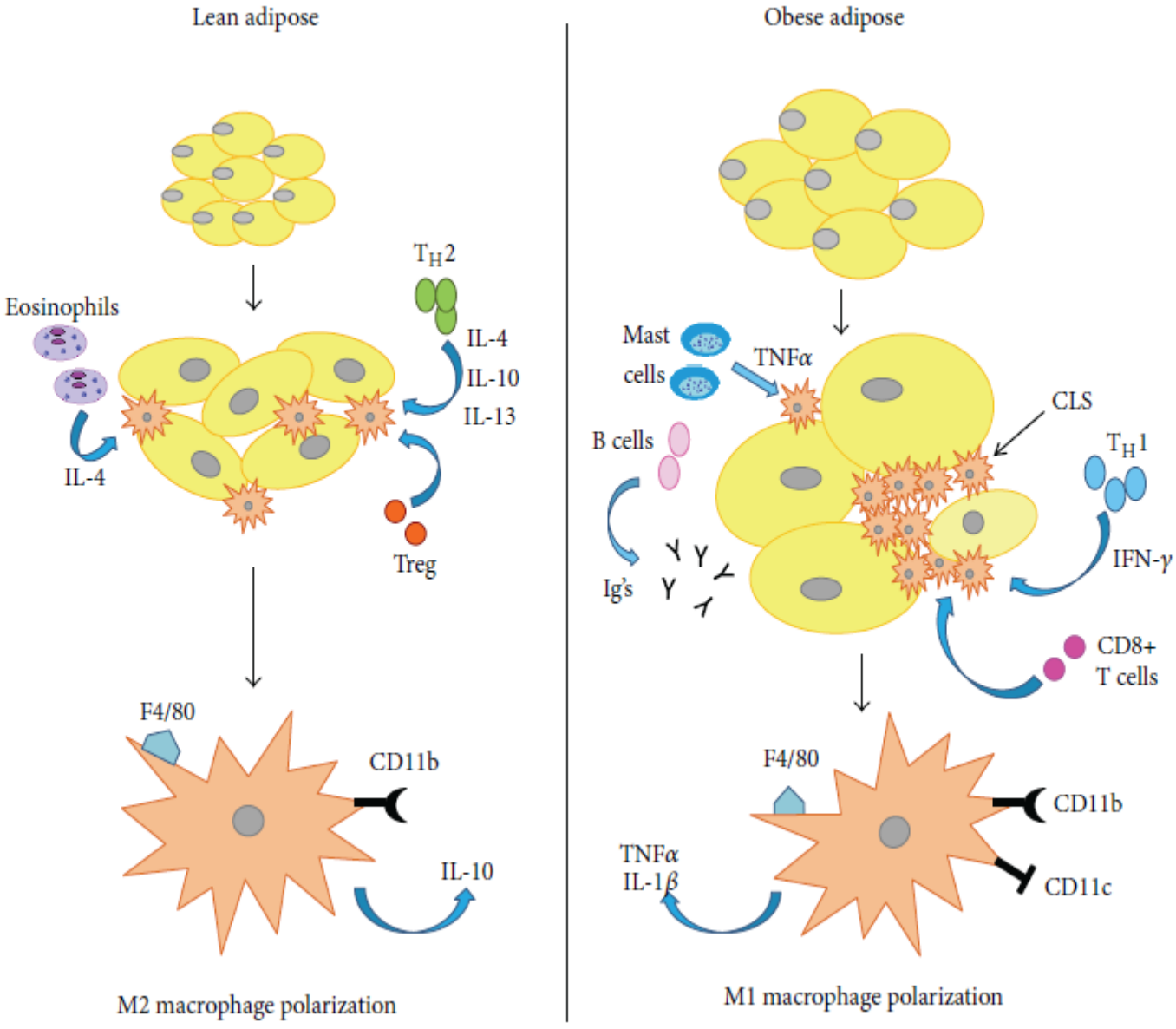


Figure 1.4: Role of the immune system in lean and obese adipose tissue. In lean adipose tissue, T-helper type 2 (TH2) cells, regulatory T cells (Tregs) and eosinophils produce anti-inflammatory cytokines such as interleukin (IL)-4, 10, and 13 which promote alternatively activated M2 macrophage polarization. M2 macrophages secrete other anti-inflammatory signals such as IL-10 which. Conversely, TH1 type cytokines such as interferon (IFN)-γ stimulate M1 macrophage polarization in obese adipose tissue. Other immune cells are also increased in obese adipose tissue including mast cells, B cells, and immunoglobulins (Igs). CD8(+) T cells promote ATM accumulation and pro-inflammatory gene expression and are also increased as well. Macrophages are not homogenously distributed throughout obese adipose tissue but rather aggregated around dead adipocytes forming crown-like structures (CLS).M1 macrophages are pro-inflammatory, secreting cytokines such as TNF-α and IL-1β.Macrophages are bone-marrow-derived myeloid cells hence both M1 and M2 macrophages express cell surface markers F4/80 and CD11b. However, only the M1 population expresses the marker CD11c.

In obese adipose tissue there several immunological events that are contributing to the polarization of macrophages to the M1 phenotype. First of all, the presence of a high number of inflammatory cells such as T helper type 1 (TH1) cells, CD8(+) T-cells, mast cells and B-cells leads to overproduction of pro-inflammatory cytokines such as INF-γ and TNFα. These cytokines are the stimuli for M1 activation. Consequently, these macrophages also produce pro-inflammatory cytokines and over express pro-inflammatory genes such as IL-6, IL-1β, TNF-α, and iNOS in order to promote further the inflammation in the adipose tissue. Their distribution is not random but they tend to form crown-like structures around dead adiposities. From the other hand cells in lean adipose tissue are usually T-helper type 2 (TH2) cells, regulatory T cells (Tregs) and eosinophils, which are producing anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 and thus the macrophages are polarized to the M2 phenotype. M2 macrophages further control the inflammation producing also anti-inflammatory cytokines and over expressing anti- inflammatory genes (IL-4, IL10 and arginase respectively).

The M1 polarization of macrophages in the adipose tissue is one of the major events that causes the creation of the inflammatory phenotype of obese individuals. So, a better understanding of the mechanism of macrophage polarization is crucial for the development of a strategy to inhibit the chronic inflammation associated with obesity.

1.3 Insulin and insulin resistance

Insulin is a peptide hormone, which is produced by beta cells in the pancreas, and its main function is to regulate carbohydrate and fat metabolism in the body. It helps the skeletal muscles cells and fat tissue to absorb glucose from the blood. Transporter proteins in cell membranes

controlled by blood insulin allow glucose from the circulation to enter a cell. High levels of insulin in the blood cause the insertion of glucose to the different types of cells. On the other hand, low levels of circulating insulin, or its absence, avert the glucose entrance.

Insulin mediates its signal through two receptors: the insulin receptor (IR) an IGF receptor (IGFR). Insulin and the insulin-like growth factors (IGFs) control many aspects of metabolism, growth and survival in a wide range of mammalian tissues (Figure 1.5). The major signaling pathways, by which IR and IGFR regulate metabolism and gene expression, are the ones involving the serine kinases Akt/PKB and MEK kinase.

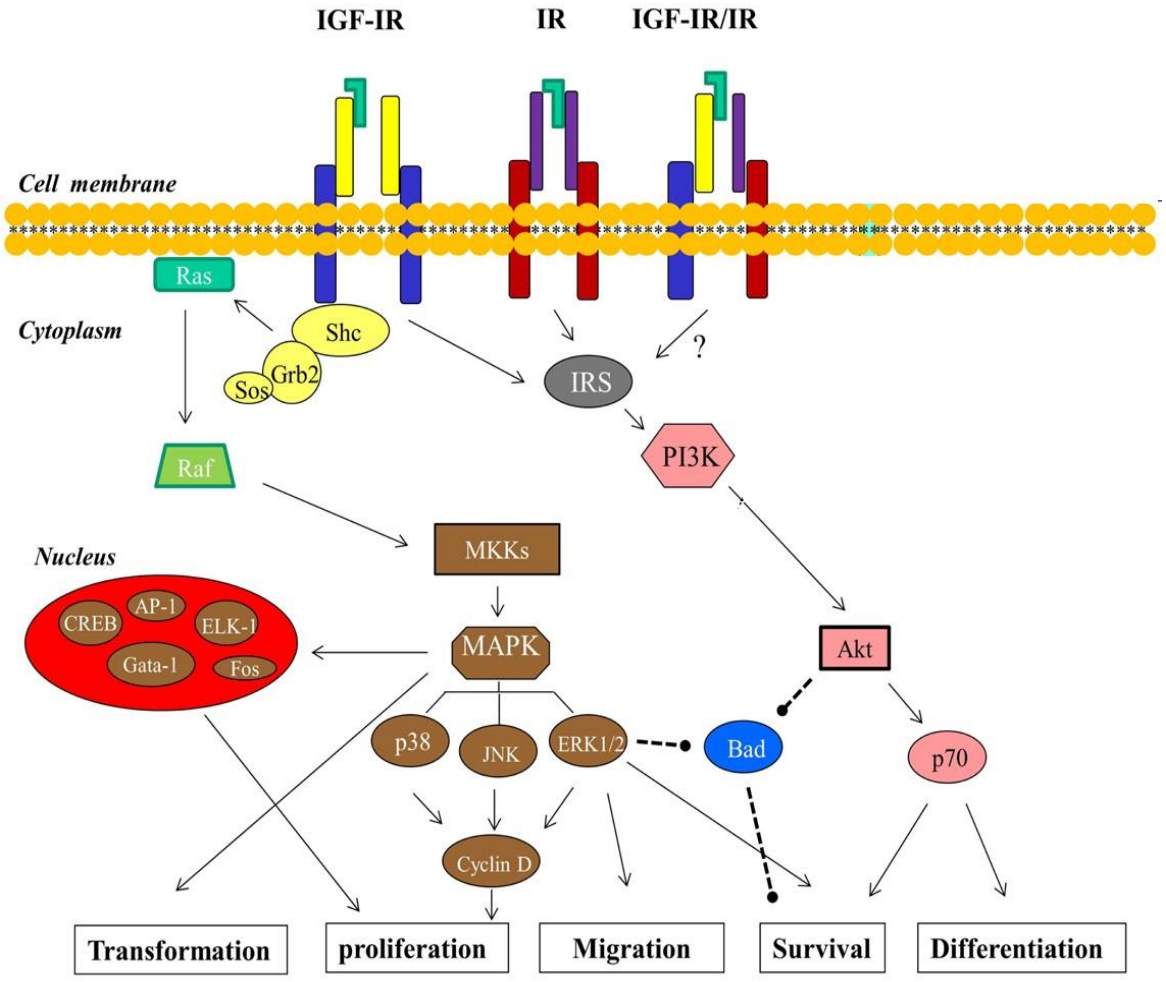


Figure 1.5: Insulin signaling pathways. Insulin mediates its signal to the cell by binging to insulin receptor (IR) or IGF receptor (IGFR).The main pathways activated by the two receptors are the ones involving the the serine kinases Akt/PKB and MEK kinase. The activation of these pathways controls the transformation, proliferation, migration, survival and differentiation of the cells.

1.3.1 Insulin signaling in macrophages

Insulin signaling on classical insulin sensitive mammalian organs, such as liver muscle and fat, has been well characterized. However, insulin signaling in macrophages are not yet well studied. Most of the molecules of the insulin signaling pathways are also expressed on macrophages except IRS1 and the glucose transporter GLUT-4 (Malide D. et al 1998). Treatment of macrophages with insulin stimulates the IR/IRS2/PI3K/AKT signaling cascade, similar to its effects in canonical insulin responsive cells (Liang CP. et al.2004). Less clear is the responses of the MAP kinase pathway to insulin in macrophages though it is known that the Ras/Raf/MEK/ERK pathway is active in these cells. Insulin signaling on macrophages effects a variety of their biological functions and properties such as viability, protein synthesis and secretion, phagocytosis and their overall action in innate immunity. Early studies documented that in the glycolytic pathway insulin increases hexokinase and citrate synthase activities in rat macrophages, while inhibiting glucose 6-phosphate dehydrogenase in the pentose phosphate pathway (Rosa LF. et al.1992). Although mouse macrophages express PI3K/AKT and Cbl/flotillin/Rho components, thought to be involved in insulin-stimulated glucose transport, insulin has no effects on 2-deoxy-Dglucose uptake by macrophages. Glucose transport in these GLUT-4–deficient cells is facilitated mainly by GLUT-1 (Gamelli R. et al.1996). Regarding macrophage innate immunity, hyperinsulinemia is known to increase phagocytic NADPH oxidase activity in human macrophages, possibly through protein kinase C, and to stimulate H_2O_2 production (Fortuno A. et al.2006). In rat macrophages, insulin can increase the activities of antioxidant enzymes such as Cu/Zn superoxide dismutase (Periera B. et al.1995). Thus insulin modulates macrophage carbohydrate metabolism, redox activity, and phagocytic capacity but it remains unclear whether insulin affects antigen processing and presentation in this cells.

1.3.2 Insulin resistance

Insulin resistance is a condition where cells have less sensitivity to insulin and it is characterized by perturbation of insulin-mediated signaling pathway resulting in systemic hyperglycemia. Even though insulin signaling is well studied, the molecular mechanisms that contribute to insulin resistance development are not well understood.

Alterations in insulin receptor expression, ligand binding, phosphorylation, and kinase activity are the most common reasons that affect the downstream signaling of insulin resulting in various clinical syndromes. Insulin receptor gene (INSR) mutations are very sporadic but at least more than 30 INSR mutations have been shown to mediate insulin receptor dysfunction, and these mutations may induce insulin resistance with various defects in its downstream signaling. Also, impaired signaling of insulin receptor may mediate insulin resistance. Decreased IR protein levels contribute insulin resistance in mice and humans. Excess insulin suppresses the expression of IR and SOCS1/3, which is induced by inflammatory cytokines, such as TNF-a, IL-6, and IL-1b enhance the degradation of IR. IR phosphorylation on serine residues is another mechanism to promote insulin resistance. IR contains numerous serine residues that are phosphorylated by kinases such as extra cellular signal regulated kinase (ERK) and cJunN-terminal kinase (JNK). More specifically, JNK phosphorylates insulin receptor substrate (IRS), a cellular molecule that connects the IR with the downstream transduction signal, on Ser-307 is a typical inhibitory signal to suppress insulin signaling. Increased TNF-α and saturated free fatty acids in obese individuals activate JNK and inhibitor of nuclear factor kB kinase b (IKKb) to phosphorylate Ser-307 of IRS (reviewed by Kwon, 2013).

!.3.3 Macrophages and insulin resistance

Various studies have demonstrated that chronic activation of intracellular pro- inflammatory pathways in insulin target cells, such as adipocytes, muscle cells and macrophages, can lead to obesity related insulin resistance. Consistent with this, TNFa and IL-6 are elevated in individuals with insulin resistance and diabetes (Tajiri Y. et al. 2005) and also neutralization of TNF-a in obese rodents improves insulin sensitivity (Hotomisligil GS. et al 1995).

As it has been previously said the adipose tissue macrophages (ATMs) have an M1 phenotype and they exhibit high levels of inflammatory markers are strongly stimulated by activating signals (Nguyen MT. et al.2007). Genetic deletion of these cells is sufficient to normalize HFD-induced inflammation, glucose tolerance and insulin resistance (Patsouris D.et al. 2008). Also, inhibition of macrophage recruitment in obesity

ameliorates the insulin resistance seen in animal models (Kanda et al. 2006; Weisberg et al. 2006, Xu et al.2003, Lumeng et al. 2007).

Furthermore, taking into account that ATMs are in lipid rich environment, there are several studies that have shown that fatty acids, especially the saturated ones, can activate the inflammatory response of macrophages and contribute to the development of insulin resistance (Lee J. et al 2001, Solinas G. et al 2007). Toll- like pattern recognitions, especially the toll-like receptor 4 (TLR4), play important role in pro-inflammatory effects of saturated fatty acids. Studies have demonstrated that circulating free fatty acid (FFA) activate inflammatory pathways and and cause insulin resistance in wild type mice but not in TLR4 knock outs (Shi H. et al 2006). Specific knockout of TLR4 in myeloid cells also have demonstrated a protection against HFD- induced insulin resistance (Saberi M. et al. 2009). This provides more direct evidence that, in vivo, macrophage TLR4 can sense saturated FFAs, and perhaps other obesity-induced signals, to engage proinflammatory pathways that lead to secretion of cytokines such as TNFα, IL-1β, and IL-6, which then block insulin signaling to produce the overall insulin-resistant state in vivo.

PPARγ is also significant for the induction of insulin resistance in macrophages. PPARγ negatively regulates a large set of inflammatory pathway genes. Thus PPARγ knock out studies have shown heightened inflammatory pathway activation, glucose intolerance and insulin resistance. Also PPARγ is a target of TZDs, which are insulin sensitizers that are used for type 2 diabetes treatment. Animals with PPARγ depletion on macrophages were less responsive to TZDs treatments, suggesting that macrophages ae important target cells for the antidiabetic actions of this class of drugs (Hevener AL. et al 2007)

The mechanisms by which macrophage mediated inflammation induces insulin resistance in insulin target cells likely due to a two-hit process in which tissue macrophages become activated, releasing cytokines and possibly other factors, which in turn activate inflammatory pathways within the neighboring insulin target cell, such as adipocytes, causing decreased insulin signaling. According to this concept, studies of conditioned media (CM) harvested from macrophages have provided direct support for this idea. Thus, when CM from wild-type primary macrophages was applied to 3T3-L1 adipocytes, inhibition of insulin-

stimulated glucose transport was observed (Solinas G. et al 2007). CM from JNK1 or TLR4 knock out macrophages does not lead to inhibition of adipocyte insulin signaling. In a similar vein, CM from PPARγ knock out macrophages displays an enhanced ability to cause cellular insulin resistance (Hevener AL. et al 2007). Thus, these studies show that macrophages secrete factors that directly inhibit insulin action and that activation of the macrophage inflammatory program enhances this effect, whereas inhibition of macrophage inflammation abrogates this effect. Taken together, these results are consistent with a two-hit mechanism in which the activated immune cell initiates an inflammatory program in the nearby insulin target cell, causing decreased insulin sensitivity. This is likely not the entire cause of inflammation-induced insulin resistance because additional events such as accumulation of fatty acid intermediates and activation of ER stress pathways within insulin target cells can also trigger proinflammatory mechanisms, contributing to decreased insulin signaling (Gregor GM. et al.2007).

To conclude, in the adipose tissue, where a strong pro-inflammatory environment exists, there is a high possibility for the development of insulin resistance. This phenomenon is mostly mediated by the pro-inflammatory cytokines which are abundantly expressed from the M1 type macrophages that infiltrate the adipose tissue. This pro-inflammatory cytokines triggers a cascade of events that leads to a further production of inhibitors of insulin signaling, such as TNF-a, from adipocytes and that contributes to the establishment of insulin resistance.

1.4 Leptin functions and its involvement in obese inflammatory environment.

1.4.1. Leptin regulation of immune system

Leptin the first adipocyte hormone identified is a 16-kd protein encoded by the ob gene. Adiposities are the most important source of leptin, and its circulating levels are highly correlated with BMI. This hormone controls the appetite and influences food intake through a direct effect on the hypothalamus. In fact, mice and humans with a mutation in the leptin (ob/ob mice) or leptin receptor (db/db mice) gene are tremendously obese. They also develop a complex syndrome

characterized by abnormal reproductive function, hormonal imbalances, and alterations in the hematopoietic and immune system.

Leptin also plays a significant role in the immune system. It is interesting that the primary amino acid sequence of leptin indicates that it could belong to the long-chain helical cytokine family such as IL-2, IL-12 and GH. In fact, leptin receptor (Ob-R) shows sequence homology to members of the class I cytokine receptor super family that includes the receptor for IL-6, leukocyte inhibitory factor and granulocyte colony-stimulating factor. Moreover, Ob-R has been shown to have the signaling capabilities of IL-6-type cytokine receptors. Ob-R expression is not limited to the hypothalamus, but is distributed widely and thus it is found in hematopoietic cells. It has been observed that the absence of leptin or leptin receptor causes a large variety of immunological dysfunctions. The immunological properties of leptin and its receptor have a dramatic influence in a variety of immune cells. It has been demonstrated that leptin can stimulate the proliferation of stem cells and hematopoiesis into lymphoid, erthyroid and myeloid lineages and it regulates the activation of human peripheral blood monocytes and lymphocytes.

Generally, adaptive immunity leptin is essential for thymic homeostasis by its anti-apoptotic functions and maintenance of thymic maturation. It enhances the proliferation and activation of T-lymphocytes and the survival of B-cells. In T-lymphocytes it boosts the production of pro-inflammatory cytokines such as IL-2, TNF-α and INF-γ. Thus, leptin modulates T-lymphocyte response towards to a more Th1 phenotype in mice through a production of a pro-inflammatory cytokines. In addition, it has been shown that leptin inhibits stress induced apoptosis of T-lymphocytes in vivo.

In innate immune system, leptin can stimulate a variety of cell types such as dendritic cells, monocytes, macrophages, neutrophils and natural killer (NK) cells. In dendritic cells it prevents apoptosis; it helps with the maturation and shifts the cytokine profile towards to a more Th1 phenotype. Furthermore, in neutrophils leptin enhances the chemotaxis and the production of hydrogen peroxide (H_2O_2). Natural killer cells are also affected by leptin. This hormone is enhances their survival and cytotoxicity by up regulating IL-2 and performing expressions through direct activation of STAT3. Apart from cytotoxicity, leptin exerts a wide

range of actions by affecting proliferation, differentiation and activation in NK cells (Figure 1.6) (reviewed by Lai 2007 and Goberna, 2003).

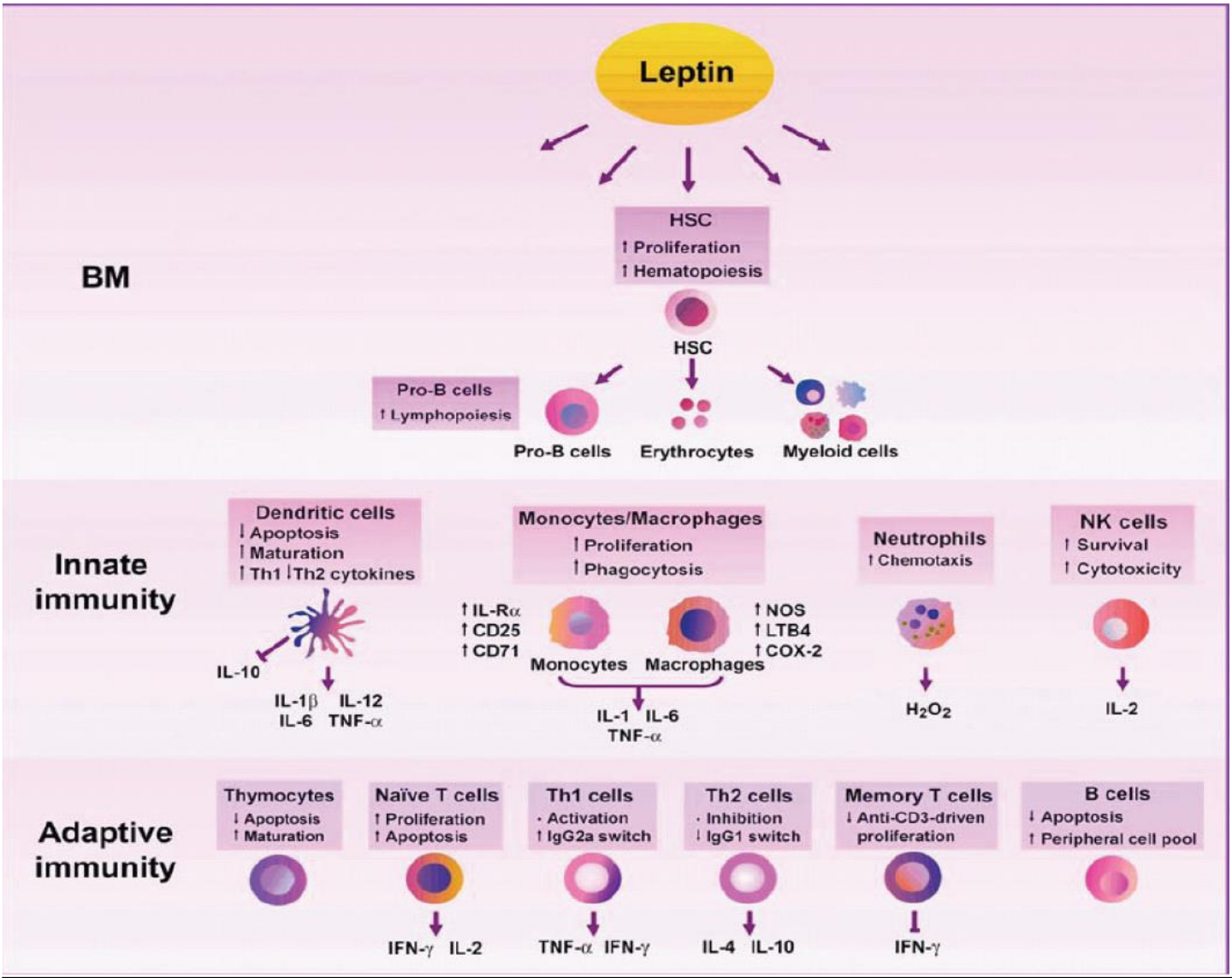


Figure 1.6: Effects of leptin in immunity. Leptin can stimulate the proliferation of stem cells and hematopoiesis into lymphoid, erthyroid and myeloid lineages. Leptin have shown direct effects in both innate and adaptive immunity. In Innate immunity can stimulate DC, monocytes, macrophages, neutrophils and natural killer (NK) cells. Leptin is involved in survival an maturation of DCs and causes a shift to a Th1 cytokines response by producing pro-infammatory cytokines such as IL-6, TNF-α, IL-12, IL-1β and inhibition of IL-10. In monocytes and macrophages, leptin has been shown to stimulate proliferation and phagocytosis, together with production of pro-inflammatory cytokines. In macrophages, leptin can induce production of factors involved in regulating immune responses such as nitric oxide, leukotriene B4 (LTB4), cholesterol acyl-transferases-1 (ACAT-1) and cyclo-oxygenase 2 (COX-2). Leptin can induce the expression of IL-1Rα, CD25 and CD71 in monocytes. It stimulates chemotaxis and release of hydrogen peroxide in neutrophils. Moreover, leptin is required for normal NK cell development and enhances their cytotoxicity.in adaptive immunity leptin is essential for the maturation and survival of thymocytes. In naïve T-cells it enhances proliferation and survival b production of INF-γ and IL-2. Th1 cells are activated and switched to an IgG2a by B cells IgG2a phenotype on leptin presence. Also they produce pro-inflammatory cytokines such as TNF-α and INF-γ. In contrast, there is an inhibition of Th2 cells. In memory T-cells leptin enhances the anti-CD3 driven proliferation. Finally, as a survival factor, leptin has been shown to suppress B cell apoptosis.

1.4.2 Leptin signaling in macrophages

In monocytes and macrophages, leptin has been shown to stimulate proliferation and phagocytosis, together with production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α. In macrophages, leptin can induce production of factors involved in regulating immune responses such as nitric oxide, leukotriene B4 (LTB4), cholesterol acyl-transferases-1 (ACAT-1) and cyclooxygenase 2 (COX-2). It has been demonstrated that leptin stimulates in vitro proliferation of peripheral blood mononuclear cells in a dose dependent manner, comparable of that of low levels LPS stimulation. Moreover, leptin has been found to stimulate the production of GM-CSF and G-CSF in macrophages and also can act as a chemoattractant for macrophage adipose tissue infiltration Gruen et al 2007 .

The main signaling pathway of leptin that induces all these procedures that take place in the immune system is a Jak/STAT pathway. When leptin binds to its receptor signal transmits through a large number of phosphorylation events that regulated the expression of several genes inside the nucleus of the cells. These phosphorylation events include the Jak tyrosine kinase and a variety of STAT molecules (STAT 1, 3, 5, 6). SOCS-3 is an inhibitor molecule of this pathway and it is associated with the leptin resistance phenomenon that is observed in obesity and several other occasions. Another pathway that is activated by leptin is a MAPK pathway which also regulates the expression of several genes that are associated with leptin function (Hegyi et al, 2004).

Although there are some gaps on how leptin acts there is enough reported evidence on the immunomodulatory effect to consider this hormone as an important signal that regulates the immune response, with a specific role in the up regulation of inflammation. On the other hand, leptin is also associated with energy storage and food intake. Thus, leptin could be considered as a link between energy stores and immune system. It has been noticed that upon starvation there is an impairment of immune system that could be an effect of leptin absence. So, leptin could be associated with immunologically mediated pathophysiological conditions. Elevated levels of leptin have been associated with an increased risk for coronary heart disease and type II diabetes. In this

context, in obesity which is characterized by high risk of developing these two conditions, overexpressed leptin is probably responsible for these diseases. Furthermore, other inflammatory disorders associated with obesity, such as atherosclerosis, are also related with high levels of this hormone. Finally, it has been suggested that there is a crosstalk between leptin and insulin signal transduction pathways, although these interaction is not yet well understood. Generally leptin is probably one of the key players in the development of the metabolic syndrome (accelerated atherosclerosis associated with insulin resistance, glucose intolerance and central obesity).

Therefore, the study of signal transduction pathways of the leptin and leptin receptor and their alterations in the immune system should contribute to a better understanding of the physiological and pathophysiological roles of leptin, its role in the development of obesity associated chronic inflammation and its contribution in the phenotypic alterations in macrophages.

1.5 Micro RNAs in immunity and inflammation

MicroRNAs (miRNAs or miRs) are a large family of highly conserved short (22 nucleotides long) single-stranded non-coding RNAs which regulate the translational inhibition of target messenger RNAs (mRNAs) by binding to their 3'-untranslated region (UTR), leading either to their inhibition or degradation. In animals, miRNAs are transcribed as primary miRNA (pri-miRNA). The pri-miRNA is processed in the nucleus to precursor miRNA (pre-miRNA) by the microprocessor complex prior to their transport into the cytoplasm. In the cytoplasm, they are further processed to mature miRNAs by Dicer and incorporated into the RNA-induced silencing complex (reviewed by Bartel, 2004).

In the past few years it has been clear that micro RNAs are involved in a variety of physiological and pathological processes. One important aspect of their action is regulation of the immune system and inflammation. There are plenty micro RNAs that has been identified as major regulators of the innate and adaptive immune system. In the innate immune system micro RNAs constitute an integrate part of the regulatory networks and the modulation of inflammation is primarily through altered expression of

specific micro RNAs in stimulated immune cells. Some of the most important micro RNAs of the innate immune system are shown in Table 1.1.

Table 1.1 miRNAs in Innate Immunity

miRNA	Targets	Effect
miR-9	NF-κB1	Negative regulator of TLR4 signaling
miR-19	TLR2	Decreases TLR2-mediated inflammation
miR-21	PDCD4, IL-12 p35	Negative regulator of TLR4 signaling
miR-27b	PPAR-γ	Enhances response to LPS
miR-105	TLR2	Decreases TLR2-mediated inflammation
miR-106a	IL-10	Decreases IL-10
miR-125b	TNF-α, IRF4	Diminishes inflammation, enhances macrophage activation
miR-145	TIRAP	Inhibits TLR signaling
miR-146a	TRAF6, IRAK1, IRAK2	Negative regulator of TLR signaling
miR-155	AID, MyD88, TAB2, Pellino-1, IKKε; SHIP-1, SOCS1, C/EBP-β	Enhances inflammation, negative feedback regulation- M1 macrophage polarization
miR-223	IKKα, Pknox1	Proinflammatory activation of macrophages
Let-7i, let-7e	TLR4	Downregulate inflammatory signaling

More specifically miR-146a is one of the first micro RNAs that was indefinite as a negative regulator of TLR signaling that is rapidly upregulated in human monocytic cells after stimulation with lipopolysaccharide (LPS) (Yang et al.2012). This upregulation is essential for endotoxin tolerance in innate immune response and insufficient induction of Mir-146a could lead to hyperactive or prolonged

inflammation (Nahid et al.2009). Mir-21 is also induced in monocytic cells by LPS, and it is downregulating the NF-kB and IL-6 expression, but enhances IL-10 production. Its anti- inflammatory response lies in targeting tumor suppressor programmed cell death protein 4, a pro-inflammatory protein, but in early time points its action is pro-inflammatory because it binds to TLR7/8. Thus the role mir-21 is maintaining homeostasis in immune responces. Also, several other micro RNAs regulate aspects of innate immune responses by targeting signaling components in inflammatory pathways. For example, miR-223, miR-105, miR-19, and let-7 family members directly target TLR2, TLR3, or TLR4. Thus, these miRNAs have an impact to the ability of immune cells to interact with TLR-specific extracellular ligands. miR-147 is induced after the activation of TLR2, TLR3, or TLR4 in both an MyD88- and TIR-domain containing adapter-inducing interferon-β-dependent manner and acts as a negative regulator to prevent excessive inflammatory responses in macrophages. miR-9 is induced by LPS in both neutrophils and monocytes and negatively regulates nuclear factor-κB–dependent inflammatory responses by suppressing the expression of nuclear factor-κB1 transcripts. Some other miRNAs, such as Let-7i and miR-125b, are downregulated in response to LPS and microbial infections. The reduced expression of these miRNAs was shown to lead to enhanced immune responses. Thus, miRNAs are important components of networks in inflammatory amplification (Liu G. et al.2014).

In addition to their role in regulating the innate immune system, micro RNAs, have been implicated in adaptive immunity by controlling the development and activation of T-cells and B-cells. The significance of miRNAs in T-cell development and activation was primarily established by using global and specific miRNA-deficient mice. Different studies showed that disruption of miRNA biogenesis by conditional removal of Dicer in the early stages of T-lymphocyte development results in reduced T-cell population in the thymus and peripheral lymphoid organs and causes abnormal T-helper (Th) cell differentiation and cytokine production. Several micro RNAs have been well characterized in the regulation of T-cell differentiation and clonal expansion (Table1.2). More specifically, miR-146a, one of the major miRNAs expressed in Treg cells, is crucial for their suppressive functions. Deficiency of miR-146a in Treg cells resulted in failure of tolerance induction. This may be a result of increased

expression and activation of signal transducer and activator transcription 1, which is a direct target of miR-146a. Another micro RNA, miR-181a has a significant influence on positive selection by enchancing T-cell receptor signaling during thymic development. Inhibition of miR-181a led to unnecessary reactions to self-peptides. Thus, miR-181a contributes to clonal selection and to preventing autoreactive T-cell clones from reaching the periphery and potentially causing autoimmune disorders. miR-17–92 cluster miRNAs are involved in cell survival. Overexpression of the miR-17–92 cluster in transgenic mice results in T-cell populations that show enhanced proliferation and less activation-induced cell death. miR-326 was shown to target Ets, a transcription factor that negatively regulates Th17 development, and overexpression of miR-326 led to the promotion of Th17 differentiation and IL-17 production (Liu G. et al.2014).

Table 1.2 miRNAs in Adaptive Immunity

miRNA	Targets	Effect
miR-10	Ncor2, Bcl6	Treg↑
miR17–92	Bim, PTEN	CD4 T-cell proliferation↑
miR-29b	T-bet, IFN-γ	Th1↓
miR-146a	IRAK1,TRAF6, Stat1	TCR signaling↓, Treg↑
miR-155	c-Maf, PU.1, SHIP-1, AID	Th1↑,Th17↑,Treg survival↑, Th2↓, antibody secretion↑ ,class-switch recombination↑
miR-181a	SHP1, 2- DUSP5, 6	TCR signaling↑
miR-182	Foxo1	CD4 T-cell expansion↑
miR-301a	PIAS3	Th17↑
miR-326	Ets	Th17↑
miR-150	c-Myb	B1 cell expansion↓
miR-181		B-cell expansion↑

1.5.1 MiR-155 and its role in the immune system

MiR-155 is a significant immunoregulatory micro RNA that plays role both in innate adaptive immune system. MiR-155 is encoded by the MIR-155 host gene or MIR-155HG contained within the noncoding B cell integration cluster (Bic) gene (Lagos-Quintana et al., 2002). The MIR-155HG is composed of three exons that span a 13 kb region within human chromosome 21 (Hsa21 band q21.3). The MIR-155HG is transcribed by RNA polymerase II and the resulting ~1,500 nucleotide RNA is capped and polyadenylated. The 23 nucleotide single-stranded miR-155, which is harbored in exon 3, is subsequently processed from the parent RNA molecule.

In adaptive immunity miR-155 regulates several aspects of T-cell and B-cell function as far as their proliferation. Studies with miR-155 dificient mice showed that miR-155 is essential for the polarization of T-cells towards Th1 responses and also that is important for Th1 and Th17 cell production during autoimmune inflammation. This inhibitory effect of miR-155 on Th2 differentiation is through targeting c-Maf, which is important for the development of Th2 cell population. MiR-155 is also important for the development of T-regs but not for their suppressive function. In B-cell population, miR-155 is also important for their activation. Deficiency of miR-155 in B cells led to reduced production of the IgM isotype and IgG-switched antibodies (Vigorito et al.2013).

In innate immune system TLR agonist stimulation (lipopolysaccharide (LPS), interferon-b (INF-β) and tumor necrosis factor (TNFa)) enhances the production of miR-155 in macrophages and dendritic cells (O'Connell et al. 2006). More specifically, macrophages treated with LPS upregulate MiR-155 through NF-kβ and AP-1 transcription factors. This upregulation of MiR-155 by LPS indicates this molecule as a pro-inflammatory (M1) marker of macrophages. The activation of MiR-155 also leads to suppression of negative regulators of inflammation, such as suppressor of cytokine signaling 1 (SOCS1) (Lu et al, 2009, Androulidaki et al, 2010).

1.6 ''Obesity paradox''

As it is previously mention obesity is a major cause for developing a variety of health disorders and generally obese individuals are prone to

have worst clinical outcomes than the normal ones. On the other hand there are several studies that suggest that obese individuals that are hospitalized in the intensive care unit (ICU) have better outcomes after infection than the normal weight ones (Ray et al,2005, Wurzinger et al, 2010, Hatagalang et al, 2011). Even though obese patients are more difficult to handle, it has been noticed that they have equal or lower mortality levels. In spite that it is very risky to make conclusions and more than one parameters should be taken under consideration. For example, the age of the penitent, the genetic background and habits like smoking could modify the outcomes of the penitents in the ICU.

This recent data though arise the question why these patients respond better and how their phenotype helps their recovery. Further analysis of the immunological response of obese individuals and their unique characteristics may answer these questions.

1.7 Aims of this Study

Since obesity alter the circulating levels of insulin and leptin, our goal was to investigate their effect on macrophage activation and the potential role of miR-155 as a mediator of their action. Specifically, aim of this study was to define the impact of insulin and leptin on miR-155 in macrophages as a potential mediator of their effects on activation and polarization. Finally, the last aim of this study was to investigate the connection between the insulin resistance phenotype and the leptin overproduction in obesity associated macrophages.

2. Materials and methods

2.1 Mouse models and treatments

For this study, wild type C57BL-6, Akt2-/- , and IGF1R-/- macrophages specific knock outs mice (LyMCre+/IGF1Rfl/fl) and High Fat Diet mice were housed in micro-isolators in pathogen-free facility. All mice that were put into this study were male. High fat diet mice were fed with a special diet purchased from Mucedola srl ™ for 5 days. All this groups were injected intraperitoneally with 2ml 4% thioglycollate for 5 days. After 5 days peritoneal macrophages were collected and cultured in DMEM or resuspended in Trizol reagent for further treatments.

2.2 Cell cultures and treatments

Insulin resistance protocol: Macrophages ($3*10^5$ cells) from 3 different mice were cultured in DMEM (Invitrogen) media containing 10% FBS, penicillin, and streptomycin (100U/ml), overnight in a 24-well Nunclon ™Delta Surface plate (Nunc A/S, Roskilde, Denmark) . The next morning, when the cells were attached to the surface of the plate they treated with 100nM Humulin® Regular (by Eli Lilly and Company). The Humulin® treatment was repeated after 8h for 48h.

Leptin treatments: 100ng/ml of Recombinant Murine Leptin from Peprotech® was used to treat peritoneal macrophages cultured in a 24-well Nunclon ™Delta Surface plate for 6 h. Leptin is tested to be endotoxin free.

LPS treatments: Macrophages were also treated with 100ng/ml Lipopolysaccharides from Escherichia Coli (SIGMA®, Saint Luis, Missouri, USA) for 6h.

IL-4 treatment: Macrophages isolated from wild type mice were treated with 10ng/ml IL-4 from R&D Systems® for 48h.

2.3 BCA Protein Assay

To measure the total protein production of the cultured macrophages we used a BCA protein assay.

Reagents and instruments:

- RIPA Buffer (50mM Tris-HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, 50mM NaF)
- Microplate well (Thermo Scientific™ Pierce™ 96 -Well Plates, Product No. 15041)
- Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Rockford, USA)
- Model 680 Microplate Reader (BioRad, Hercules, California, USA)

Procedure:

First, cells from cultures were resuspended in 50µl of RIPA buffer. Next, all standards were prepared with a working range of 20-2000 µg/mL. Then, the total number of wells had to be calculated in order to determine the amount of working reagent (WR) needed. The calculation formula is: (n standards + n unknowns) × (n replicates) × (volume of WR per sample) = total volume WR required. To make a working reagent, BCA reagents A and B were mixed in a ratio 50:1. 25µL of each sample and standard was added to the respective microplate well. Then, 200µL of the working reagent were added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was shielded and incubated at 37°C for 30 minutes. Finally, the plate was cooled to RT and measured at 550 nm on a plate reader.

2.3 ELISA

For the detection of the levels of Interleukin-6 that were produced from cultured macrophages Enzyme Linked ImmunoSorbent Assay was performed.

Reagents and Instruments:

- Mouse IL-6 or TNFa ELISA Ready-SET-Go! ® [containing 250x Capture Antibody, 250x Detection antibody, 250x Detection enzyme (Avidin-HRP), ELISA/ELISPOT Coating Buffer Powder, 5x concentrated Assay Diluent, Human IL-6 Standard] (eBioscience, Inc., San Diego, California, USA)
- 1x Tetramethylbenzidine (TMB) Substrate Solution (eBioscience, Inc., San Diego, California, USA)
- Stop Solution (2N H_2SO_4)
- Wash Buffer (1x PBS, 0.05% Tween-20)
- 96-well plate (Nunc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)
- Model 680 Microplate Reader (BioRad, Hercules, California, USA)

Procedure:

A 96-well plate (Nunc C8 White LockWell MaxiSorp) was coated with 100 µL/well of 1x Capture Antibody in Coating Buffer (prepared by diluting ELISA/ELISPOT Coating Buffer Powder in appropriate volume of distilled water) and left to incubate overnight at 4 ^{o}C. The plate was washed three times with Wash Buffer and it was blocked with 200 µL/well of 1X Assay Diluent followed by incubation at room temperature for 1 hour. Next, the plate was washed once with Wash Buffer and the samples as well as the standards (diluted according to manufacturer's protocol) were added (100 µL/well) and left to incubate overnight at 4^{o} C on a rocker. After four washes with Wash Buffer, incubation of 100 µL/well of 1x Detection antibody for 1 hour at room temperature, four washes with Wash Buffer and subsequent incubation of 100 µL/well of 1x Detection enzyme (Avidin-HRP) for 1 hour at room temperature, 100 µL/well of 1x Tetramethylbenzidine (TMB) Substrate Solution to each well were added and the plate was left to incubate at room temperature for 15 minutes. The reaction was stopped by adding 50 µL of Stop Solution to each well and the plate was read at 450 nm and 570 nm at a Model 680 microplate reader. Values at 570 nm were subtracted from those of 450 nm and data were analyzed by Graph Pad Prism 5 software.

2.4 RNA Extraction

For the extraction of the RNA from the macrophages a Trizol protocol was performed.

Reagents:

- TRI Reagent® (SIGMA Life Science, St. Louis, USA).
- Chlorophorm
- Isopropanol
- Ethanol

Procedure:

$3*10^5$ macrophages were resuspended in 250 µl of TRI Reagent ® and 50 µl of chlorophorm were added. The samples were centrifuged at 12.000g at 4 ^{0}C for 15 min. After the centrifuging the aqueous phase was removed and transferred to a new Eppendorf tube in which 125 of 100% isopropanol were added. The samples were shaken for 15 seconds and incubated at room temperature for 10 min. The incubation followed by another centrifuge at 12.000g at 4^{o}C for 10 min. The supernatant was removed from the tubes and the pellet was washed with 250 µl of 75% ethanol. Last centrifuging was performed at 12.000g at 4 0C for 15 min. Finally the pellet was dried for 10-15 min and resuspended in 20µl of RNase- free water. The same procedure was used also for the macrophages directly isolated from the mice. The T amounts of the cells were about $1*10^6$ and thus they were resuspended in 500µl of TRI Reagent. The amounts of the other reagents were adjusted respectively.

2.5 DNase treatment:

To remove the DNA that may precipitate with the RNA in the samples DNase treatment was performed.

Reagents:

- DNase I (RNase free) (New England Biolabs®$_{Inc}$), concentration 2000 units/ml.
- DNase I reaction Buffer 10x (New England Biolabs®$_{Inc}$).

Procedure:

0,5µl of DNase I and 1µl of DNase I reaction Buffer were added in each sample. The sample were incubated at 32 ^{0}C for 30 min and then at 75 ^{0}C for 10 min.

2.6 c-DNA synthesis

To synthesize the first strand c-DNA from the isolated RNA a PrimeScript™ 1st strand cDNA Synthesis Kit (TAKARA BIO INC.) was used.

Procedure:

First a mix of the following reagents was made:

Component	**Volume**
Random 6mers50µM	1 µl
dNTPs 10 mM each (mix)	0.5 µl
Template RNA	5,75 µl *
Total	**7,25 µl**

* The RNA should be <5µg. If 5,75 µl of RNA exceed 5µg then the volume is adjusted and the remaining is made up with H_2O.

This mix was then incubated for 5 min at 65 ^{0}C. After the incubation, the following reagents were added to the above mix:

Component	**Volume**
5x Prime Script Buffer	2 µl
RNase Inhibitor (40U/µl)	0,25 µl
Prime Script RTase (200U/µl)	0,5 µl
Total	**10 µl**

For the first strand cDNA synthesis a thermo cycler Alpha Unit™ Block Assembly for PTC DNA Engine™ Systems was used and the above programmed was followed:

Step	**Time**	**Temperature (°C)**
Hold	10 minutes	30°C
Hold	50 minutes	42°C

Hold	5 minutes	95°C
Hold	∞	4°C

2.7 Quantitative PCR (qPCR)

To detect the gene expression profile a quantitative PCR was performed.

Materials:

- KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPABIOSYSTEMS, BOSTON, Massachusetts, United States).
- MicroAmp® Optical 8-Cap Strip (Applied Biosystems® by Life technologies, USA).
- 7500 Fast® Real Time PCR System (Applied Biosystems® by Life technologies, USA).

Presedure:

In the MicroAmp® Optical 8-Cap Strip the following mix was made:

Components	**Final concentration**	**Master mix volume per 10-µL reaction**
KAPA SYBR®FAST qPCR Master Mix (2x) Universal	1x	5µl
Forward Primer (10µM)	200nM	0,2 µl
Reverse Primer (10µM)	200nM	0,2 µl
ROX Low	-	0,2 µl
Template DNA	<10ng	1 µl
Water	-	3,4 µl
Total		**10 µl**

The MicroAmp® Optical 8-Cap Strips were then inserted into the 7500 Fast® Real Time PCR System and qPCR performed by following the steps bellow:

Step	**Temperature**	**Duration**	**Cycles**

Enzyme activation	95°C	3min	Hold
Denature	95°C	15sec	40
Anneal/extend	60°C	60sec	

The data were analyzed by Graph Pad Prism 5 software.

2.8 TaqMan® MicroRNA Assay

To detect the miR-155 expression TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems® by life technologies, USA) was used to synthesize the first strand cDNA of the RNA isolated from macrophages.

Procedure:

A mix of the reagents provided with the kit was made following the amounts below:

Component	**Master mix volume per 10-µL reaction**
dNTP mix (100mM total) with dTTP	0.1 µl
MultiScribe™ Reverse Transcriptase, 50 U/µL	O,67 µl
10× Reverse Transcription Buffer	1 µl
5x Taq Man Micro RNA RT Primer	2 µl
RNase Inhibitor, 20 U/µL	0.126 µl
RNA	<100ng
Nuclease-free water	
Total	**10 µl**

To perform reverse transcription a thermo cycler Alpha Unit™ Block Assembly for PTC DNA Engine™ Systems was used. The conditions are described below:

Step	Time	Temperature (°C)
Hold	30 minutes	16°C
Hold	30 minutes	42°C
Hold	5 minutes	85°C
Hold	∞	4°C

After the cDNA synthesis a quantitive Quantitative PCR (qPCR) amplification was performed to detect the miR-155 levels in each sample.

Materials:

- TaqMan® Small RNA Assays Quick Reference Card (Applied Biosystems® by Life technologies, USA).
- MicroAmp® Optical 8-Cap Strip (Applied Biosystems® by Life technologies, USA).
- 7500 Fast® Real Time PCR System (Applied Biosystems® by Life technologies, USA).

Reaction set-up:

Component	Volume (µL)
TaqMan® Universal PCR Master Mix II, no UNG	5 µl
Nuclease-free water	3.17 µl
TaqMan® Small RNA Assay (20✕)	0.5 µl
Product from RT reaction	1,33 µl
Total	**10 µl**

Reaction conditions:

Step	Optional AmpErase® UNG activity	Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time	2 minutes	10 minutes	15 seconds	60 seconds

The data were analyzed by Graph Pad Prism 5 software.

2.8 Western Blot

To perform western blot analysis 12% polyacrylamide gels were prepared:

Materials:

- For the separating gel: H2O, 30% acrylamide mix, 1,5 M Tris (pH 8,8),10% SDS, 10% ammonium persulfate, TEMED
- For the staking gel: the same solutions as separating except of using 1M Tris (pH 6,8)

The amounts of each solution depended on the number of the gels that were prepared each time. The samples were loaded and run for about 2h at 120V.

SDS-page gels were transferred on nitrocytarine membranes by using a wet transfer apparatus. Each time, one whatman paper and one sponge were used on the top of the gel and under the membrane. The membrane, the gel and the papers were incubated in transfer buffer 1X. The transfer was performed for 1h at 400mA constant. After the transfer was complete, the membrane was incubated into a blocking buffer (5% BSA into 1X PBST) for 1h at room temperature. After the incubation the membrane was washed with PBST 1X (X3, for 15 min each). At the same time the primary antibody (anti- phospho-Akt-473 specific) was diluted 1:1000 into 3% BSA and after the washes the membrane was incubated over night at 4 ^{o}C. The next day the membrane was washed again 3x with PBST (15min each). After the washes a secondary antibody (anti-rabbit) was diluted 1:7500 in 5% BSA and incubated with the membrane for 1h at room temperature. This step followed by another round of washes with PBST. . Finally, LCL was added on the top of the membrane (2ml→1ml of buffer A and 1ml of Buffer B). Lass 3000 was used for the detection.

For the detection of the amount of total Akt the membrane was stripped by using a stripping buffer.

Stripping buffer:

The membrane was incubated with the stripping buffer for 10 min at 55 ^{o}C. An anti-Akt (1:1000) antibody was used for the detection of total Akt in the samples. The same procedure and secondary antibody, as above, was followed.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com and p value < 0.05 was considered as indicative of statistical significance. Furthermore, One-way ANOVA analysis was performed. Tolerance tests were analyzed by two way ANOVA test with Conferring post-test.

3. Results

3.1 Down regulation of miR-155 in insulin resistant peritoneal macrophages.

To test the expression levels of miR-155 in insulin resistant macrophages we isolated the peritoneal macrophages directly from wild type, high fat diet (HFD), Akt2-/- and IGFR-/- macrophages specific knock outs. Akt2-/- mice tent to develop insulin resistance, since Akt2 is a significant molecule downstream of insulin signaling. Furthermore, IGFR--/- macrophages specific knock outs mice, develop insulin resistance specifically in their macrophages since insulin signaling is also transduced through IGF receptor. The levels of miR-155 were measured by Q-PCR as described in material and methods section 2.8. As shown in Figure 3.1, there is a significant downregulation of miR-155 in all insulin resistance macrophage phenotypes.

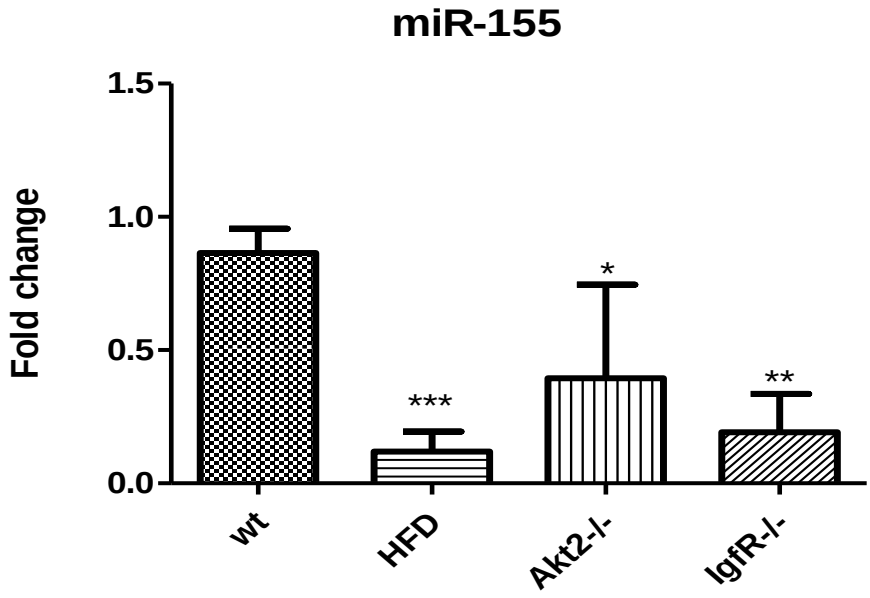


Figure 3.1: Downregulation of Mir155 in insulin resistant peritoneal macrophages. Peritoneal macrophages from wild type, HFD, Akt2-/- and IGFR-/- macrophages specific knock outs were isolated from mice. Each group is consisting of 3 mice in duplicates. The levels of Mir155 were detect by Q-PCR. ***p<0,0002.

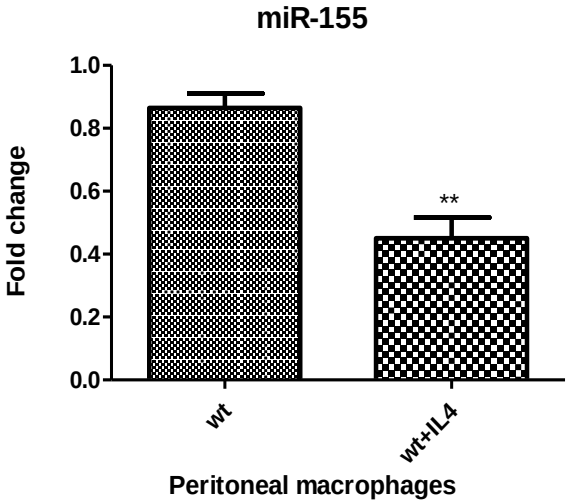


Figure 3.2: Downregulation of Mir 155 in IL-4 treated peritoneal macropgages. Cells from three mice were used and cultured without treatment or with IL-4 (10ng/ml) for 4h. The levels of Mir155 were detect by Q-PCR.***p<0,0002

In agreement with a previous study in our lab (Arranz A. et al, 2012), peritoneal macrophages from Akt2-/- mice, that down regulate the pro-inflammatory molecule miR-155 tend to have an M2 phenotype. Also another coworker (Eleftheria Ieronymaki) showed that peritoneal macrophages from these types of mice upregulate typical M2 markers such as Arg1 and Ym1. To further investigate if macrophages that have M2 phenotype down regulate MiR-155 we also treated wild type macrophages with IL-4 (10ng/ml) for 48h. Is previously known that IL-4 treatment tent to polarize macrophages to the M2 phenotype (Ji Y. et al, 2012). As shown in Figure 3.2, IL-4 treatment also down regulates MiR-155 expression.

These results indicate that MiR-155 which is a pro- inflammatory molecule is deregulated in peritoneal macrophages of all insulin resistance models and thus these macrophages tend to be polarized in to the M2 phenotype.

3.2 Insulin resistance *in vitro* model also down regulates the MiR-155 expression

To investigate if the in vitro insulin resistance model that we use has the same phenotype as the in vivo models we perform a Q-PCR for the Insulin Receptor (IR), IGF Receptor (IGFR) and Akt2 gene. As it was previously referred, in the Insulin resistance models these molecules are down regulated since insulin uses the IR and IGFR pathways for cell signaling and Akt2 is a downstream signal transduction molecule. As it

shown in figure 3.3 after 48h of insulin treatment (6 doses every 8h) peritoneal macrophages there is a down regulation of IR about 5 fold, IGFR about 2 fold and Akt2 about 3 fold (Figure 3.3). Thus the in vitro insulin resistance model resamples the in vivo phenotype.

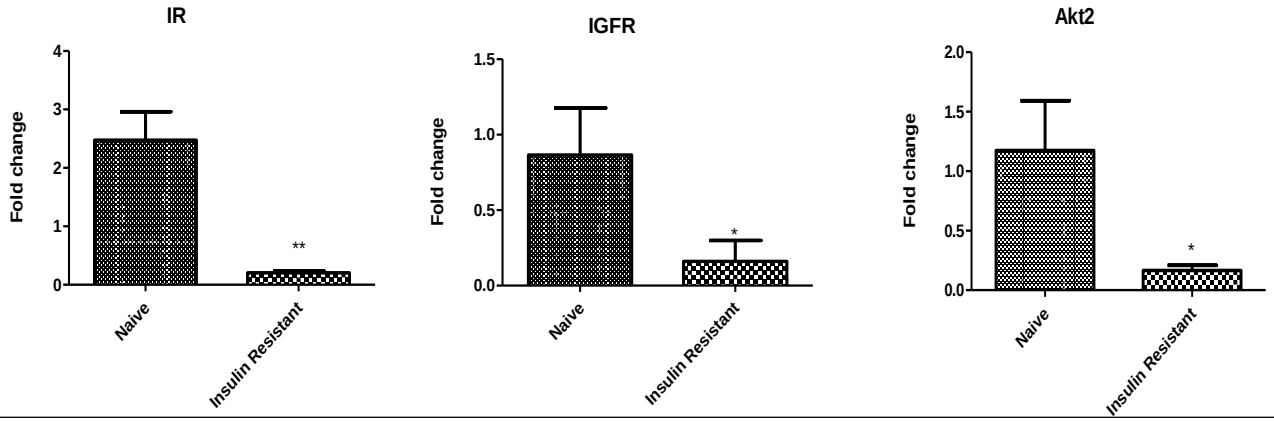


Figure 3.3: Downregulation of insulin receptor (IR)(A), IGF receptor (IGFR)(B) and Akt2 (C) after insulin treatment. For each treatment 3 mice were used. Insulin treatment was in 6 doses every 8h for 48h. The levels of the genes were detect by Q-PCR. *p<0,0232,**p<0,0013.

To evaluate further that the insulin resistant phenotype leads to low levels of MiR-155 we tested by Q-PCR the expression levels in our *in vitro* insulin resistance model. MiR-155 was significantly down regulated (approximately 6 fold) after insulin treatment (Figure 3.4).

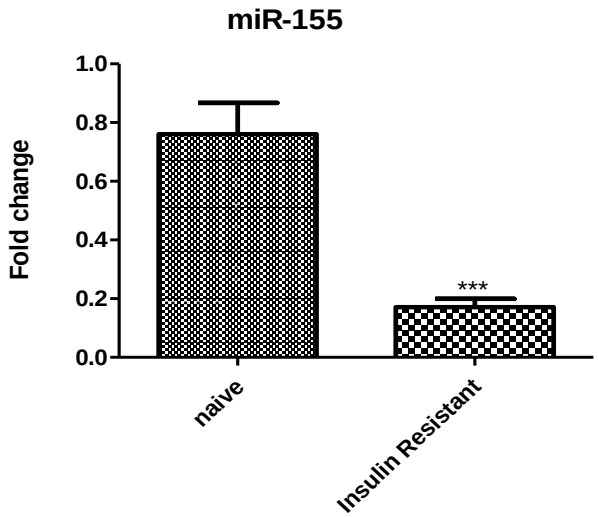


Figure 3.4: Downregulation of miR-155 in insulin resistant macrophages. Cells from three mice were cultured without treatment or with insulin treatment (100U/ml) for 48h (6 doses every 8h). The levels of miR-155 were detect by Q-PCR.**p<0,0002

3.3 Insulin resistance reduces immunological responses after LPS treatment.

To further answer the question regarding how insulin resistance affects a systemic inflammation we treated peritoneal macrophages with either LPS alone or LPS together with insulin. Intriguingly, when an ELISA was performed we observed lower levels of inflammatory cytokines (IL-6 and TNFα) in LPS and Insulin treated macrophages than the LPS treated alone.

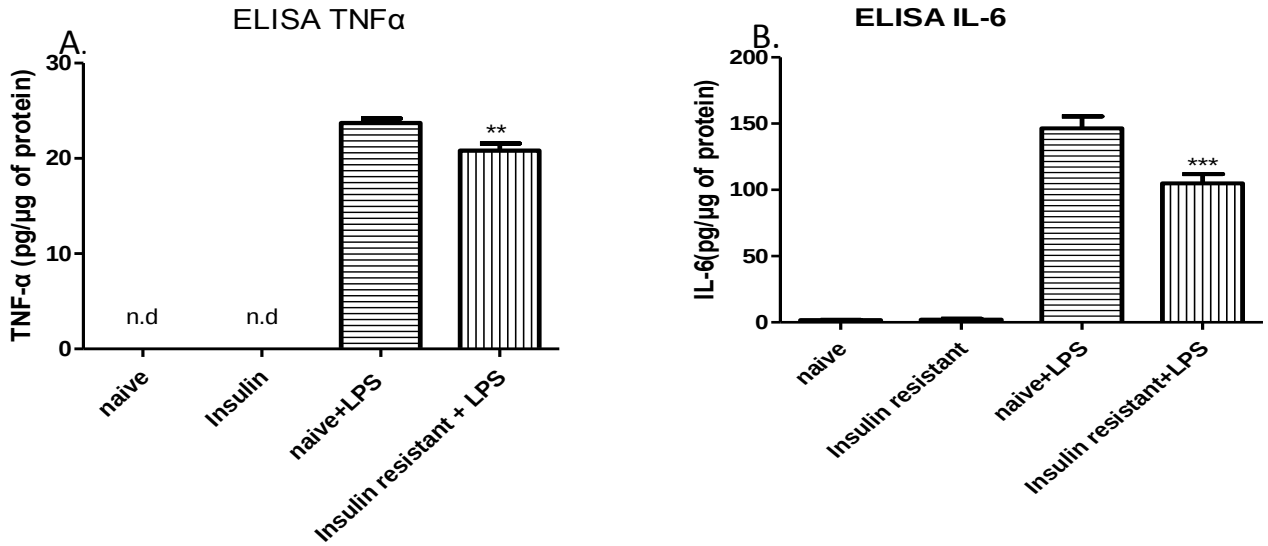


Figure 3.5: insulin treatment reduces the production of pro-inflammatory cytokines after LPS treatment. A) TNFα cytokine production is reduced in Insulin resistance macrophages after treatment with LPS (100ng/ml) compare to insulin sensitive as measured by ELISA assay. Macrophages were treated for 6h with LPS and 48h (6 doses, each every 8h) with insulin. Supernatants from 3 different mice in dublicates were used for each group.**p<P<0.0001.B) IL-6 production is also reduced in insulin resistant macrophages. The treatment were the same as for TNF-α. The plot represents an average of 3 independent experiments each consisting of supernatants of three mice in duplicates.***p<0.0001.

It is previously known that LPS induces the production of pro-inflammatory molecule MiR-155. Thus, to answer the question how insulin resistance may affect the expression levels of miR-155 after LPS treatment we performed a Q-PCR. RNA from peritoneal macrophages that were treated either with LPS for 6h or LPS and insulin for 6 and 48h, respectively, was isolated and a Q-PCR was performed. The results showed that not only insulin reduces the miR-155 expression levels in

uninfected macrophages but also there is a down regulation about 2 folds of the micro RNA after LPS treatment (Figure 3.6).

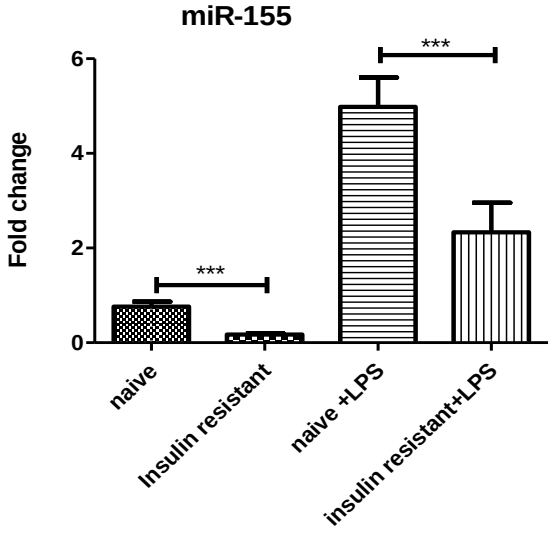
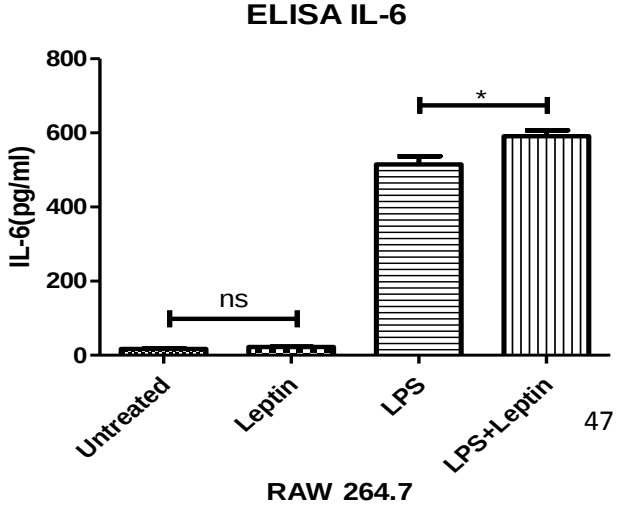


Figure 3.6: LPS treated insulin resistant macrophages are reducing the MiR-155 expression levels. Cells from 2 mice in duplicates were treated with either insulin (48h) or LPS (6h) or both LPS and insulin. The levels of MiR-155 were detect by Q-PCR.*p< 0,0001

3.4 Leptin alone fails to induce pro-inflammatory genes but it primes macrophages to be more responsive to Lipopolysaccharides.

To investigate the role of leptin, which is well documented to contribute to excessive inflammation in obese individuals (Reily et al., 2004, Lago et al., 2008) in macrophages we test the expression of pro-inflammatory mediators in peritoneal macrophages and in murine macrophage cell line RAW 264.7. These two cell types were both treated with either LPS (100ng/ml) for 6h, Leptin (100ng/ml) also for 6h or both LPS and Leptin. As it has been previously shown (Vaughan et al., 2010), leptin alone was not sufficient for robust induction of IL-6 in both peritoneal macrophages and RAW 264.7 murine macrophage cell line, but after combined Leptin and LPS treatment there is a significantly elevated induction of IL-6 in both cell types (Figure 3.6).



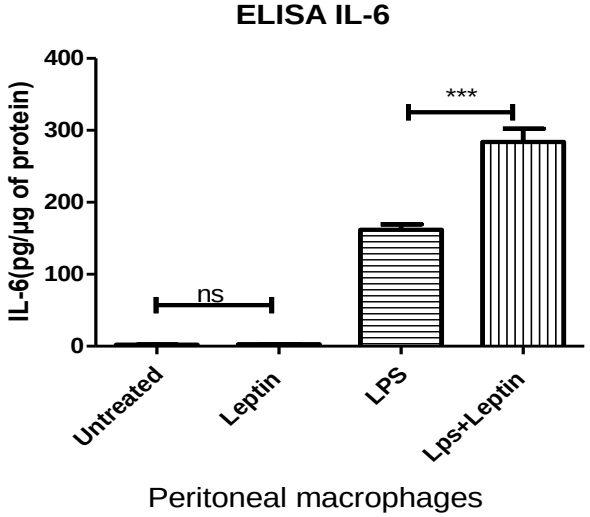


Figure 3.7: Leptin induces the production of IL-6 in macrophages after LPS treatment. Supernatant of peritoneal macrophages and RAW 264.7 cells were collected after treatment with LPS (100ng/ml), leptin (100ng/ml) or both LPS and leptin. Leptin treatment alone fail to significantly induced IL-6 production but in combination with LPS, leptin enhanced further the IL-6 production. The Levels of IL-6 were mesuered by ELISA.***p<0,0001.

3.5 Leptin induces the production of pri-miR-155 in RAW 264.7 and miR-155 in peritoneal macrophages

To answer the question how may leptin affect the production of Mir 155 in peritoneal and RAW264.7 macrophages we treated cells with leptin for 6h and then measure by Q-PCR the levels of Pri- MiR-155 and MiR-155 from cultured RAW 264.7 and peritoneal macrophages respectively. Pri-MiR-155 is a precursor of MiR-155 and its levels are in comparison with the levels of mature MiR-155. Our results indicated that leptin enhances about 3 folds the levels of Pri-MiR-155 in Raw 264.7 cells and about 2 folds the levels of Mir 155 in peritoneal macrophages.

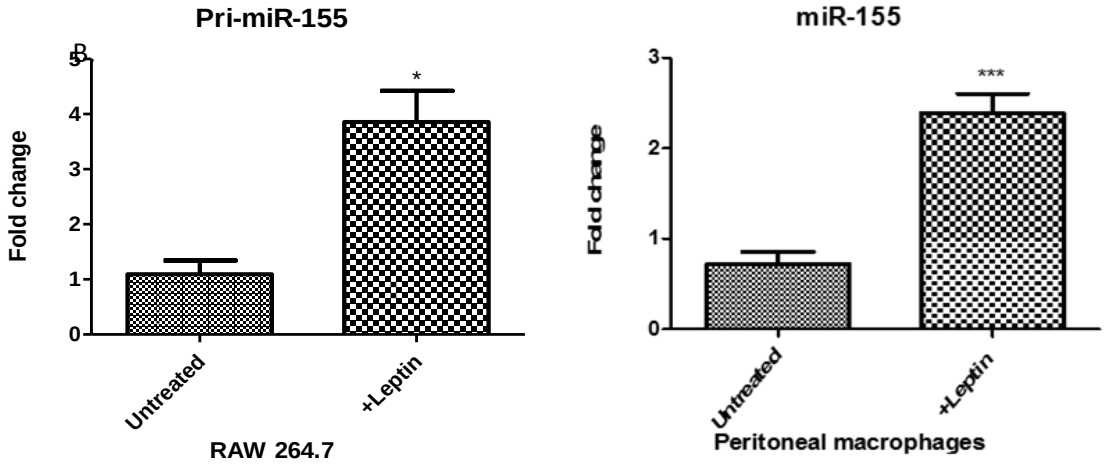


Figure 3.8: Upregulation of Pri- miR155 and miR-155 after LPS treatment. A)Raw 264.7 cells were cultured and treated with 100ng/ml leptin for 6h. Leptin upregulates the levels of Pri-miR-155 as shown by Q-PCR. *p<0,0113.B)Peritoneal macrophages from three different mice treated with 100ng/ml leptin for 6h. Q-PCR showed an upregulation of miR-155 levels .***p<0,0001

3.6 Leptin further induces the production of Pri-miR-155 and miR-155 in Raw 264.7 cells and peritoneal macrophages respectively after LPS treatment.

It has been shown that Lipopolysaccharides induces the production of miR-155 in peritoneal macrophages and Raw 246.7 cells. In Raw cells the induction is usually about 30-40 fold and in peritoneal macrophages about 3-5 fold. To investigate how leptin affects the miR-155 we measured its production after a co-treatment of leptin (100ng/ml) and LPS (100ng/ml) for 6h. The result showed that leptin further induces the production of Pri-miR-155 and mir 155 in Raw 246.7 cells and peritoneal macrophages respectively. As shown in figure 3.9, the induction is elevated about one fold in peritoneal macrophages and 30 fold in Raw 246.7 cells.

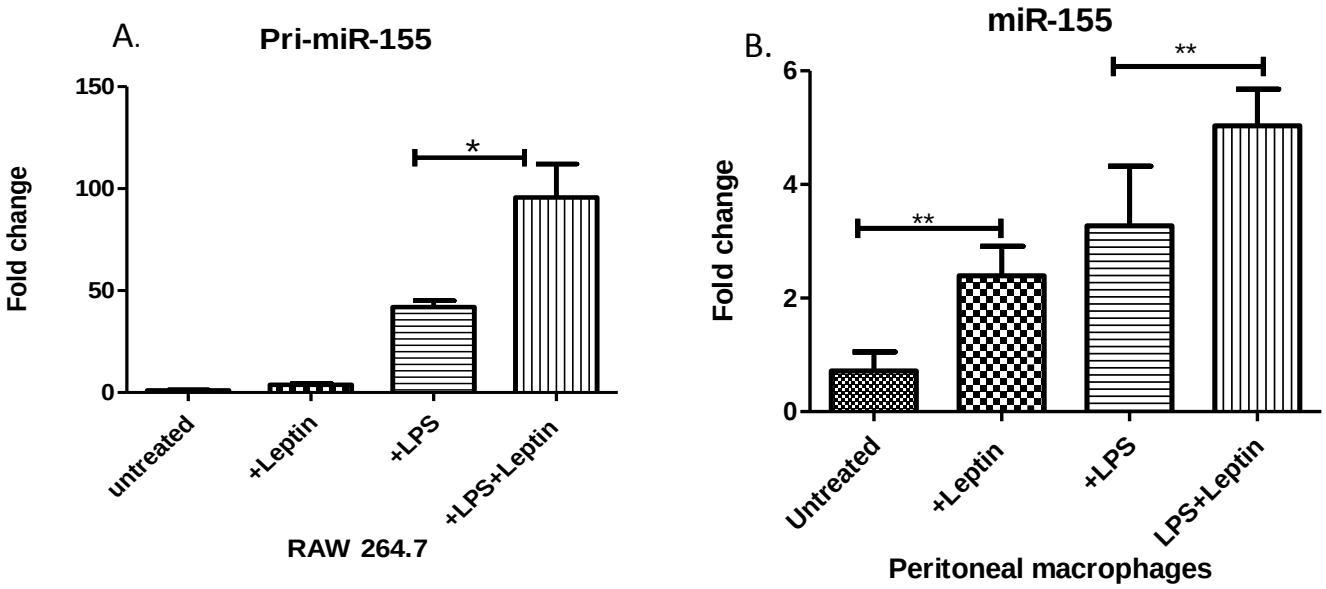


Figure 3.9: Upregulaton of Pri-Mir155 and Mir155 in Raw 264.7 cells and peritoneal macrophages. A) Raw 264.7 cells were treated either with leptin (100ng/ml), LPS(100ng/ml) or both for 6h. Q-PCR showed elevated levels of Mir 155 after co- treatment of leptin and LPS compare to LPS alone. B)Peritoneal macrophages cells were treated either with leptin (100ng/ml), LPS(100ng/ml) or both for 6h. Q-PCR showed elevated levels of Mir 155 after co- treatment of leptin and LPS compare to LPS alone. *p<0,001

3.7 Leptin induces Serine phosphorylation of Akt but does not modify its insulin-induced phosphorylation.

Downstream the Insulin pathway a major modification of the signal transduction is a serine 473 phosphorylation of the Akt. The phosphorylation of this molecule indicates the activation of the insulin pathway. To check if insulin-induced phosphorylation in serine 473 is affected by leptin we pre- treated peritoneal macrophages over night

with 100 ng/ml leptin. After the treatment the media was replaced with starvation media (without FBS) for 2h followed by 30 min of insulin treatment (100nM). The levels of phosphor-Akt were measured by western. The results showed that leptin induces the phosphorylation of Akt by it do not affect its phosphorylation by insulin (Figure 3.9). Insulin and leptin independently increase the phosho-Akt levels about 0.5 fold and the co-treatment had no effect.

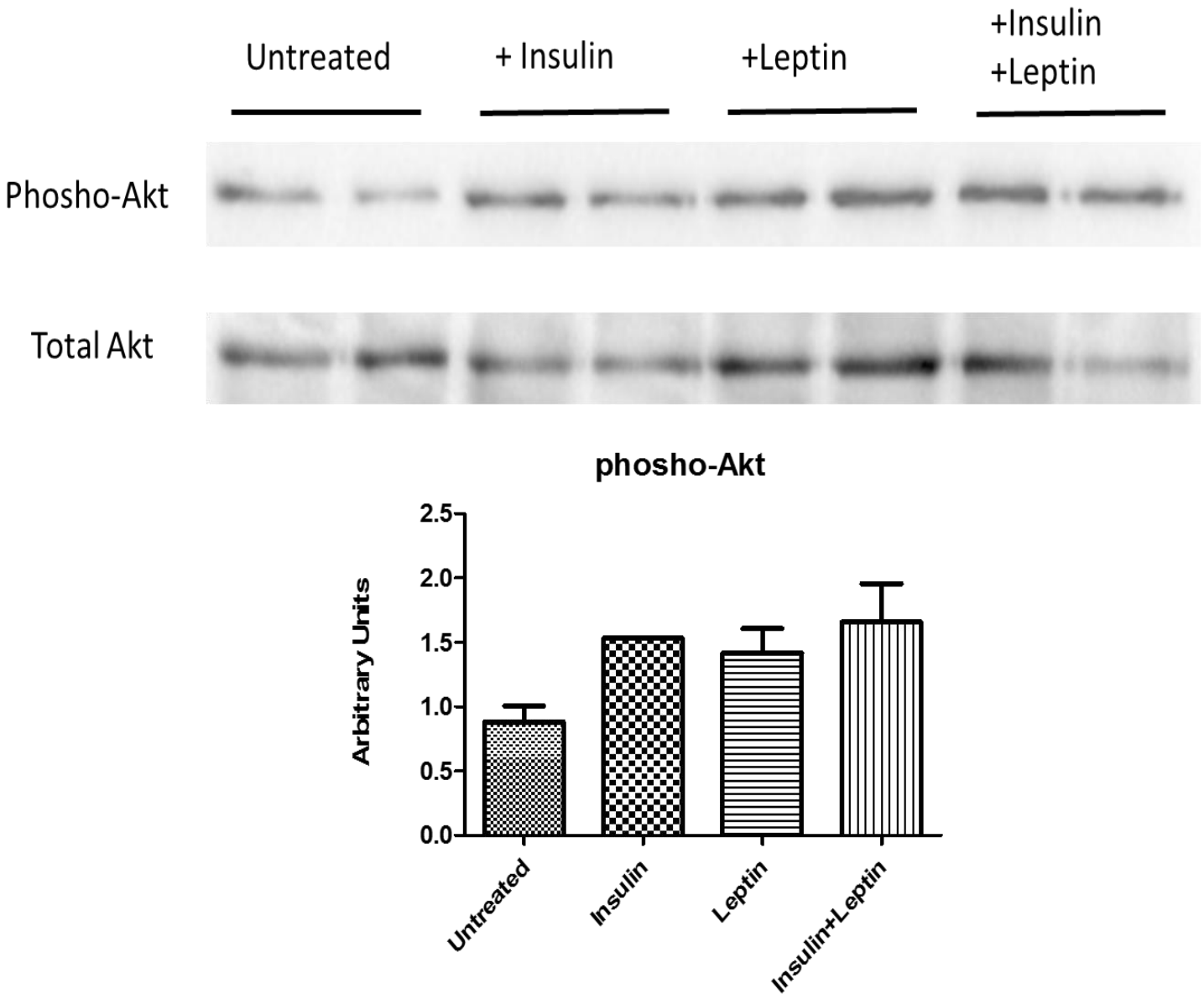


Figure 3.9: Leptin induces Serine 473 phosphorylation of Akt but does not modify its insulin-induced phosphorylation. Peritoneal macrophages from two mice were cultured and treated with either insulin (100U/ml), leptin (100ng/ml) or both. The leptin treatments were performed o/n. After the leptin treatments the media was removed and replaced with fresh starvation media for 2h. After the 2h the cells were treated for 30min with insulin. The cells were collected in RIPA buffer including phosphatase and proteinase inhibitors. The levels of phospho -Akt were detected by western and then the membrane was striped and used for total Akt detaction. The absolute levels were canculated by using IMAge Lab™Software.

3.8 Leptin downregulates the expression of IGFR and IR in peritoneal macrophages.

To test how leptin affects the insulin signaling pathway we tested the levels of expression of IGF receptor and insulin receptor in peritoneal macrophages. These two receptors are the major receptors for insulin binding and signal transduction and there levels are collated with the insulin signaling inside the cells. Peritoneal macrophages from wild type mice were cultured either without any treatment or treated with 100ng/ml leptin for 6h. Q-PCR showed that the levels of IGF receptor were decreased about 9 folds and the levels of insulin receptor also were decreased about 2 folds after leptin treatment (figure 3.10).

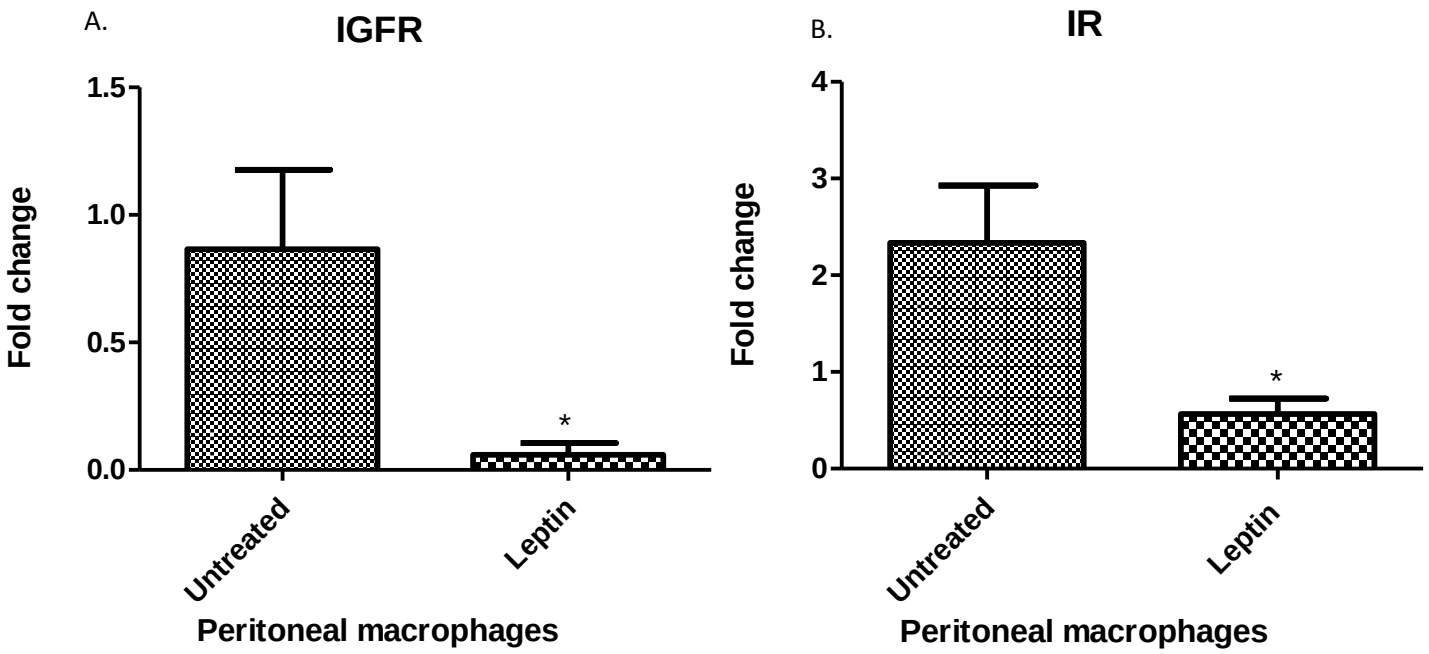


Figure 3.9: Leptin promotes downregulation of insulin receptor and IGF receptor in peritoneal macrophages. A) Levels of IGFR measured by Q-PCR in untreated and leptin treated macrophages (100ng/ml for 6h)*p<0,04. B) Levels of IR measured by Q-PCR in untreated and leptin treated macrophages (100ng/ml for 6h).*p<0,01.

4. Discussion

Even though, obesity is characterized as a high risk factor of multiply medical conditions, obesity paradox reveals another aspect of this condition. The controversial survival rate of ICU patients (Ray D. et al.2005, Marsh C. et al.2006, Werzinger B. et al. 2009, Hatagalang R. et al. 2011) sets numerous questions that have not been answered yet. Obese patients are characterized by increased levels of leptin and insulin in their blood (Considine RV et al.1996, Kahn B. et al.1998) and the study of the role and mechanism of those two molecules might reveals significant clues for the pathophysiology of obese individuals.

Macrophages have been identified as major players of the innate immunity in the adipose tissue (Fantuzzi et al.2005). Their polarization to an M1 phenotype induces a cascade of invents that leads to a low grade inflammation locally of the adipose tissue. As it has been previously shown (Reily et al. 204, Lago et al.2008) leptin induces the proinflammatory phenotype of the macrophages by inducing the production of pro- inflammatory cytokines and molecules. On the other hand, as it is known from another study in our research team, insulin resistance tend to prime macrophages to a more regulatory phenotype (M2 phenotype). So the combination of those two events, raise several questions. First of all, how this two molecules control the immunological events in the adipose tissue and secondly which is the mechanism and the key molecules that control their action.

In this study using insulin resistant macrophages derived either from obese, Akt2-/-, LyMCre+/IGF1Rfl/fl mice or from chronic in vitro high insulin treated macrophages we showed that insulin resistance tent to reduce the pro- inflammatory action of macrophages by downregulating the expression of a significant pro- inflammatory micro RNA, miR-155, that has been previously characterized by our research group to be strongly associated with the M1 phenotype (Arranz A. et al 2012). On the other hand we showed that leptin tend to up regulate MiR-155, indicating that this hormone tend to prime macrophages to a M1 phenotype.

Although insulin and leptin seem to have an opposite effect on macrophages there are indications that there is a cross talk between the

pathways and molecules that they regulate (Hegui et al.2004). More specifically we showed that leptin down regulates the expression of the insulin receptor and IGF receptor, the main receptors of insulin transduction signal. So, taken into account this results, there is an indication that leptin tend to induce insulin resistance in peritoneal macrophages by down regulating its receptors on the surface of the cells. This result comes to agreements with the fact that adipose tissue inflammation is an important contributor to systemic insulin resistance (Weisberg SP. et al. 2003, Lumeng CN. et al. 2007 Huo Y. et al. 2010).

Furthermore, we showed that insulin resistant macrophages tend to have less pro- inflammatory responses after infection. LPS treated insulin resistant macrophages have less production of pro- inflammatory cytokines, such as TNF-a and IL-6, than the wild type ones and also they significantly down regulate the expression of miR-155. Thus, Insulin resistant macrophages are less ‘’aggressive’’ not only in their naïve state but also when they are stimulated after an infection.

Taken together this findings we can conclude that insulin resistance phenotype of individuals reduces the pro- inflammatory action of macrophages and that can explain partially the outcomes of ICU patients. On the other hand, leptin induces inflammation locally of the adipose tissue, which can explain the low grade chronic inflammation of obese individuals, but by inducing insulin resistance in the periphery of the organism it reduces the inflammation grade after infection. Thus obese ICU patients tend to control better the inflammatory responses by macrophages after inflammation due to high blood levels of leptin and insulin. A better understanding of the mechanisms behind this phenomenon may be beneficial to improve the outcomes of ICU patients.

Obesity is a condition that has multiply impacts not only in everyday life of individuals but also it dramatically affects their immune system. The alternations and specific characteristics of the immune system that are associated with this condition is a new field of immunology and further investigation will be needed. There are many questions regarding how miR-155 is important for leptin induced inflammatory response and how may this micro RNA be significant for leptin induced insulin resistance. Thus complementary experiments are necessary to answer these questions. As future directions in our study, will be to use miR-155-/-

peritoneal macrophages to test whether there are any differences in the production of pro-inflammatory cytokines, such as il-6, after leptin treatments compere to wild type peritoneal macrophages. Also the same knock out macrophages will be used to address if miR-155 is important for the downregulation of insulin and IGF receptors and as a consequence, leptin- induced insulin resistance. Taken together these findings with our previous work a potential therapeutic approach may be revealed. Targeting miR-155 may be beneficial to minimize the inflammatory and insulin resistant phenotype observed in obese individuals

5. Acknowledgments

Undoubtedly, this project could not be completed without the contribution of several people who supported me during my research.

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