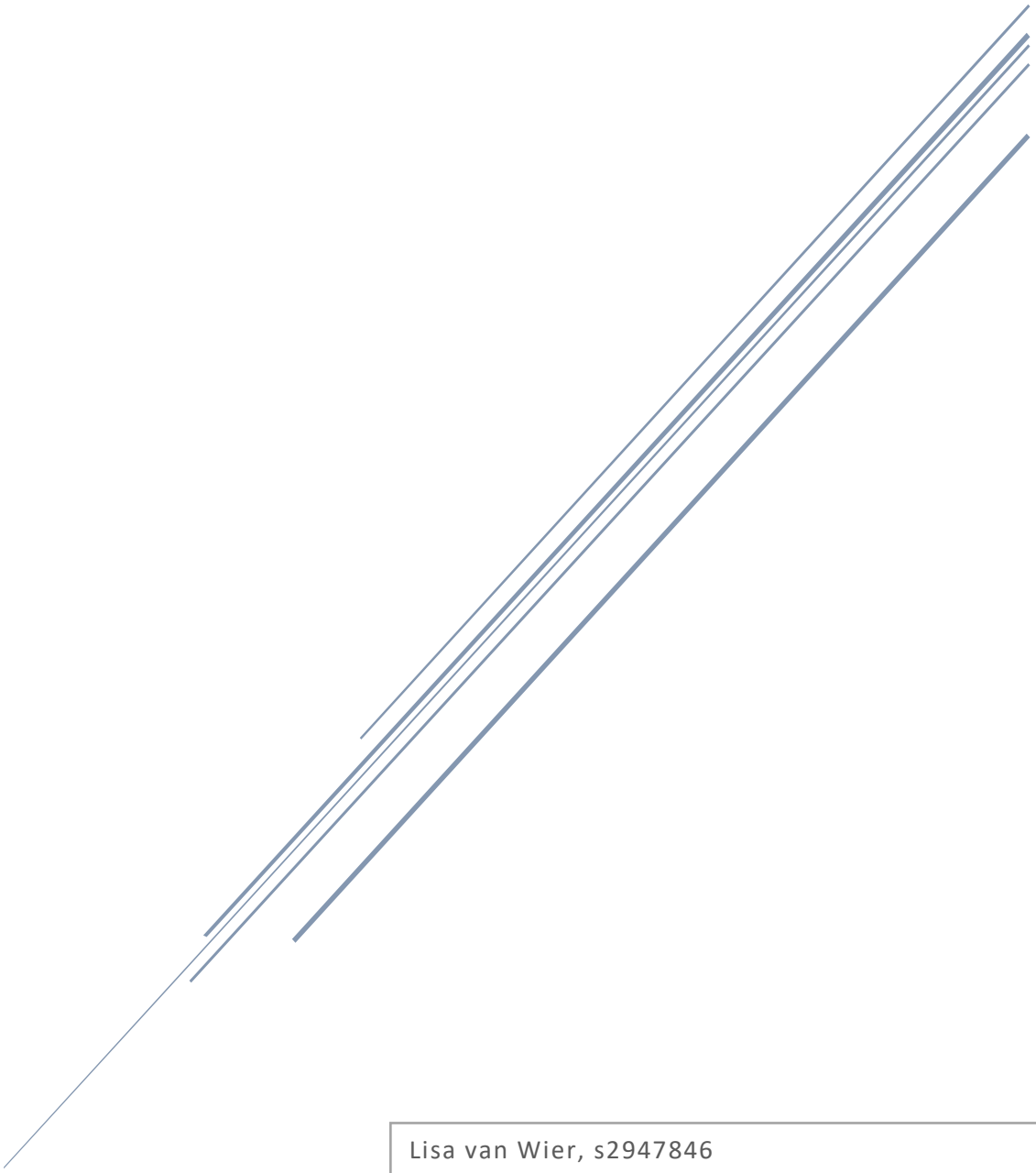
The effect of macrophage stimulation on cytokine levels in relation to the development of Diabetes Mellitus type II



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Abstract

Diabetes mellitus type 2 is a metabolic disorder defined by impaired insulin secretion and insulin resistance as well as chronic inflammation. The precise mechanism behind macrophage polarization in relation to diabetes mellitus type 2 is still unknown. In this thesis, the role of macrophages, protein (de)acetylation, acetyl-CoA, glucose and lipids to the development and pathogenesis of diabetes mellitus type 2 are discussed.

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Introduction

Diabetes Mellitus type II

According to the World Health Organization the global prevalence of diabetes mellitus has almost doubled since 1980, from 4,7% in 1980 to 8,5% in 2014. There were 422 million adults living with diabetes mellitus in 2014, compared to 108 million adults in 1980. Diabetes mellitus is a chronic, progressive disease characterized by hyperglycemia. It is characterized by hyperglycemia resulting from defects in insulin action, insulin secretion or both. Diabetes mellitus is classified into two types: type 1 diabetes (DMT-I) and type 2 diabetes (DMT-II). DMT-I is characterized by an absolute deficiency of insulin secretion. The body is unable to produce and secrete insulin. It is thought that the underlying cause of DMT-I is an autoimmune reaction to the insulin-producing beta cells of the pancreas. Due to the lack of insulin, the body is not able to process glucose. This causes hyperglycemia, a high level of glucose in the blood. Hyperglycemia can cause long-term damage, dysfunction, and failure of different organs. DMT-II is characterized by a combination of insulin resistance and inadequate insulin secretion. This combination also leads to faulty processing of glucose leading to hyperglycemia. DMT-II is caused by several factors such as overweight and obesity. DMT-II accounts for approximately 95% of the people with diabetes. (1,2) It is thought that low grade inflammation is the connection between insulin resistance and obesity. (3) The pathogenesis of DMT-II is not fully understood. Recent studies indicate that macrophages play an important role in the pathogenesis of DMT-II.

Macrophages

Macrophages are originated from circulating monocytes (adult-derived macrophages) or during embryonic development (embryonically derived macrophages). Embryonically derived macrophages originate from the yolk sac during embryonic development. They have a self-renewal capacity and they are maintained independently of circulating monocytes. Studies have showed that embryonic macrophages are involved in tissue remodeling, and that adult-derived macrophages primarily play a role in host defense. Each organ has its own unique combination of embryonically derived and adult-derived macrophages. During inflammation, circulating monocytes migrate into the tissue and differentiate, depending on local factors, into macrophages. (4) Macrophages along with dendritic cells are ate the forefront of initiating an innate and an adaptive immune response, respectively. Their function consists of phagocytosis, antigen presentation and production of different types of cytokines. The secreted cytokines induce an inflammatory or anti-inflammatory phenotype. Macrophages play an important role in the progression of inflammatory diseases. Overweight and obesity can be accompanied by inflammation, which could lead to the development of DMT-II. (5)

Macrophages can be polarized into two phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2). Macrophage polarization is induced by specific microenvironmental stimuli and signals. They are cells with a high functional plasticity as they can switch from one phenotype to another. This phenomenon is called repolarization. Exposure of M1 macrophages to M2 signals can lead to repolarization and vice versa. By adapting their metabolic phenotype they are able to fulfill a specific function. (4-6)

Classically activated macrophages are pro-inflammatory, and induced by Th1 cytokines such as interferon gamma (IFN-γ), tumor necrosis factor (TNF-α) or by lipopolysaccharides (LPS). M1 macrophages trigger inflammatory responses by producing pro-inflammatory cytokines like interleukin 1 beta (IL-1ß) and TNF-α. The inflammatory response of M1 macrophages can lead to a disturbance of tissue homeostasis leading to tissue damage. In a non-inflammatory state there is a balance between tissue destruction and tissue repair. Unfortunately, in an inflammatory state this balance is disturbed and so it causes impaired tissue regeneration and wound healing. To protect against tissue damage, the chronic inflammatory response is inhibited by regulatory mechanism driven by the anti-inflammatory function of M2 macrophages. M1 macrophages also contribute to the

elimination of pathogens during infection, by activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, and subsequent production of effector molecules such as reactive oxygen species (ROS). All these functions lead to a potent anti-microbial and anti-tumoral activity of M1 macrophages.

M2 macrophages are anti-inflammatory, and polarized by Th2 cytokines. Activating STAT4 through IL-4 receptor alpha leads to this polarization. Besides that, they can also be activated by IL-10. IL-10 acts on the IL-10 receptor which then activates STAT3. As mentioned before, M2 macrophages inhibit the pro-inflammatory activity of M1 macrophages. They also produce cytokines that differ from M1 macrophages. M2 macrophages produce a low level of IL-12 and a high level of IL-10 and TGF-ß. Besides the anti-inflammatory function M2 macrophages execute phagocytosis, and promotes tissue repair and wound healing. They possess pro-angiogenic and pro-fibrotic properties, which when overactivated could lead to undesirable effects. The outcome of overactivation of fibrosis is scarring and thickening of the affected tissue which leads to inadequate function of the organ. M2 macrophages can be further divided into four groups: M2a, M2b, M2c, and M2d. To which subset a M2 macrophage belongs depends on activating stimuli. M2a subset macrophages are induced by cytokine IL-4 and cytokine IL-13, they are defined as alternative activated macrophages. M2a macrophages are play a role in tissue remodeling and immunoregulation. M2b subset macrophages are induced via stimulation by immune complexes and agonists of Toll-like receptors (TLR) or IL-1 receptor ligands. They are defined as type 2 macrophages. TLRs can be induced by LPS, which also induces M1 macrophages. On top of that, IL-1 receptor ligands also induce M1 macrophages. So, the stimuli that induce the M2b subset are similar to the stimuli of M1 macrophages. M2b macrophages have a immunoregulatory function as they promote Th2 differentiation and production of humoral antibodies. M2b macrophages produces pro- and anti-inflammatory cytokines: IL-1ß, IL-6, IL-10 and TNF-α. M1 macrophages also produce IL-1ß and TNF-α. These overlapping properties suggest that M1 macrophages and M2b have a similar pro-inflammatory function. M2c macrophages are induced by glucocorticosteroids and IL-10. They have a strong anti-inflammatory effect, and are defined as deactivated macrophages. The last subset, M2d, is activated by adenosines and IL-6. M2d macrophages are defined as M2-like macrophages and their they have pro-tumoral and pro-angiogenic capacities. (4,5,7,8)

Protein acetylation

Protein acetylation is one of the major post-translational modifications (PTM) in eukaryotes. Protein acetylation can exert multiple effects on proteins, affecting the activity and structure, protein-protein interactions, protein-nucleic acid interactions, protein subcellular localization and trafficking, and following protein modification and stability. By having such an impact on proteins, lysine acetylation is a main regulator of protein function in humans.(9)

During protein acetylation, the acetyl group from acetyl-CoA is transferred to a specific site on the polypeptide chain. Thus, acetyl-CoA is essential for the execution of protein acetylation. There are two different forms of protein acetylation classified on the specific site of the polypeptide chain that they act on. The first form is protein acetylation of the N-terminus of polypeptide chains. This form of protein acetylation is catalyzed via Nt-acetyltransferases (NATs). The second form of protein acetylation is acetylation of the ε-amino group of lysines. It is, next to phosphorylation, the most prevalent and important PTM in cell signaling and metabolism. It is also involved in a variety of cellular pathways. This type of protein acetylation is catalyzed by lysine acetyltransferases (KATs) which is also known as histone acetyltransferases (HATs). Lysine acetylation is strongly regulated and it is, in contrast to Nt-acetylation, reversible. KATs are counteracted by lysine deacetylases (KDACs), also known as histone deacetylases (HDACs). There is a balance between KATs and KDACs and they regulate important cellular processes like glucose metabolism, energy homeostasis, and gene expression. Malfunctioning of protein acetylation can lead to severe diseases.(10)

Acetylation of the ε-amino groups of lysines is next to phosphorylation the most prevalent and important PTM in cell signaling and metabolism. KDACs and KATs are crucial for the activation and deactivation of particular pathways. There are three major families of KATs: GNAT, MYST and p300/CBP. Most KATs belong to multiprotein structures. The multiprotein structures contain a variety of subunits which determine the specifications of the substrate, and the catalytical activities. Diverse transcription factors and transcriptional co-regulators are modified by KATs, resulting in regulation of their activity.

As stated before, deacetylations are carried out by KDACs. KDACs can be divided into two super families. The first family of KDACs contain a bound Zn^+ ion, they are called HDACs. The second family require nicotinamide-adenine nucleotide (NAD^+) as a cofactor. The latter are called sirtuins (SIRTs). Furthermore, there are four classes of KDACs: class I, class II, class III and class IV. The classification is based on the structural homology of the KDACs. Class I includes HDAC1, 2, 3, and 8. Class II is subdivided into IIa (HDAC4, 5, 7, 9) and IIb (HDAC6, 10). Class IIa possess highly homologous N-terminal extensions involved in specific protein-HDAC interactions, class IIb lacks these extensions. Class III includes SIRT1 through SIRT7 which are further subdivided based on their phylogeny. Class IV consists solely of HDAC11, which possesses features characteristic of both class I and class II. KDACs can be subjected to many post-translational modifications: phosphorylation and various modifications of lysine residues, including acetylation, methylation, ubiquination, and SUMOylation. Next to chromatin modification and control of gene expression, KDACs also play a role in various metabolic processes as their shared role of the metabolic cofactor NAD^+.(11)

In general, a high degree of histone acetylation correlates with increased transcriptional activity. Histone acetylation destabilizes the DNA-histone interaction. This leads to a lightly packed, open chromatin structure, resulting in an increase of gene transcription. KDACs repress transcription by histone deacetylation. The level of induction of the transcriptional activity depends on the complexes they form and the specific site they act on. (10)

Targeted acetylation of lysine residues of signaling proteins and in particular histone proteins at specific genomic loci is linked to a tight regulation of essentially all types of DNA-templated processes such as transcription, replication, recombination, repair, translation and formation of specialized chromatin structures. (12) This implies that protein lysine acetylation plays a huge role in various cellular signaling pathways as well as among other things metabolism and stress responses. Thus, dysregulation of protein acetylation could lead to dysregulation of cellular signaling pathways, metabolism and stress responses resulting in development of unwanted effects as well as diseases.

Studies have shown that KDACs play a regulatory role in the signaling of insulin. Different types of HDACs are regulators of insulin signaling. (10) HDAC2 has been reported to be a key regulator of diabetes in mice. Similar to HDAC2, the role of HDAC3 has also been explored in insulin resistance and DMT-II. It was reported that HDAC3 contributes to an increased prevalence of DMT-II in the Chinese Han population. Because HDACs play a part in the integration and coordination of lipid metabolism, HDAC gene polymorphism can lead to the development of DMT-II. HDAC3 polymorphisms led to fatty liver and higher blood sugar. It was shown that that HDAC3 is not related to BMI, high blood triglyceride and high total cholesterol meaning that it is an independent factor for the development of DMT-II. As a result of this study it can be hypothesized that modulation of HDAC3 expression could prevent or treat DMT-II. Previous work by Sun et al. demonstrated that liver-specific deletion of HDAC3 in C57BL/6 mice results in hepatosteatosis along with improved insulin sensitivity without affecting the insulin signaling or body weight. HDAC3 has also been reported to regulate PPARγ function in adipocytes. Moreover, HDAC3 inhibitors have been suggested to play a role in improving insulin sensitivity and protecting ß-cells from cytokine-induced apoptosis. All these reports suggest the potential of HDAC3 inhibition in regulating insulin signaling and improving insulin sensitivity.(13-17)

Acetyl-CoA

Most of the cellular ATP is derived from acetyl-CoA. Acetyl-CoA is a metabolite derived from glucose, fatty acids, citrate and acetate. (10) Glucose is converted into pyruvate through glycolysis in the cytoplasm. Pyruvate is transported into the mitochondria and converted into acetyl-CoA by the pyruvate dehydrogenase complex. (18) Acetyl-CoA is produced via ß-oxidation of fatty acids, by acetyl-CoA-citrate lyase from citrate, and from acetate catalyzed by acetyl-CoA synthetase.
Pyruvate dehydrogenase, the citric acid cycle, and the machinery catalyzing ß-oxidation are found in the mitochondria. Acetyl-CoA synthetases are feedback-regulated. Lysine acetylation inhibits the activity of acetyl-CoA synthetase in response to a high concentration of acetyl-CoA. Acetyl-CoA is found in the cytosol and mitochondria, and deacetylated by SIRT1 and SIRT3.

Under fed states (high glucose levels), acetyl-CoA is directed into the cytosol and nucleus for use in histone acetylation or lipid synthesis. When in fasted state (low glucose levels), acetyl-CoA stays in the mitochondria for synthesis of ketone bodies and ATP. (19) High fat concentrations could lead to activation of a metabolic inhibitory mechanism via SIRT3. A low concentration of acetyl-CoA leads to low histone acetylation and gene expression.(10) Acetyl-CoA serves as a substrate for KATs. As mentioned before, KATs utilize acetyl-CoA to acetylate proteins which has an impact on cellular metabolism, and cell signaling pathways. As KATs use acetyl-CoA to fulfill their function, it has been hypothesized that the availability of acetyl-CoA can influence the extent of protein acetylation. The concentration of acetyl-CoA fluctuates along with the energy state of the cell. KDACs can us other substrates than acetyl-CoA. KDACs use NAD^+ or Zn^+ for deacetylation of proteins. So, it is independent of acetyl-CoA.(10) As DMT-II is a metabolic disorder and changes in the metabolic pathway induce changes in the concentration of acetyl-CoA, it is expected that this could affect protein acetylation, gene expression, and energy metabolism. The activity of KATs will decrease while the activity of KDACs is not disturbed as they are not dependent on acetyl-CoA. Furthermore, it could lead to inflammation and macrophage polarization. This hypothesizes that there is a vicious circle resulting in chronic inflammation and severe outcomes. Zeng et al hypothesized that a reduced level of acetyl-CoA affects the pyruvate carboxylases activity. This way the flux of pyruvate to glucose production is limited which leads to reduced glucose output and insulin hypersensitivity. This could be a trigger for DMT-II. (15)

Glucose, lipids and the activation of macrophages

Glucose is a critical component in the pro-inflammatory response of macrophages. M2 macrophages primarily utilize fatty acids to function. Classically activated macrophages utilize glucose metabolism to function. Freemerman et al demonstrated that metabolic reprogramming is driven by GLUT1-mediated glucose uptake and subsequent enhanced glucose metabolism. (20) This suggest that glucose metabolism plays a role in the DMT-II associated chronic inflammation.

It has been suggested that the pro-inflammatory effects of macrophages may be regulated by molecules such as glucose, insulin, and palmitate, which are elevated in patients with metabolic diseases. (21) Kratz et al. showed that treating macrophages with glucose, insulin, and palmitate produces a metabolically-activated phenotype (MMe) distinct from classical activation. This suggests that metabolic disease-specific pathways drive the polarization of macrophages into pro-inflammatory macrophages via different mechanisms than those during infection. It has been demonstrated that IL-1ß and TNF-α expression are induced in human monocyte-derived macrophages treated with media conditioned by visceral adipose tissue from obese human or mice. Analysis of adipose tissue macrophages (ATMs) from obese mice produced also the findings that IL-1ß and TNF-α expression was increased. In addition, it has been confirmed that high levels of palmitate could stimulate the MMe phenotype in DMT-II patients. Patients with metabolic diseases have an elevated level of palmitate, insulin and glucose. MMe is mediated by at least two mechanisms: binding of palmitate to cell surface TLRs that drives pro-inflammatory cytokine production, and palmitate internalization which activates p62 and PPARγ. The activation of p62 and PPARγ promotes lipid metabolism and limits inflammation.

Altogether, the balance between the two mechanisms determines a pro- or anti-inflammatory response. Next to that, it produces a macrophage phenotype on the spectrum between M1-like and M2-like states. This suggests that there are mixed macrophage phenotypes in humans. This would lead to the hypothesis that metabolic diseases, like DMT-II, could be treated by decreasing the activation towards M1-like states, and induce the activation of the M2-like state. Kratz et al predicted that accumulation of excessive palmitate decreases inflammation via an elevated activation of p62 and PPARγ. Which leads to a more M2-like macrophage phenotype. (22) Kratz et al also predict that excessive accumulation of palmitate within the macrophage would promote an M2 ATM phenotype, and attenuate inflammation. The switch in ATMs from M2 to the M1 phenotype in obese mice are reversed when feeding a high-fat diet for a long period of time.(22,23) This suggests that it could be possible to treat DMT-II patients with a high fat diet, to reduce the inflammation that is associated with DMT-II.

Macrophage stimulation and DMT-II

Metabolic homeostasis of the human body is maintained by metabolic organs such as liver, pancreas, and apidose tissue. By regulation of their interaction, it is possible for humans to adapt to changes in their environment and nutrient intake. During infection, activation of macrophages results in secretion of pro-inflammatory cytokines which promote peripheral insulin resistance to decrease nutrient storage. This metabolic adaption is necessary for mounting a robust and effective response against infections in which all activated immune cells use the glycolysis pathway to provide energy for the function of the host response. This strategy of nutrient reallocation becomes maladaptive in the case of diet-induced obesity, which is characterized by macrophage-mediated inflammation. (4)

One of the main driving forces for the development of DMT-II is inflammation originating from the adipose tissue. Adipose tissue macrophages are a major component of the stromal vascular fraction of adipose tissue and they play an important role in obesity-associated pathology. Adipocytes release mediators that promote macrophage recruitment and activation. Inflammatory cytokines counteract the insulin-sensitizing action of adiponectin and leptin, which leads to insulin resistance. (4) Macrophages play an important role in the regulation of adipose tissue functioning. As stated previously, it has been suggested that pro-inflammatory macrophages in adipose tissue contribute to insulin resistance. The cause of the elevated number of pro-inflammatory macrophages is obesity-induced inflammation. The adipose tissue produces and secretes a variety of adipokines. Adipokines enables the communication between cells and tissues as well as it regulates energy metabolism and satiety. Next to that, adipocytes are also able to produce types of adipokines that attract circulating monocytes. These monocytes can than develop into macrophages. On top of that, there are pro-inflammatory adipokines. During the development of obesity, a significant alteration in the number and composition of immune cells takes place. This is also the case for adipokines, they change along with the development of obesity and DMT-II. Along with hypertrophy of adipocytes this promotes a chronic low-grade inflammatory state. This chronic low-grade inflammation is related to the development of insulin resistance and DMT-II.(24)

The number of adipose tissue macrophages (ATMs) increase in obesity and participate in the pro-inflammatory pathways in the adipose tissue of people with obesity. In lean mice and humans, ATMs represent less than 10% of all adipose tissue cells. This percentage increases to over 50% in extremely obese mice and near 40% in obese humans. Next to the increase in the number of ATMs, their localization also changes. Increase of the number of ATMs lead to an increase in the production of pro-inflammatory cytokines. (25) A different study showed that ATMs that were isolated from white adipose tissue from lean mice showed hallmarks of polarization toward the M2 phenotype. Among the markers of M2 macrophages was cytokine IL-10. (23) There is significant evidence that macrophages change their phenotype under the influence of the microenvironment. (22,26)

As stated before, it has been suggested that macrophages undergo a phenotypic switch during weight gain. In lean adipose tissue, the macrophages have been described as anti-inflammatory M2 macrophages. They maintain adipose tissue homeostasis by lipid buffering and cleaning cellular debris. (27) Macrophages in obese adipose tissue have been described as pro-inflammatory M1 macrophages. They are believed to be the major contributors of obesity-induced insulin resistance, which leads to DMT-II. The development of DMT-II is due to the increased number of macrophages along with an increased production of pro-inflammatory cytokines, like TNF-α, IL-1ß, IL-6, and IL-8. (28) Macrophages also contribute to DMT-II-associated complications such as nephropathy, neuropathy, retinopathy, and cardiovascular disease through release of pro-inflammatory cytokines, chemokines, and proteases. These substances induce pro-inflammatory cell recruitment, cell apoptosis, angiogenesis, and matrix protein remodeling. Therefore, M1 macrophages have an important role in tissue inflammation, development of insulin resistance DMT-II patients. (3,4)

There are two main transcription factor complexes which orchestrate macrophage inflammatory polarization in contribution to DMT-II pathogenesis: activator protein-1 (AP-1) and nuclear factor κB (NF-κB). They are both known to promote an M1 phenotype in macrophages. AP-1 regulates cell growth, differentiation, apoptosis as well as other cellular processes, in response to physiological stress. (29) AP-1 activity is regulated by post-translational modifications, composition of its DNA binding dimer and through interactions with various binding partners. (29) Cell extrinsic stimuli that regulate AP-1 activity include cytokines and growth factors. Next to that, in the context of DMT-II, palmitate has been shown to activate AP-1 in macrophages as well as the downstream release of pro-inflammatory cytokines. (30) NF-κB is highly expressed in adipose tissue macrophages after their pro-inflammatory polarization and during the development of insulin resistance. Furthermore, cytokine secretion of M1 and MMe macrophages results in NF-κB activation in other leukocytes recruited to the site of inflammation, increasing the magnitude of the inflammation in DMT-II. Macrophages express a wide range of nuclear receptors like peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR) and glucocorticoid (GC) receptors. Nuclear receptors regulate the inflammatory pathways in macrophages. They do that by controlling the gene expression and macrophage polarization. (31)

It is evident that macrophages play a major role in the development of DMT-II and the accompanied chronic inflammation.

Discussion

The aim of this research and the upcoming project is to investigate the effect of macrophage stimulation on cytokine levels in relation to the development of DMT-II. DMT-II is associated with pro-inflammatory macrophage polarization and changes in energy metabolism. Chronic inflammation is an important mediator of the development of DMT-II, and macrophages play an important role in the regulation of inflammation. (4) Kratz et al predict that excessive accumulation of palmitate within the macrophage promotes an M2-like ATM phenotype, and decreases inflammation. It is suggested that a repolarization from the M1 phenotype to the M2 phenotype takes place. (22) Thus, by adding high lipid concentrations to the cell cultures it will be expected that the pro-inflammatory cytokines will decrease and that the anti-inflammatory cytokines will be elevated. Freemerman et al demonstrated that high glucose levels plays a role in the chronic inflammation associated with DMT-II. (20) Therefore, when applying high glucose levels it is expected to observe an induction of pro-inflammatory cytokines.

To investigate the influence of glucose and lipid concentration in the presence or absence of KDAC inhibitors, different cytokines will be measured. The cytokines that will be measured are IL-10, IL-1ß and TNF-α. TNF-α and IL-1ß are pro-inflammatory cytokines and IL-10 is an anti-inflammatory cytokine.

TNF-α and IL-1ß are both produced by M1 macrophages. It is well known that IL-1ß is the main pro-inflammatory cytokine in pancreatic islets that causes an increased expression of other pro-inflammatory cytokines and chemokines leading to further macrophage recruitment to the islets. This inflammation decreases insulin secretion and induces ß-cell apoptosis which leads to islet mass reduction. (3,32)
IL-10 is a major anti-inflammatory cytokine which has which levels has been reduced in DMT-II patients. A previous study showed that there was a significant decrease in IL-10 plasma concentrations of individuals with DMT-II. It has been suggested that insulin resistance seems to be the major determinant of IL-10 plasma concentrations and that the low level is related to decrease insulin sensitivity. (33) These findings contribute to the hypothesis that IL-10 levels will be decreased when macrophages are stimulated with high glucose and lipid concentrations. High glucose levels will lead to M1 macrophages and thereby decreases the level of IL-10.
By measuring the levels of TNF-α, IL-1ß and IL-10 it is possible to determine whether the macrophages are polarized into a more M1-like or a M2-like phenotype. As TNF-α and IL-1ß cytokines are both pro-inflammatory, high levels of both will indicate that there is an inflammation. As IL-10 levels decrease in case of DMT-II, it is expected that there is a low level of IL-10 accompanied with a high level of pro-inflammatory cytokines. If there is no inflammation, it is expected that there will be a low level of TNF-α as well as a low level of IL-1ß cytokines.

As described earlier, KDACs (or HDACs) play a role in the induction of DMT-II. It has also been suggested that KDAC inhibitors could play a role in improving insulin sensitivity. It is thus expected that KDACs inhibitors will lower chronic inflammation in DMT-II. This will be characterized by low TNF-α and IL-1ß levels secreted by the macrophages.

There are a few things to keep in mind during the planned research project. Macrophages have a high plasticity, they adapt their phenotype and possibly also their metabolic state throughout different phases of disease, which could hamper metabolic targeting. (34) It could also be very helpful to understand the metabolic mechanisms and to target specific mechanisms. But it is also possible that the metabolic state is changing and that other cytokines will show which could lead to an incorrect assumption.

Most of the studies, as well as the upcoming project, have been done *in vitro* in cell culture. *In vivo*, macrophages are dealing with a specific microenvironment. During *in vitro* studies, it is impossible to recreate these specific microenvironments. It is thus not possible to take all the *in vivo* influences into account. To translate the in vitro findings to the *in vivo* level requires specific technologies. Next to that, each human or animal has a different microenvironment which could be of an impact on the development of DMT-II. It 2 be possible to administer the same levels of glucose, lipids and KDAC inhibitors in vivo and to determine the outcome. The outcomes can be compared to the in vitro outcomes and further analyzed. This way perhaps more insights on the pathogenesis and possible treatments of DMT-II will be discovered.

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