EFFECTS OF **OBESITY-INDUCED INFLAMMATION** ON DIABETES MELLITUS

EDWIN VAN ASSELDONK

EFFECTS OF
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ON DIABETES MELLITUS

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Edwin Josef Petrus van Asseldonk
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Promotoren
Prof. dr. Cees J.J. Tack
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Prof. dr. Marc Y. Donath (Universitätsspittal Basel, Zwitserland)

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General introduction and outline of the thesis

Introduction

The worldwide prevalence of obesity is sharply increasing and obesity has become one of the greatest global health challenges of the 21th century. The World Health Organisation reports that about 50% of European citizens are overweight and 20-23% are obese. (1) Obesity predisposes to many disorders, including type 2 diabetes mellitus.

Obesity is one of the most common acquired risk factors for the development of insulin resistance. As a consequence of the obesity epidemic, the global prevalence of diabetes mellitus has increased from 153 million in 1980 to about 382 million affected people in 2014. (2) Age-standardized adult diabetes prevalence increased to 9.8% for men and 9.2% for women in 2008. (3)

Diagnosis and classification of diabetes mellitus

Diabetes mellitus is a group of disorders characterized by hyperglycemia. The American Diabetes Association defines diabetes mellitus as the presence of plasma glucose levels higher than 11.1 mmol/l, randomly measured or 2 hours after an oral glucose tolerance test using 75 grams of glucose, or a fasting glucose higher than 7.0 mmol/l. Normal values are substantially lower. Some individuals display glucose levels above normal but not fulfilling the criteria for diabetes mellitus. Patients with a fasting glucose level between 5.6 and 7.0 mmol/l have impaired fasting glucose. Patients with a glucose level between 7.8 and 11.1, 2 hours after ingestion of 75 grams glucose, are categorized as impaired glucose tolerance. (4) Together, this group of disorders is sometimes also listed as prediabetes.

Once diabetes has been diagnosed, the disease can be classified in different groups. The vast majority of cases can be divided in two categories. Patients with type 1 diabetes mellitus have an absolute deficiency of insulin due to (auto-immune mediated) beta-cell destruction. Patients diagnosed with type 2 diabetes mellitus have a combination of insulin resistance and an insufficient insulin production to compensate for the insulin resistance that can ultimately progress to beta cell failure.

Obesity and insulin resistance

Although both obesity and type 2 diabetes mellitus are associated with insulin resistance, most obese individuals do not develop diabetes mellitus, or only after many years. In first instance, beta-cells will produce more insulin to overcome decreased insulin sensitivity. As long as the beta-cells are able to compensate for the increased insulin demand, glucose tolerance is preserved. Some individuals are more susceptible to develop type 2 diabetes mellitus then others. To unravel why some obese individuals are more prone to develop diabetes mellitus than others it is important to identify the factors that link obesity to diabetes mellitus. These interactions, however, are complex and incompletely understood. Multiple potential pathways

describe how nutrient excess leads to insulin resistance. Among the factors involved are increased fatty acid supply, biochemical adaptations induced by nutrient overload, microhypoxia in adipose tissue, endoplasmatic reticulum stress, secretion of adipocyte- and macrophage-derived cytokines, chronic tissue inflammation, genetic predisposition and fat distribution. In most patients with diabetes mellitus it is impossible to point out one specific factor as causative agent. All mentioned pathways do influence the development of diabetes mellitus and no common factor or set of factors is applicable to all patients. Inflammation might play a role in many of the earlier mentioned pathways and high levels of pro-inflammatory markers have been shown in insulin resistant individuals. (5) The present thesis focuses on the influence of inflammation in the pathogenesis of diabetes mellitus.

Inflammation and the adipose tissue

Obesity is the consequence of a long-term positive energy balance. Nutrient excess and subsequent storage in adipose tissue results in expansion of the adipose tissue mass and adipocyte size. This hypertrophy leads to local hypoxia. The combination of microhypoxia and nutrient excess leads to induction of the hypoxia-inducible factor (HIF)-1 gene program as well as ER stress within the adipocyte. This leads to cell death and an inflammatory response characterized by the influx of various pro-inflammatory immune cells in obese adipose tissue. (6) Due to dysfunction of adipocytes, free fatty acids are released. Free fatty acids have been suggested to be ligands of Toll like receptor (TLR)-2 and -4. (7-9) Activation of TLR- receptors induces an immune response. (10)

Pattern recognition receptors

While both TLR-2 and -4 seem to be involved in the pro-inflammatory state of the adipose tissue it is likely that also other receptors are involved as may other TLRs are expressed in adipocytes and pre-adipocytes. (7)

An inflammatory reaction initiates when a pathogen (exogenous) or danger (endogenous) associated molecular pattern (PAMP or DAMP) is recognized. Pathogen recognition receptors (PRRs) play a pivotal role in the initiation of an immune response.(11) PRRs are present on specialised immune cells like macrophages and dendritic cells but also on other non-immune cell types. (11)

Four different classes of PRRs are distinguished. The best-known group of PRRs are the TLRs a family consisting of 10 members (in humans) that have the capacity to recognize different PAMPs or DAMPs. TLR-1, -2 and -6 are activated by bacterial lipopeptides, TLR-4 recognizes enterobacterial lipopolysaccharides, the ligand for TLR-5 is bacterial flagellin and TLR-3, -7, -8 and -9 detect nucleic acid motifs. Other groups of PRRs are nucleotide-binding oligomerization domain (NOD)-like receptors NLRs). NLRs are cytoplasmic proteins; more than 20 NLR genes have been detected and most of them function as PRRs as recognize bacterial components. The NLR family member pryin domain containing protein (Nlrp)-3 is involved in the activation of IL-1β and IL-18 in adipose tissue via the inflammasome. (12-14)

The third group of PRR's are the C-type lectins (CLRs). CLRs contain carbohydrate recognition domains. CLRs are important for the recognition of fungi and bacteria. Retinoic-acid-inducible gene 1(Rig)-like helicases (RLRs) are the last group of PRRs. These cytoplasmatic receptors are important for the recognition of viral nucleic acids.

Although the role of TLR's in inflammatory signalling during infection and inflammation is well established, this is not the case for their role in adipose tissue inflammation. As mentioned earlier, TLR-2 and -4 are activated by free fatty acids. TLR-3 deficiency has been reported to lead to increased insulin sensitivity and a reduction in hepatic steatosis in mice. (15) Furthermore TLR-3 is possibly involved in the pathogenesis of type 1 diabetes mellitus. (16, 17)

Macrophages and other immune cells

Once PRR's are activated a pro-inflammatory response develops. Adipokines and chemoattractants like monocyte chemotactic protein-1 (MCP-1) are released, resulting in influx of macrophages into the adipose tissue. (18) Eventually this leads to clearance of cell debris originating from damaged or dying adipocytes, which enables remodelling of the adipose tissue. This pro-inflammatory process induces the release of other cytokines like tumour necrosis factor α (TNFα) IL-1 and IL-6. (19)

In obese, insulin resistant patients more dead adipocytes might be present due to adipose tissue hypertrophy resulting in local hypoxia. Indeed, the number of macrophages in adipose tissue correlates with body mass and adipocyte size. (20) The causal link between macrophage infiltration and damaged adipocytes is also suggested by the crown-like structures that are characterized by aggregated macrophages surrounding dying adipocytes. (19, 20) Macrophages that infiltrate the adipose tissue during the development of obesity are suggested to differ from residual macrophages, which are present in adipose tissue of lean individuals (*figure 1*). (21)

In general, macrophages can be divided in two groups. Residual macrophages in lean adipose tissue are considered to have a more anti-inflammatory phenotype, these, alternatively-activated macrophages, are coined M2-macrophages. M2-macrophages are well equipped to preserve normal function of adipose tissue by promoting tissue repair. M2-macrophages are characterised by increased expression of IL-10, arginine-1, mannose receptor, c type 2 (Mrc2), IL-1 receptor 2 (decoy receptor) and low expression of IL-12. (22) Infiltrating macrophages in an overfeeding state display a more pro-inflammatory phenotype. These, classically activated, macrophages are named

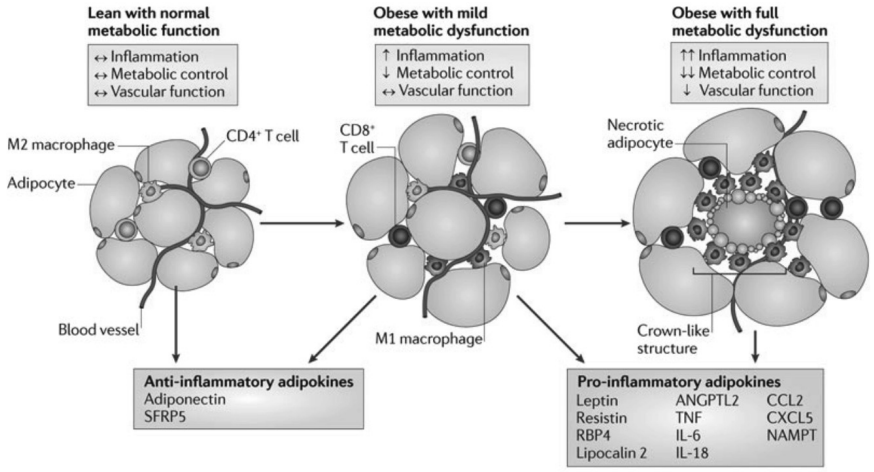

Figure 1 Obesity induced inflammation.

Adipose tissue of lean individuals is characterized by small adipocytes. The adipose tissue is populated by anti-inflammatory macrophages (M2-phenotype), the adipokines that are secreted exhibit an anti-inflammatory profile (left picture). When obesity develops, adipose tissue and individual adipocytes become hypertrophic. Local hypoxia occurs due to insufficient vascularisation. Adipocytes become dysfunctional and necrotic. Cell debris is cleared by pro-inflammatory macrophages (M1 phenotype), which sometimes form crown-like structures. Adipokines with a more pro-inflammatory profile recruits more pro-inflammatory immune cells (right picture). (Figure adapted from Ouchi et al (27))

M1-macrophages. M1-macrophages express high levels of IL-12, IL-1 receptor 1 (IL-1R1) and IL-1 receptor associated protein (IL-1RAcP) and low IL-10. (22) The activation state of macrophages in the adipose tissue does likely form a continuum between M1 and M2 state. Next to macrophages other immune cells like CD4+, CD8+, and regulatory T-cells also contribute to determining the inflammatory trait of adipose tissue. (23-26)

Interleukin-1 family

IL-1 is a key player in the local and systemic response to infections and inflammatory challenges. (28, 29) The release of IL-1 has major effects on virtually all organ systems. IL-1 release causes fever, triggers an acute phase response, and induces the expression of adhesion molecules and chemokines in endothelial cells, leading to recruitment of cells of the immune system. (28) The lifespan and effector function of neutrophils and macrophages increases. (30) Here the IL-1 family is described with special focus on its metabolic effects.

The IL-1 family comprises at this moment of 11 members. 7 IL-1 family members have a pro-inflammatory effect, there are three receptor antagonists and one, IL-37, has an anti-inflammatory function. (31) The IL-1 receptor family also includes 11 molecules. The receptor molecules form 4 receptor complexes (IL-1Receptor, IL-18 receptor, IL-33 receptor and IL-36 receptor), 3 IL-1 receptors are decoy receptors and TIR-8 does have no known ligand. (31)

Two prominent member of the IL-1 family are IL-1α and IL-1β. IL-1α is constitutively present in its active form. When cells become necrotic, IL-1α is directly released in the circulation and initiates an immune reaction. Il-1β is mainly, but not exclusively, produced by cells of the innate immune system. IL-1β is translated as pro-IL-1β, which is not immunologically active. Pro-IL-1β needs to be cleaved into its biologically active form. Cleavage is mediated by caspase-1, which is in turn controlled by a protein-complex called the inflammasome. The inflammasome contains a molecule (mostly an NLR) that recognizes DAMPs and PAMPs. For activation of IL-1β typically two signals are necessary. A first signal, for example an invading pathogen that activates a PRR, which in turn induces IL-1β and NLR transcription. A second signal activates the inflammasome. This might be a PAMP, but reportedly also uric acid, fatty acids, high glucose levels, islet amyloid polypeptide and adenosine triphosphate, can serve as second signal. (30, 32-37) Notably, monocytes do need only one signal to release active IL-1β, as these cells do contain constitutively active caspase-1. (38)

IL-1α and IL-1β both bind to the IL-1R1 and their biological effects are similar. IL-1 and IL-1R1 form a complex with the IL-1 receptor accessory protein (IL-1RAcP, also IL-1R3), after which an intracellular signalling cascade eventually leads to a potent immune reaction. IL-1R2 lacks the intracellular domain of IL-1R1, which is necessary for further signalling and functions as a decoy receptor for IL-1. It binds IL-1 with high affinity. (29, 30, 39) IL-1R2 does also prevent processing of pro-IL1β into its active form by caspase-1. (40) Further IL-1 activity is kept in control by the competitive binding of IL-1 receptor antagonist (IL-1RA) to IL-1R1. The affinity of IL-1RA for IL-1R1 is higher than the affinity of IL-1 to IL-1R1. Binding of IL-1RA to IL-1R1 does not result in recruitment of IL-1RAcP, thus no further intracellular responses occur. (31) IL-1RA also binds IL-1R2, although its affinity is quite low in comparison to IL-1. (31) IL-1RA is produced in response to the same stimuli as IL-1.

Interleukin-1F6 and Interleukin-1F8

IL-1F6 and IL-1F8 are nowadays designated IL-36α and IL-36β. (41) Both are interleukin-1 family members that signal via IL-1 receptor related protein 2 (also IL-1R6) and IL-1RAcP. (29) IL-1F5 (also IL-36RA) is a receptor antagonist of IL-1F6 and Il-1F8. (42) Il-1F6 and IL-1F8 do induce the production of proinflammatory cytokines and lead to Th1 and Th17 cell polarisation. (43-45) It has been shown that IL-1F6 and IL-1F8 are

involved in immunity of the skin and the lung. (46-48) The effects of IL-1F6 and IL-1F8 on adipocytes and insulin metabolism have not yet been investigated.

Interleukin-1 and insulin sensitivity

IL-1β treatment reduces adipocyte differentiation, as shown in murine 3T3-cells and human SGBS cells. Treatment with IL-1β reduces GLUT-4 expression and translocation to the cell membrane and reduces expression of the insulin sensitivity markers PPARγ and adiponectin, and decreases intracellular lipid accumulation. When IL-1β effects are antagonized by the IL-1 receptor antagonist (IL-1RA), differentiation of 3T3 cells increases. IL-1β knockout mice are more insulin sensitive than their wild-type littermates. (13) Altogether, these results argue that IL-1 contributes to the development of insulin resistance of adipocytes.

Blockage of caspase-1 using pralnacasan also resulted in improved adipocyte differentiation, as shown by higher expression of PPARγ, adiponectin and GLUT4. As caspase-1 does also activate IL-18, this effect might theoretically be IL-18 mediated, but IL-18 does not affect adipocyte differentiation. (13)

Interestingly, IL-18 knockout mice are more insulin resistant and obese than their wildtype littermates. (49) So, although IL-1β and IL-18 are both substrates of caspase-1, their effects on adipocyte differentiation and insulin sensitivity appears to be opposite.

Interleukin-1 and Beta-cell function

Although insulin resistance is preceding decreased beta-cell function in type 2 diabetes mellitus, type 2 diabetes mellitus does not develop as long as beta-cell function compensates for the level of insulin resistance. Beta-cells respond to IL-1 via the IL-1R1. High glucose levels result in the activation of caspase-1 and the release of IL-1. (32, 50) High IL-1 levels induce beta-cell apoptosis via activation of the FAS-receptor (50, 51) and insulin synthesis and release is inhibited by high IL-1 levels. (50, 52, 53) Beta cells can be rescued from hyperglycaemia-induced apoptosis by addition of IL-1RA. (50) Children with newly diagnosed type 1 diabetes mellitus treated with interleukin-1 receptor antagonist for 28 days had lower insulin need as compared to historical controls. (54) Thus, IL-1 appears to be involved in the development of decreased beta-cell function.

Glucose toxicity

Once diabetes has emerged, chronically elevated glucose levels can further amplify beta-cell dysfunction and insulin resistance. This glucose toxicity is best characterized at the level of the beta cell and held responsible for the progressive deterioration of beta-cell function. (55, 56) Glucose toxicity is also known to affect insulin sensitivity as shown in elegant studies in patients with type 1 diabetes that develop insulin resistance after a period of 24 hours of hyperglycemia. (57) Both fasting glucose levels and HbA1c are inversely related to peripheral insulin sensitivity. (58) In patients with type 1 diabetes mellitus, insulin sensitivity is normal when glucose control is optimized. (57)

Interleuking-1 and hyperglycemia

As noted above, inflammation may play a causal role in glucose toxicity both at the level of insulin sensitivity and beta cell function. It has been shown that high glucose levels increase the production of acute phase reactants and pro-inflammatory cytokines by adipocytes. (32, 59, 60) Hyperglycemia is shown to induce caspase-1 mediated interleukin-1β (IL-1β) in beta-cells. (37) Hence, chronic hyperglycemia may accelerate the inflammatory response, both at the level of insulin secretion and at the level of insulin action.

Anakinra

As IL-1 is important in the development of insulin resistance as well as in decreased beta-cell function, it might be beneficial to block IL-1 mediated actions. A number of potential interventions is illustrated in figure 2. First, blocking caspase-1 should reduce the activation of IL-1, but currently no caspase-1 inhibitor is available for clinical use. Another possibility would be to directly block the binding of IL-1 to its receptor. As described earlier, IL-1 actions are constrained by IL-1RA. Anakinra is a recombinant human IL-1RA. It has been used as a treatment of rheumatoid arthritis and other IL-1 mediated auto-inflammatory diseases. (61) The most frequent side effect of anakinra is a transient injection site reaction. This reaction is the effect of one of the solvents of anakinra and is dose dependent. (61) Most patients do not experience site reactions after continuing beyond 3 weeks with daily injections. Another adverse event is leukocytopenia. Infections appear to be a non-frequently occurring adverse event (61, 62). In 2007 Larsen *et al.* treated patients with type 2 diabetes mellitus with anakinra 100 mg daily for 13 weeks. Patients treated with anakinra had better glycemic control compared to placebo treated patients. HbA1c levels and fasting glucose levels decreased. Beta-cell function improved. However, no increase in insulin sensitivity was detected by euglycemic-hyperinsulinemic clamp, as well as by insulin sensitivity index calculated by the homeostasis model assessment. (63) So far, the results of this trial have not been reproduced and clearly more detailed information regarding the exact effect of the intervention on insulin secretion and sensitivity is needed.

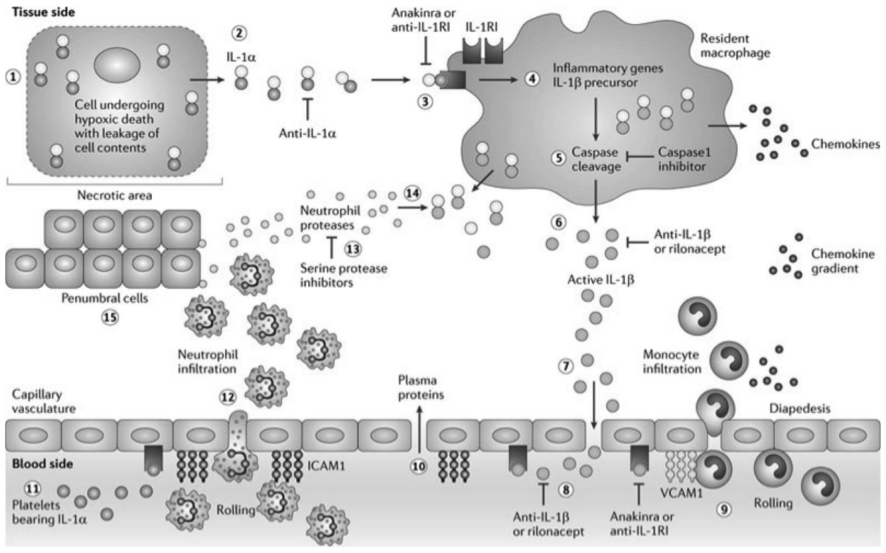

Figure 2 Treating inflammation by blocking Interleukin-1.

1. In case of hypoxia, adipocytes become necrotic and leak cell contents. **2**. IL-1α is released in the circulation. IL-1α-blockers can block the inflammatory process at this stage. **3**. IL-1α binds to the IL-1R1 on resident macrophages. This process can be blocked by anakinra of anti-IL-1R1 antibodies. **4**. Inflammatory genes are activated and the IL-1β precursor is produced. **5**. The IL1-β precursor is cleaved by caspase-1 into active IL-1β. Caspase-1 inhibitors can block this cleavage into the active form. **6**. Active IL-1β is secreted into the extracellular compartment where it can be blocked by anti-IL-1β antibodies or by rilonacept. **7**. IL-1β enters the circulation as the vascular integrity at the necrotic side diminishes. **8**. IL-1β bind to IL-1R1 at the capillaries and vascular cell adhesion molecule 1 (VCAM-1) is induced. **9**. Circulating monocytes bind to VCAM-1 and enter the necrotic area. More IL-1β is produced by the monocytes. **10**. Plasma proteins enter the ischemic side. **11**. Platelet-derived IL-1α binds to IL-1R1 and activates intercellular adhesion molecule 1 (ICAM-1). **12**. Neutrophils enter the ischemic side. IL-1 prolongs neutrophil-life. **13**. Neutrophil proteases are released. **14**. IL-1β precursor released in the extracellular space is cleaved by neutrophil proteases, resulting in active IL-1β. Serine protease inhibitors can block this cleavage. **15**. Increasing numbers of neutrophils enter the ischemic side to scavenge cell debris. Neutrophil proteases damage penumbral adipocytes resulting in increased loss of function of the adipose tissue. *(*Figure adapted from Dinarello et al. (64))

Objectives and outline of the thesis

The overall objective of this thesis is to obtain more insight in the role of inflammation in affecting insulin sensitivity and beta cell function. First we set out to acquire more clarity in the instigation of the pro-inflammatory reaction in the adipose tissue. TLR-2 and TLR-4 have been mentioned to recognize fatty acids, so these, but also other PRR's might be involved in the initiation of the inflammatory response. We first measured the expression of a large selection of PRR's in human subcutaneous adipose tissue. The observed expression pattern for TLR-3 was remarkable, as it was predominantly present in the adipocyte fraction, as compared to the stromal vascular fraction whereas other PRRs showed predominant expression in the stromal vascular fraction. In **chapter 2** we describe the role of TLR-3 in adipocytes and additionally show effects of TLR-3 deficiency on insulin sensitivity and adipose tissue inflammation using an in vivo approach.

After investigating the instigation of the pro-inflammatory reaction in the adipose tissue did we focus on the effects of novel, possibly important, cytokines on adipose tissue inflammation. In **chapter 3** have we investigated the effects of two IL-1 family members, IL-1F6 and IL-1F8. It has been shown earlier that other members of the IL-1 family do have important effects on adipose tissue inflammation. Blockade of IL-1, using anakinra, resulted in improved fasting glucose levels and is known to result in decreased adipocyte differentiation and insulin sensitivity. Further, IL-18 deficiency leads to insulin resistance and obesity. IL-1F6 and IL-1F8 activation is known to result in the release of pro-inflammatory cytokines. IL-1F6 and IL-1F8 are involved in skin and lung immunity. We determined the presence and functional activity of IL-1F6 and IL-1F8 in adipocytes.

As IL-1 is involved in insulin resistance as well as in decreased beta-cell function, blockade should result in (partial) reversal of these effects. At the start of the research described in this thesis, anakinra was the only, commercially available drug inhibiting IL-1 activity.

To determine the direct effects of IL-1β blockade on insulin resistance, we selected patients with the metabolic syndrome, a condition strongly associated with insulin resistance. By selecting patients with the metabolic syndrome, but without diabetes, we were able to measure the effect of Anakinra both on insulin sensitivity and beta-cell function without interfering effects of changes in glycemic levels. The results of this trial are reported in **chapter 4**.

In **chapter 5** we focused on insulin sensitivity and Anakinra treatment. For this study patients with long-standing type 1 diabetes mellitus were included. As these patients do not have residual beta-cell function, we were able to specifically monitor effects on insulin sensitivity. For this study, overweight patients with suboptimal regulated diabetes (HbA1c > 58 mmol/mol) were included. Thus, this study should not only be able to detect Anakinra-induced effects on insulin sensitivity by reducing the obesity-associated inflammation but also by interfering with the chronic hyperglycemia-related inflammation.

Patients with impaired glucose tolerance have a high risk to develop type 2 diabetes mellitus over time. Impaired glucose tolerance is an early manifestation of an underlying insulin secretion defect. Based on the in vitro data, we postulated that inflammatory changes in general and over activity of the IL-1β pathway in particular may contribute to this early insulin secretion dysfunction. This hypothesis was tested in the study described in **chapter 6** where we determined the effects of IL-1 blockade on beta-cell function, again using the IL-1receptor antagonist Anakinra. We selected subjects with a family history for diabetes mellitus who had impaired glucose tolerance on glucose tolerance testing. As these patients have relatively normal glucose levels an indirect positive effect on beta-cell function by decreased glucose toxicity is less likely. In this trial we also measured effects of anakinra treatment on the incretin hormone glucagon-like peptide-1 (GLP-1).

Chapter 7 provides a summary and discussion of the findings of this thesis.

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2

TLR3 is present in human adipocytes, but its signalling is not required for obesity-induced inflammation in adipose tissue in vivo

Dov B. Ballak, Edwin J.P. van Asseldonk, Janna A. van Diepen, Henry Jansen, Anneke Hijmans, Leo A.B. Joosten, Cees J. Tack, Mihai G. Netea, Rinke Stienstra

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Abstract

Innate immunity plays a pivotal role in obesity-induced low-grade inflammation originating from adipose tissue. Key receptors of the innate immune system including Toll-like receptors-2 and -4 (TLRs) are triggered by nutrient excess to promote inflammation. The role of other TLRs in this process is largely unknown. In addition to double-stranded viral mRNA, TLR-3 can also recognize mRNA from dying endogenous cells, a process that is frequently observed within obese adipose tissue. Here, we identified profound expression of TLR-3 in adipocytes and investigated its role during diet-induced obesity.

Human adipose tissue biopsies (n=80) and an adipocyte cell-line were used to study TLR-3 expression and function. TLR-3-/- and WT animals were exposed to a high-fat diet (HFD) for 16 weeks to induce obesity.

Expression of TLR-3 was significantly higher in human adipocytes compared to the non-adipocyte cells part of the adipose tissue. *In vitro,* TLR-3 expression was induced during differentiation of adipocytes and stimulation of the receptor led to elevated expression of pro-inflammatory cytokines. *In vivo*, TLR-3 deficiency did not significantly influence HFD-induced obesity, insulin sensitivity or inflammation. In humans, TLR-3 expression in adipose tissue did not correlate with BMI or insulin sensitivity (HOMA-IR).

Together, our results demonstrate that TLR-3 is highly expressed in adipocytes and functionally active. However, TLR-3 appears to play a redundant role in obesity-induced inflammation and insulin resistance.

Introduction

Obesity is a worldwide problem that profoundly affects global health (1). Obesity is associated with type 2 diabetes mellitus, cardiovascular disease, hepatic steatosis and obesity-related cancers (2). Development of obesity leads to morphological and functional changes in adipose tissue. Adipocytes become hypertrophic and immune cells, such as macrophages (3, 4), T- and B-cells (5), infiltrate into the adipose tissue (6). This inflammatory trait is associated with insulin resistance and subsequently type 2 diabetes mellitus. Much remains to be learned about the factors that initiate and propagate adipose tissue inflammation.

An inflammatory response is initiated when exogenous (pathogen associated molecular pattern, PAMP) or endogenous (danger associated molecular pattern, DAMP) ligands are recognized by a pattern recognition receptor (PRR). Activation of PRRs triggers intracellular signalling cascades, eventually leading to production of chemokines and pro-inflammatory cytokines and recruitment and activation of immune cells. TNFα, interleukin (IL)-1β, IL-6 and IL-8 are the best-known cytokines involved in adipose tissue inflammation and the subsequent development of insulin resistance (7-9). Potential DAMPs in adipose tissue, released by dying adipocytes or certain lipids, are present in increased concentrations in obesity (10). It is therefore of high interest to identify which pattern recognition receptors mediate the link between obesity and adipose tissue inflammation.

Toll-like receptors (TLRs) are a large class of transmembrane PPRs and well known for their role in inflammatory signalling during infection and inflammation (11). In humans, ten members of the TLR-family have been described (12). Amongst these, TLR-2 and TLR-4 have emerged as important regulators of pro-inflammatory signalling in response to saturated fatty acids (13-16), thereby mediating at least part of the HFD-induced adipose tissue inflammation (17). In contrast, the role of other TLRs in adipose tissue inflammation remains largely unknown.

In the present study we screened the expression of all TLR-family members in adipose tissue. Remarkably, we identified TLR-3 as being highly expressed in the adipocytes, in contrast to other TLRs that were mainly present in the stromal vascular fraction of the adipose tissue. Interestingly, TLR-3 can bind mRNA that is released from dying cells (18) aside from double-stranded viral mRNA (19), hence may be activated by apoptotic adipocytes that are frequently observed within obese adipose tissue (20). Therefore, we were prompted to investigate the role of TLR-3 in adipose tissue inflammation, using complementary *in-vitro* and *in-vivo* experimental models, as well as human adipose tissue biopsies.

Materials and methods

Human subjects

Paired subcutaneous (SAT) and visceral adipose tissue (omentum) (VAT) samples were obtained according a standardized procedure from 4 patients (two females and two males) to investigate expression of TLRs in mature adipocytes (MA) versus the stromal vascular fraction (SVF). Tissue biopsies were taken during elective cholecystectomy. Subjects were between 40–60 years old with a body mass index (BMI) of 25–28 kg/m^2. Subjects were normoglycemic. Metabolic, endocrine and chronic and/or acute inflammatory diseases were excluded.

Moreover, adipose tissue samples were obtained from 80 healthy human subjects who were recruited through advertisements in local newspapers. We included healthy subjects between 40 and 70 years old (see Supp. Table 1). Subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies 6–10 cm lateral to the umbilicus. Samples were taken after an overnight fast. TLR-3 expression was measured in the adipose tissue biopsies of all subjects. Subsequently, metabolic parameters were compared to TLR-3 expression: BMI (<25, n=33; BMI >30, n=18), Homeostatic model assessment for insulin resistance (HOMA-IR) levels (<2, n=40; >2 n=26, hsCRP plasma levels (<0.5mg/l, n=19; >1.7mg/l, n=19) and number of crown-like structures in the adipose tissue (no, n=53; yes, n=19). Furthermore, associations were made between adipose tissue TLR-3 expression and the lowest and highest quartiles, with respect to mRNA expression levels, of IL-8, monocyte chemoattractant protein (MCP)-1, fatty acid binding protein (FABP)4 and adipocyte size in the adipose tissue. These are markers for either adipose tissue inflammation or adipocyte health. The tissue samples were collected after written informed consent from all individual participants, and the protocol was approved by the ethical committee of the Radboud University Nijmegen Medical Centre and in accordance with the Declaration of Helsinki.

Stromal vascular and mature adipocyte fractions

The collected SAT and VAT samples were disaggregated using collagenase type 1 (Gibco, Life Technologies) digestion to isolate adipocytes and the stromal vascular fraction (SVF) as described before (21). Purity of the two different fractions was confirmed using FABP 4 (adipocytes), and CD45 (hematopoietic cells) (*table 1*). The cellular fractions were subsequently used for RNA isolation, followed by real-time PCR analysis, as described below.

Histochemistry

Morphometry of individual fat cells was assessed using digital image analyses as described previously (22). In short: for each subject, the adipocyte cell diameter of all fat cells in five to ten microscopic fields of view were counted and measured. For detection of crown-like structures, a CD68 antibody (AbD Serotec, Oxford, UK) was used in human samples. In mouse samples, an antibody against F4/80$^+$ (AbD Serotec, Oxford, UK) was used. Visualization of the complex was done using 3,3'-diaminobenzidene for 5 min. Negative controls were used by omitting the primary antibody.

Animals

16 Male TLR-3-/- and 20 male WT mice on a C57Bl/6 background were obtained from Jacksons Laboratory and housed in a pathogen-free environment in the animal facility of the Radboud University, Nijmegen. The mice were purchased at Jackson and housed individually in filter top cages in a separate room, with water and food *ad libitum*. The housing temperature was held at 23°C and a 12:12h light-dark cycle was maintained. After a 1-week run-in period on a low-fat diet, the mice were divided in a low-fat diet (LFD) and a high-fat diet (HFD) group. The diet contained either 10% or 45% energy derived from fat, lard-oil was replaced by palm-oil (D12450B or 12451; Research Diet Services, Wijk bij Duurstede, The Netherlands). This diet was continued for 16 weeks. Bodyweight of the animals was recorded weekly. After sacrifice, liver and epididymal adipose tissue weight were measured. All animal procedures were conducted under protocols approved by the animal experimentation committee of Radboud University Medical Centre, Nijmegen.

Insulin tolerance test (ITT)

An insulin tolerance test was performed after a 4 hours fasting period. 0,75 U of insulin per kilogram bodyweight was injected intraperitoneally. Blood glucose levels were determined with an Accu-chek glucosemeter (Roche Diagnostics, Almere, The Netherlands) at indicated time points after insulin administration.

qPCR

Total RNA was isolated from adipose tissue using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. RNA was reverse-transcribed (iScript cDNA Synthesis Kit, Bio-Rad Laboratories). RT-PCR was performed using specific primers (see **S2 Table**), power SYBR green master mix (Applied Biosystems, Foster City, CA) using the Step-one Real-Time PCR system (Applied Biosystems, Foster City, CA). For mice samples, we used 36B4 as housekeeping gene. For human samples we used B2M as a housekeeping gene.

Insulin

Insulin levels in humans were measured by the clinical laboratory unit of the Radboud University Medical Centre, Nijmegen.

Plasma glucose

Glucose (Liquicolor, Human GmbH, Wiesbaden, Germany) was measured enzymatically following manufacturers' protocols.

Cell lines

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were cultured as described earlier (23). After full differentiation cells were treated with poly(I:C) 12.5 μg/ml or lipopolysaccharide 50 ng/ml to stimulate respectively TLR-3 or TLR-4.

Western Blot

Human subcutaneous adipose tissue samples were analysed for TLR-3 expression. Lysates prepared using a lysis buffer [50mM Tris (pH7.4), 150mMNaCl, 2mMEDTA,1%Nonidet P-40, 50mMNaF,and 0.25% sodium deoxycholate with phosstop phosphatase-inhibitor cocktail tablet (Roche) and complete, EDTA-free protease-inhibitor cocktail tablet (Roche). The homogenate was centrifuged at 4˚C for 10 min at 18.000 rcf and the supernatant was used for Western blot analysis. Equal amounts of protein, as determined by a BCA protein assay (Thermo FisherScientific, Rockford, IL) were separated using a polyacrylamide SDS page gel. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Biorad) following manufacturer's instructions. The membrane was blocked with 5% (wt/vol) milk powder in Tris-buffered saline (TBS)/Tween 20 for 1 h at room temperature followed by incubation overnight at 4 C with a TLR-3 antibody (Abcam, ab62566), in 5% (wt/vol) milk powder/TBS/Tween 20. After overnight incubation, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, A0545, Sigma Aldrich) at a dilution of 1:5000 in 5% (wt/vol) milk powder in TBS/Tween 20 for 1 h at room temperature and subsequently developed with ECL plus (Thermo Scientific) according to the manufacturer's instructions. Bands were visualized using a ChemiDoc System (Biorad) and quantified using Image lab software (Biorad).

Small interfering RNA

To specifically suppress TLR-3 expression in differentiated adipocytes, cells were transfected (X-tremeGENE siRNA Transfection Reagent, Roche) with small interfering (si)RNA against TLR-3 (Thermo Scientific). As a nonspecific control, scrambled siRNA (Thermo Scientific) was used. After 72 hours of incubation, gene expression was determined.

Statistical analysis

Data are shown as means ± SEM. Differences between groups were analyzed using Student's *t* test, differences among 4 groups were analyzed with ANOVA followed by *post-hoc* Bonferroni tests and correlations were evaluated with regression analysis in Graphpad Prism 6.0. p-values < 0.05 were considered significant.

Results

TLR-3 is highly expressed in adipocytes and functionally active

We investigated expression patterns of all TLR-family members in human adipose tissue. Adipose tissue biopsies of both subcutaneous and visceral depots were used and separated in stromal vascular fraction (SVF) and mature adipocytes (MA). Separation of adipocyte and stromal vascular fraction was adequate as determined by expression of CD45 and FABP4 in the two fractions (*S1 figure a, b*). Most TLR-family members were more robustly expressed in the SVF fraction compared to the MA fraction (*e.g.* TLR-1, TLR-2), or similarly expressed between the SVF and MA fraction (*e.g.* TLR-6, TLR-7, TLR-8). Remarkably, the expression profile of TLR-3 was different from the expression pattern observed for the other TLRs, as it was significantly higher expressed in the MA fraction compared to SVF (*figure 1a*). The only additional receptor that was relatively high expressed in adipocytes was TLR-5. However, its expression was just 4 times higher in only the visceral adipocytes compared to the stromal vascular cells, where TLR-3 had a 6-fold higher expression in adipocytes. This difference was apparent for both subcutaneous and visceral adipose tissue. Moreover, TLR-3 protein expression in human subcutaneous adipose tissue was confirmed by Western Blot (*S1 figure c*). Additionally, we found that TLR-3 was highly induced during differentiation of SGBS adipocytes *in vitro* (*figure 1b*), in a similar fashion as PPAR-γ, a marker for adipocyte differentiation (*figure 1c*).

Next, we tested whether TLR-3 activation in adipocytes was functionally active. Therefore, we treated differentiated adipocytes with poly(I:C), a synthetic TLR-3 ligand and compared this to E.coli LPS, a TLR-4 ligand. Similar to LPS, poly(I:C) significantly increased transcription of pro-inflammatory cytokine IL-8 after 24 hours. In addition, poly(I:C) induced expression of MCP-1, a key molecule that mediates macrophage infiltration in adipose tissue (24), while IL-1β expression was not increased after 24 hours of LPS or poly(I:C) treatment. Furthermore, adipogenic genes adiponectin and PPARγ were significantly decreased after Poly(I:C) stimulation (*figure 2a*). To ensure that the effects of poly(I:C) on adipogenic and pro-inflammatory cytokine gene expression were specifically mediated by TLR-3, the expression of TLR-3 was blocked using small interfering RNA (siRNA). TLR-3 was blocked with a knockdown of almost 75% (*S2 figure*). These data reveal that poly(I:C) increased expression of IL-8 and MCP-1 in a TLR-3 dependent manner, since blockage of TLR-3 with siRNA greatly inhibited the poly(I:C) mediated induction of these genes (*figure. 2b, c*). Gene expression levels of IL-1β were unaffected (*figure 2d*). In contrast,

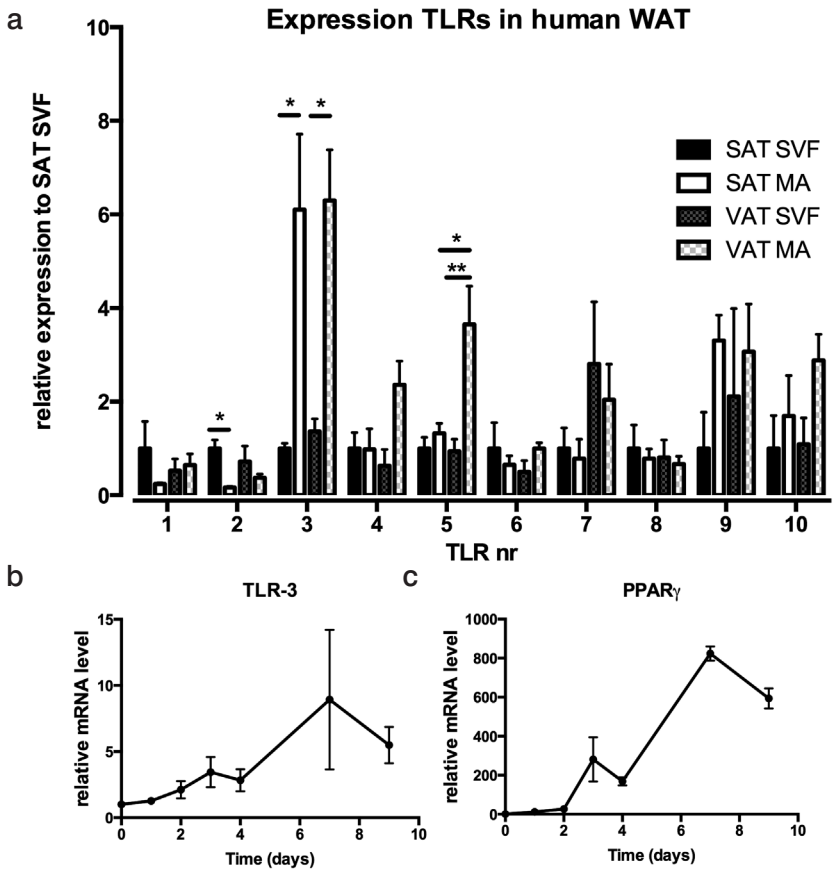

Figure 1 TLR-3 is predominantly expressed in adipocytes.

(**a**) Biopsies from visceral- (VAT) and subcutaneous adipose tissue (SAT) were obtained from 4 healthy subjects and TLR expression was determined in stromal vascular fraction (SVF) and mature adipocytes (MA). mRNA levels of (**b**) TLR-3 and (**c**) PPAR-γ were measured during differentiation of human SGBS adipocytes. * $p < 0.05$, ** $p < 0.01$. Data are shown as means ± SEM.

downregulation of adiponectin and PPARγ was not affected by blockage of TLR-3, suggesting that other signalling routes besides TLR-3 are likely to play a role in down-regulating adipogenesis upon Poly(I:C) stimulation (*figure 2e, f*).

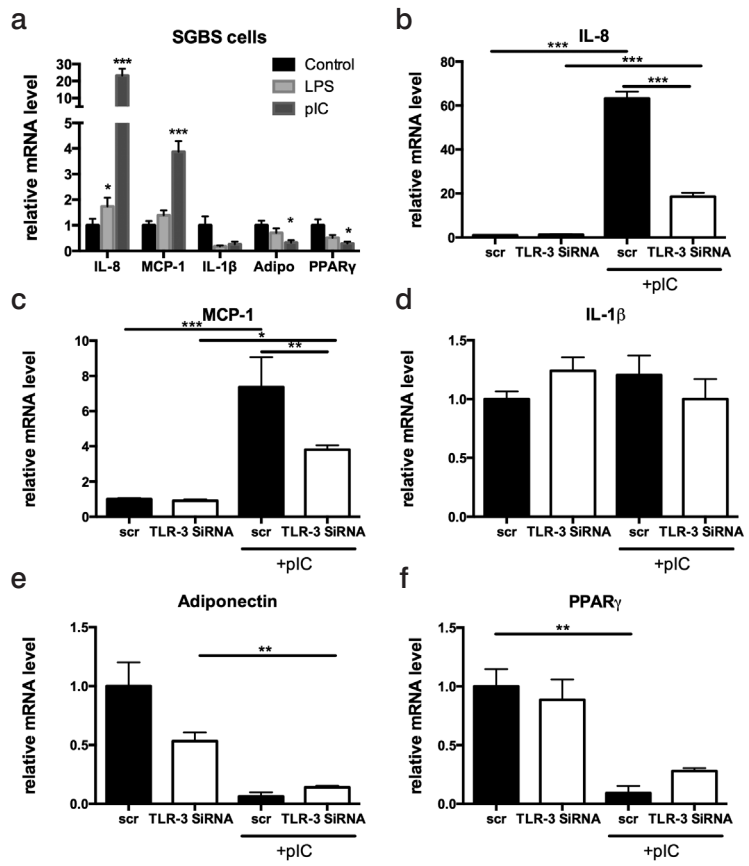

Figure 2 TLR-3 is functionally active in adipocytes.

(**a**) Differentiated SGBS adipocytes were stimulated with either a TLR-3 (poly:IC 12.5μg/ml) or TLR-4 (LPS 50ng/ml) agonist. mRNA levels were measured for IL-8, MCP-1, IL-1β, adiponectin and PPAR-γ. (b-f) SGBS adipocytes were treated with SiRNA against TLR-3 or scr SiRNA and stimulated with poly:IC. mRNA levels of (**b**) IL-8, (**c**) MCP-1, (**d**) IL-1β, (**e**) adiponectin, (**f**) PPAR-γ were subsequently measured. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are shown as means ± SEM.

TLR-3 deficiency does not affect weight gain or insulin sensitivity after HFD-feeding

To test whether TLR-3 is functionally relevant in the process of diet induced obesity and insulin resistance, TLR-3-/- mice and WT controls were fed a HFD for 16 weeks. TLR-3 deficiency did not influence HFD-induced obesity, compared to WT mice (*figure 3a*). Similarly, liver and epididymal adipose (eWAT) tissue weights were not

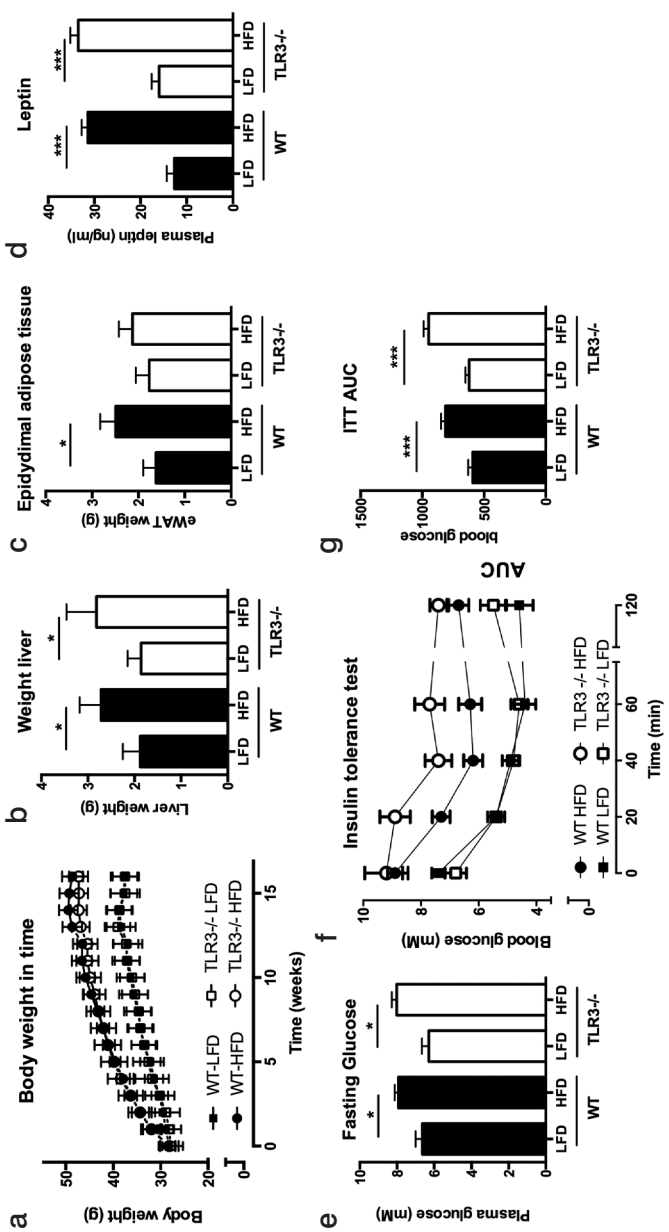

Figure 3 TLR-3 deficiency does not protect mice against metabolic abnormalities.
Wild-type (WT) and TLR-3-/- mice were subjected to 16 weeks of low fat diet (LFD) or high fat diet (HFD). (**a**) development of the bodyweight, (**b**) liver weight, (**c**) epididymal adipose tissue weight, (**d**) plasma leptin levels, (**e**) fasting glucose levels, (**f**) insulin tolerance test (ITT), (**g**) area under the curve for ITT. * $p < 0.05$, *** $p < 0.001$. Number of mice per group: WT-LFD n=10; WT-HFD n=10; TLR-3-/-LFD n=7; TLR-3-/-HFD n=9. Data are shown as means ± SEM.

statistically different in TLR-3-/-mice as compared to WT animals (*figure 3b, c*). The latter was confirmed by a similar increase of plasma leptin levels in both genotypes due to HFD feeding (*figure 3d*). Next, we tested whether TLR-3-/- mice were protected from HFD-induced insulin resistance. Fasting glucose levels (8 hours) were similar in both genotypes, after LFD and HFD-feeding (*figure 3e*). An insulin tolerance test performed 15 weeks after starting the diet intervention showed a tendency towards a reduction in insulin sensitivity in HFD-fed TLR-3-/- mice, however this difference did not reach statistical (*figure 3f, g*).

TLR-3 deficiency does not influence obesity-induced adipose tissue inflammation in mice

In a following set of experiments, we assessed whether TLR-3 deficiency affected the development of obesity-induced adipose tissue inflammation. Therefore, histological sections of eWAT were stained with F4-80, a macrophage marker (*figure 4a*). No differences were found in the number of crown-like structures between TLR-3-/- and WT animals (*figure 4b*). This result was confirmed by similar gene expression levels of two macrophage markers, F480 and CD68, as detected by qPCR analysis (*figure 4c, d*). Only monocyte chemoattractant protein (MCP)-1 was slightly higher expressed in the absence of TLR-3 (*figure 4e*). Together, these data suggest that TLR-3 deficiency does not affect macrophage infiltration. Additionally, we did not find differences in macrophage activation as determined by expression of the pro-inflammatory cytokines TNFα and CXCL1 in adipose tissue of TLR-3-/- and WT mice, (*figure 4f, g*). Interestingly, TLR-3 expression itself was not different between HFD- and LFD-treated WT mice (*figure 4h*), while expression of other TLRs was upregulated (TLR-6) or tended to increase (TLR-1 and TLR-2) in response to HFD, except for TLR-4 (*S3 figure a-d*). However, expression of these TLRs was not different between TLR-3-/- and WT mice fed either a LFD or a HFD, revealing no compensatory up-regulation of other TLRs in adipose tissue in the absence of TLR-3. Furthermore, expression of 9 TLRs in chow fed mice was measured in stromal vascular cells compared to mature adipocytes (*S4 figure*). Most TLRs, except 2, 4 and 5 were more expressed in stromal vascular cells. Thus TLR-3, opposed to human adipose tissue, displayed a lower expression level in mouse adipocytes.

TLR-3 in human subcutaneous adipose tissue

Next, we correlated the gene expression levels of TLR-3 in subcutaneous adipose tissue samples from 80 healthy donors, with markers of obesity, inflammation and insulin sensitivity. TLR-3 expression in subcutaneous adipose tissue was similar between lean and obese individuals (*figure 5a*). Similarly, no difference in TLR-3 expression was observed between subjects with high insulin sensitivity levels versus low insulin sensitivity levels, as determined by the homeostatic model assessment for

insulin resistance (HOMA-IR) (*figure 5b*). Subjects with higher systemic inflammation (high sensitive C-reactive protein (hs-CRP) levels) or increased adipose tissue inflammation (number of crown-like structures) also showed an equal expression of TLR-3 (*figure 5c, d*). In contrast, we found that adipose tissue of subjects with higher expression of the pro-inflammatory cytokine IL-8 showed a tendency for lower mRNA levels of TLR-3 (*figure 5e*). A similar tendency was seen for MCP-1 and TLR-3 expression (*figure 5f*). On the contrary, FABP4 showed a positive association with TLR-3 expression in adipose tissue (*figure 5g*), although this did not reach statistical significance. Finally, despite a high expression of TLR-3 in mature adipocytes, its expression did not associate with mean adipocyte cell size in adipose tissue (*figure 5h*).

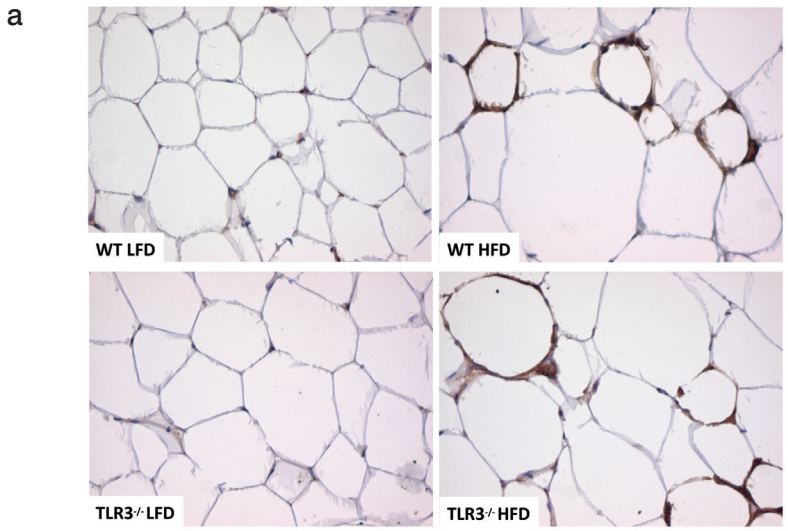

Figure 4 TLR-3 deficiency does not ameliorate adipose tissue inflammation.

After 16 weeks of low fat diet (LFD) or high fat diet (HFD) intervention, adipose tissue of wild-type (WT) and TLR-3-/- mice was investigated for inflammatory parameters. (**a**) Adipose tissue of mice stained for F4/80, magnification 20x, (**b**) number of crown-like structures in adipose tissue. Inflammatory markers were measured (**c**) F480, (**d**) CD68, (**e**) MCP-1, (**f**) TNFα, (**g**) CXCL1. (**h**) mRNA levels of TLR-3 in WT mice fed a LFD or HFD for 16 weeks. * $p < 0.05$, ** $p < 0.01$. Number of mice per group: WT-LFD n=10; WT-HFD n=10; TLR-3-/-LFD n=7; TLR-3-/-HFD n=9. Data are shown as means ± SEM.

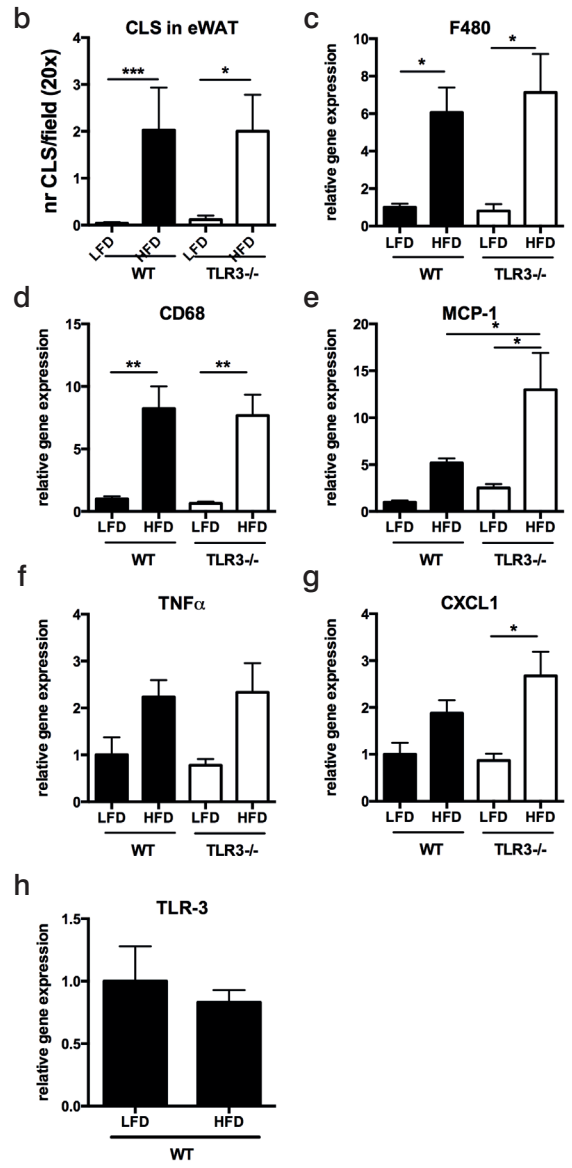

Figure 4 Continued.

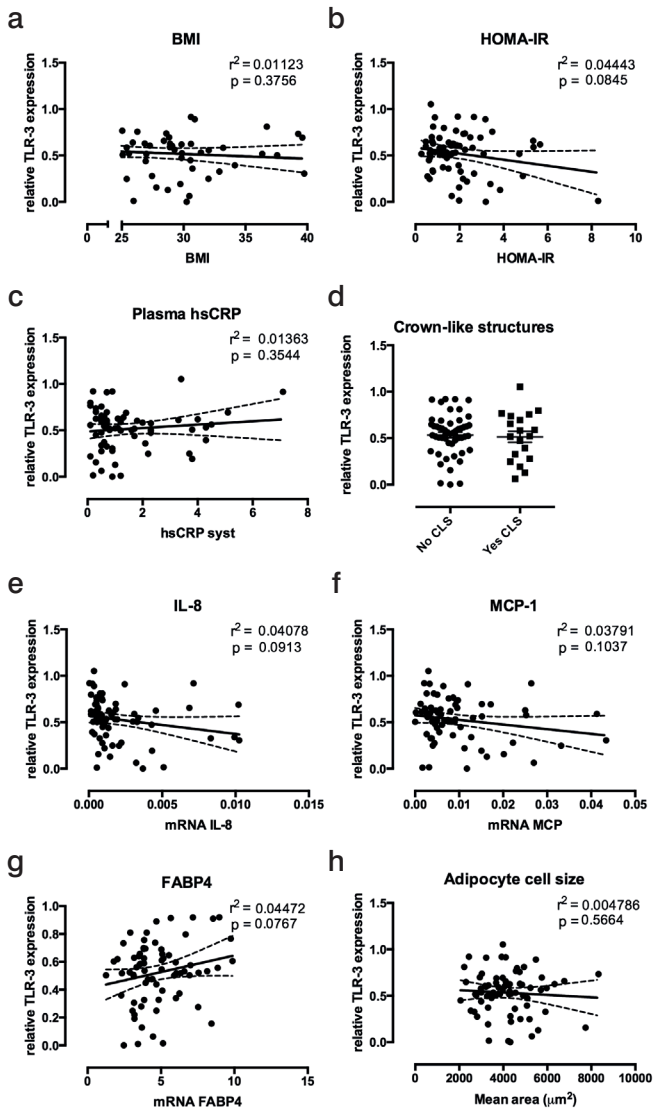

Figure 5 TLR-3 in human adipose tissue.

Subcutaneous adipose tissue samples of 80 healthy individuals were obtained. TLR-3 mRNA levels were associated with (**a**) BMI, (**b**) HOMA-IR, (**c**) plasma CRP-levels, (**d**) number of crown-like structures in adipose tissue. Association of MAP3K8 mRNA expression in human subcutaneous adipose tissue with mRNA expression of (**e**) IL-8, (**f**) MCP-1, (**g**) FABP4 and (**h**) adipocytes cell size. * $p < 0.05$. Data are shown as means ± SEM. HOMA-IR = Homeostatic Model Assessment for insulin resistance.

Discussion

Innate pattern recognition receptors (PRRs) are increasingly recognized as direct or indirect sensors of excessive nutrients instigating a chronic inflammatory response in adipose tissue during obesity, with possibly detrimental effects on insulin sensitivity levels (10). During this metaflammation (7), the function of TLR-3, stimulated by double-stranded mRNA and yielding production of type-1 Interferons (19), remains largely unknown. In the current study we examined whether TLR-3-signalling is involved in the development of adipose tissue inflammation and insulin resistance during the development of obesity. To study a potential role of TLR-3 triggered by its remarkable high expression in adipocytes, as compared to other members of the TLR family. We show that TLR-3 is active in adipocytes upon stimulation with specific ligands. However, TLR-3-/- mice showed no significant amelioration in adipose tissue inflammation and insulin sensitivity after HFD-feeding. Additionally, data obtained from 80 human adipose tissue biopsies showed a limited association of TLR-3 gene expression levels with various metabolic abnormalities including insulin resistance and adipose tissue inflammation. Together, these data suggest that although TLR-3 is predominantly expressed in adipocytes and functionally active, it has a limited role in mediating 16 weeks HFD-induced adipose tissue inflammation and systemic insulin sensitivity in mice, nor a profound association with metabolic abnormalities in humans.

Interestingly, TLR-3 is preferentially expressed on adipocytes as compared to stromal vascular cells suggestive of a functional role of the receptor specifically in these cells. Indeed, our data demonstrate that stimulation of TLR-3 increases pro-inflammatory cytokine expression, an effect that was abolished by silencing of TLR-3 in adipocytes using siRNA. Thereby, we confirm that adipocytes can produce cytokines after specific TLR-3 stimulation (25). Moreover, TLR-3 was up-regulated during differentiation of adipocytes. The high expression levels led us to believe that TLR-3 may exert a more dominant role in adipose tissue as compared to other TLR-family members.

However, despite its prominent expression and function in adipocytes, in vivo studies using TLR-3-/- mice revealed no amelioration in adipose tissue inflammation or insulin sensitivity levels as compared to WT mice after 16 weeks of HFD-feeding. The difference in expression pattern of TLR-3 in adipocytes vs stromal vascular fraction between mouse and human adipose tissue may explain at least some of our apparent conflicting observations between *in vitro* and *in vivo* results. In addition, we observed a different effect of TLR-3 deficiency on MCP-1 expression between the *in vitro* and *in vivo* experiments. These divergent responses may be explained by differences in model-complexity or a different response to TLR-3 signalling in human versus murine cells (26).

Our *in vivo* results are partly in contrast to a recent report demonstrating that the absence of TLR-3 or its blockage using a specific antibody led to improvements in glucose tolerance (27). However, in line with our results, a glucose tolerance test performed by Wu *et al* after 14 weeks of HFD revealed no differences between WT and TLR-3-/- animals compared to WT mice. Only after 26 weeks of HFD-feeding, the absence of TLR-3 led to an improvement in glucose tolerance. Moreover, the absence of TLR-3 protected against the development of hepatic steatosis after 33 weeks of HFD and reduced expression levels of TNFα in liver. Our research advances these studies by specifically focussing on establishing a role for TLR-3 in adipose tissue. Possibly, TLR-3 exerts differential effects in the liver versus adipose tissue during the development of HFD-induced obesity. More likely, the opposite outcomes are explained by differences in study design. The diet intervention in our study was done using a low fat diet and high fat diet containing 45% of energy derived from fat. Wu *et al* used a high fat diet with 60% of energy from fat with chow diet as control feeding. Additionally, the intervention period lastly for a total period of 33 weeks whereas our study design included only 16 weeks of HFD-feeding.

Hence, these outcomes may suggest that the absence of TLR-3 ameliorates metabolic abnormalities induced by high fat diet feeding only after a prolonged time of diet intervention. One might speculate that the ligand responsible for activation of TLR-3 only becomes available after long term and very high fat diet feeding.

Although many TLRs have both endogenous and exogenous stimuli, the endogenous activator of TLR-3 remains to be established. Based on current knowledge, TLR-3 primary function is to recognize double stranded RNA and contributes to the host defence against viruses. What type of endogenous activator in adipose tissue might propel TLR-3 activation is currently unknown, however it has been shown that TLR-3 also recognizes single-stranded mRNA (18). In obesity, dying adipocytes may therefore release mRNA, which is then recognized by TLR-3. However, in spite of its high expression levels in human adipocytes that may suggest otherwise, our results do not provide evidence for a vital, important role of TLR-3 in adipose tissue during diet-induced obesity. The high expression of TLR3 in the adipocyte fraction of human adipose tissue, however, suggest a possible other role in human adipocyte function. Recently, it was discovered that activation of TLR-3 reduces adipocyte differentiation (28) and that this resulted in inhibited insulin and glucose signalling (29). As TLR-3 may be involved in the recognition of dying adipocytes, the necrotic cells may thereby directly influence surrounding pre-adipocytes and inhibit their differentiation and suppress glucose and insulin signalling. Moreover, although especially TLR-2 and TLR-4 have been implicated in mediating the pro-inflammatory actions of free fatty acids, we cannot rule out that TLR-3 mediates some of these free fatty acids related effects.

As it is hypothesised that viruses play a role in the pathogenesis of type 1 diabetes, it might be interesting to investigate the role of TLR-3 in the pancreas. Several studies have showed a link between TLR-3 induced islet inflammation and type 1 diabetes (22-24). Because 16 weeks is not long enough to induce islet inflammation in mice in contrary to adipose tissue inflammation, our study was able to investigate the effects of TLR-3 without confounding effects of the pancreas.

In line with the results from the animal study that did not reveal a vital role for TLR-3 in mediating obesity-induced inflammation and the development of insulin resistance, the function of the receptor in human adipose tissue appears to be relatively small. However, as shown in literature (27), TLR-3 may exacerbate metabolic abnormalities in the liver after 33 weeks of HFD in mice as opposed to adipose tissue. Moreover, we observed no clear associations in humans, suggesting that TLR-3 has a limited role in mediating adipose tissue inflammation and the development of insulin resistance. However, these results do not completely rule out any function of TLR-3-signalling in adipose tissue during the presence of obesity. Noticeably, our results were obtained using subcutaneous adipose tissue biopsies and not the metabolically more active visceral depot. In addition to gene expression levels, future studies should also determine the activity of TLR-3-dependent signalling routes within adipose tissue by measuring TLR-3 protein levels and possible intracellular downstream targets.

Altogether, these data show that TLR-3 is highly expressed in adipocytes as compared to the stromal vascular cells and is functionally active. Nevertheless, in mice after 16 weeks of HFD and in humans, the data does not support a fundamental role for TLR-3 in the development of obesity-induced adipose tissue inflammation and insulin resistance.

Statement on the Welfare of Animals

Ethical Approval: All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Supporting information

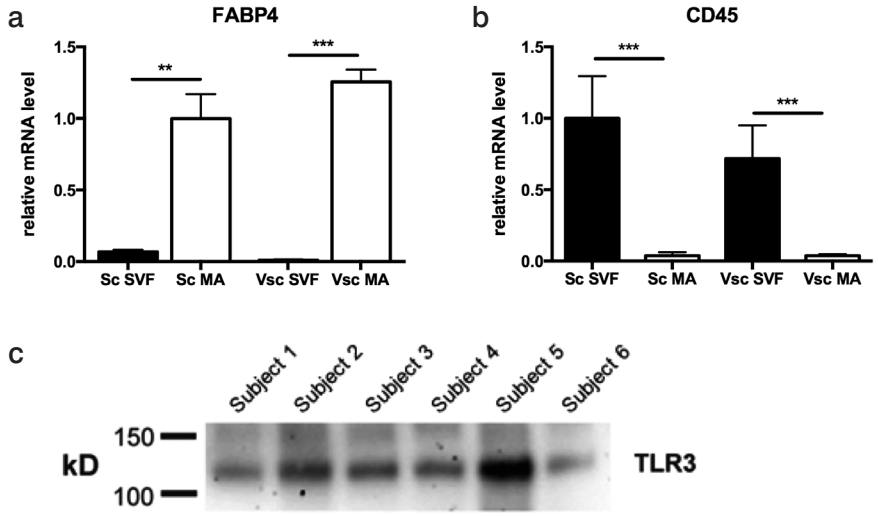

Figure S1 Human adipose tissue.

Biopsies from visceral- (VAT) and subcutaneous adipose tissue (SAT) were obtained from 4 healthy subjects and TLR expression was determined in stromal vascular fraction (SVF) and mature adipocytes (MA). mRNA levels of (**a**) FABP4 (**b**) CD45. (**c**) Western blot was used to confirm TLR-3 protein expression in human subcutaneous adipose tissue. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are shown as means ± SEM.

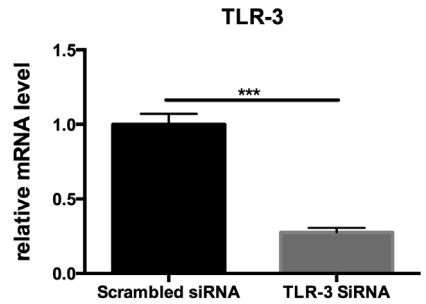

Figure S2 Knockdown of TLR-3 in SGBS cells.

SGBS cells where transfected with small interference RNA against TLR-3 to reduce expression of TLR-3. Gene expression was determined after 72 hours. *** $p < 0.001$. Data are shown as means ± SEM.

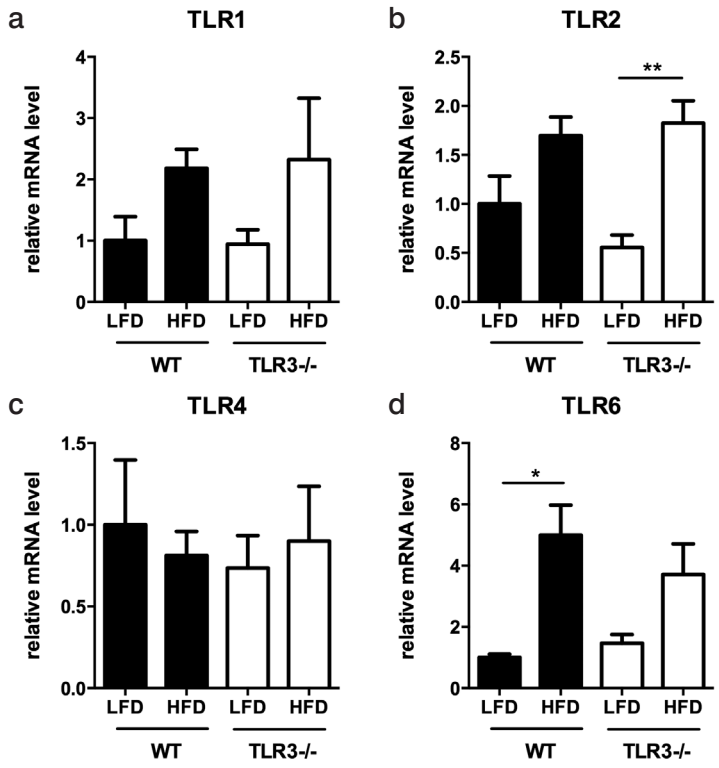

Figure S3 TLR expression in epididymal adipose tissue after HFD.

After 16 weeks of low fat diet (LFD) or high fat diet (HFD) intervention, adipose tissue of wild-type (WT) and TLR-3-/- mice was investigated for TLR expression. mRNA levels of (**a**) TLR-1, (**b**) TLR-2, (**c**) TLR-4 and (**d**) TLR-6 were measured. * $p < 0.05$, ** $p < 0.01$. Number of mice per group: WT-LFD n=10; WT-HFD n=10; TLR-3-/-LFD n=7; TLR-3-/-HFD n=9. Data are shown as means ± SEM.

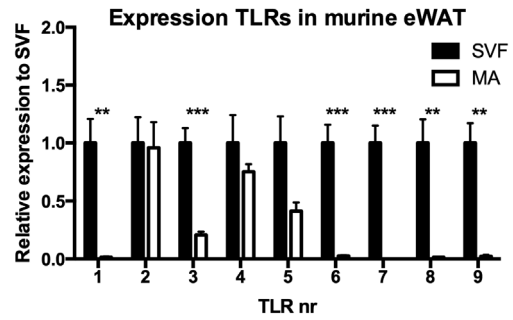

Figure S4 TLR expression in epididymal adipose tissue in chow-fed mice.

mRNA levels of 9 different TLRs were measured adipocytes versus stromal vascular cells in chow fed mice. Number of mice per group: adipocytes n = 9, stromal vascular cells n = 9. Data are shown as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are shown as means ± SEM.

Supplementary Table 1 Subject characteristics.

This table lists information on the subjects of which adipose tissue biopsies were investigated.

	Subcutaneous adipose tissue biopsies
N	80
% male	48%
Age (years) ± SD	54,7 ± 8,4
BMI (kg/m2) ± SD	27,7 ± 4,8
Fasting glucose (mmol)	5,1 ± 0,6
HOMA-IR ± SD	2,3 ± 2,1
hs-CRP (mg/L) ± SD	1,6 ± 1,7

Supplementary Table 2 Primer Sequences.

This table lists all sequences of the primers that where used in this investigation.

Primer name	Sequence Forward (5'> 3')	Sequence Reverse (5'> 3')
m36B4	AGCGCGTCCTGGCATTGTGTGG	GGGCAGCAGTGGTGGCAGCAGC
mCD68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC
mF4/80	CTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
mMCP-1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
mTNF-α	CAACCTCCTCTCTGCCGTCAA	TGACTCCAAAGTAGACCTGCCC
mCXCL-1	TGGCTGGGATTCACCTCAA	GAGTGTGGCTATGACTTCGGTTT
mTLR-3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
mTLR-1	TCAAGCATTTGGACCTCTCCT	TTGTACCCGAGAACCGCTCA
mTLR-2	AACCTCAGACAAAGCGTCAAATC	ACCAAGATCCAGAAGAGCCAAA
mTLR-4	TTCCTTCTTCAACCAAGAACATAGATC	TTGTTTCAATTTCACACCTGGATAA
mTLR-5	GCAGGATCATGGCATGTCAAC	ATCTGGGTGAGGTTACAGCCT
mTLR-6	AGGAACCTTACTCATGTCCCC	TGTTGTGGGAGAGTCTCAGAA
mTLR-7	TCTTACCCTTACCATCAACCACA	CCCCAGTAGAACAGGTACACA
mTLR-8	GAAAACATGCCCCCTCAGTCA	CGTCACAAGGATAGCTTCTGGAA
mTLR-9	TGAAGTCTGTACCCCGTTTCT	GTGGACGAAGTCAGAGTTGT
hTLR-1	CAGGCCCTCTTCCTCGTTAGA	TTCCTAAAGGTAGAAGCTGTTCTTCA
hTLR-2	GAATCCTCCAATCAGGCTTCTCT	GCCCTGAGGGAATGGAGTTTA
hTLR-3	TTTGCGAAGAGGAATGTTTAAATCT	CACCTATCCGTTCTTTCTGAACTG
hTLR-4	GGCATGCCTGTGCTGAGTT	CTGCTACAACAGATACTACAAGCACACT
hTLR-5	TGCTAGGACAACGAGGATCATG	GTTGCAGAAACGATAAAAGGCTATT
hTLR-6	CACAGAACAGCATTCCCAACA	AAAGAGCCCACGTTTGCTTTT
hTLR-7	GATTGAAACCTGACCAATTTGCT	AAATACGACATCGCCAATCTAAGG
hTLR-8	GGTCCTCTGCTCAGGGTGTCT	TGAATCCAGAAAACAACCACATG
hTLR-9	GGCCCCCGGCTTCTT	CAGGAGTGGTCCACTGTCTTGA
hTLR-10	GCATTCCCACCAGGTATCATAAAC	AAAGCCCACATTTACGCCTATC
hPPARg	ATTGACCCAGAAAGCGATTCC	TCTTCCATTACGGAGAGATCCAC
hIL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
hMCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
hIL-1β	CAGCTACGAATCTCCGACCAC	GGCAGGGAACCAGCATCTTC
hAdiponectin	ATCGGTGAAACCGGAGTACC	GCATGTTGGGGATAGTAACGTAA
FABP-4	AGCACCATAACCTTAGATGGGG	CGTGGAAGTGACGCCTTTCA

3

The effect of the interleukin-1 cytokine family members IL-1F6 and IL-1F8 on adipocyte differentiation

Edwin J.P. van Asseldonk, R Stienstra, Tim B. Koenen, Lambertus J.H. van Tits, Leo A.B. Joosten, Cees J. Tack, Mihai G. Netea

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Abstract

Obesity is characterized by chronic low-grade inflammation originating from expanding adipose tissue. In the present study we examined the adipogenic expression levels of IL-1F6 and IL-1F8, both members of the IL-1 family of cytokines, and their effects on adipose tissue gene expression. Whereas IL-1F6 is primarily present in adipose tissue-resident macrophages and induced by inflammation, IL-1F8 is absent. IL-1F6, but not IL-1F8, reduces adipocyte differentiation, as shown by a significant decrease in PPARγ gene expression. Finally, both IL-1F6 and IL-1F8 are able to induce inflammatory gene expression in mature adipocytes. In conclusion, we demonstrate for the first time that IL-1F6 is present in adipose tissue and that IL-1F6 and IL-1F8 are involved in the regulation of adipose tissue gene expression. Importantly, IL-1F6 inhibits PPARγ expression which may lead to reduced adipocyte differentiation suggesting metabolic effects of this cytokine.

Keywords
Interleukin-1F6, Interleukin-1F8, adipose tissue, inflammation

Introduction

There is strong evidence that inflammation represents an important link between obesity and the development of insulin resistance. (1) Obesity not only results in macrophage infiltration promoting inflammation, it also shifts the activation state of adipose tissue macrophages (ATM's) to a pro-inflammatory M1 phenotype. (2)

The Interleukin (IL)-1 family, with its most prominent members IL-1 and IL-18, is a central player of obesity-induced inflammation and insulin resistance. (3)

IL-1F6 and IL-1F8 are new members of the IL-1 family of cytokines, and display pro-inflammatory effects reminiscent of IL-1 activity. (4) We hypothesized that IL-1F6 and IL-1F8 are expressed in adipose tissue and influence adipocyte differentiation.

Materials and methods

Human adipose tissue

Human subcutaneous abdominal adipose tissue, obtained from patients undergoing reconstructive surgery, was cultured in DMEM (GIBCO) and treated with lipopolysaccharide (LPS) at 10 ng/ml (Escherichia coli serotype 055:B5) (Sigma). Additionally, adipose tissue was separated in adipocytes and stromal vascular cells using collagenase (Sigma, St Louis, MO) digestion.

Animal study

Male C57/Bl6 mice were injected intraperitoneally with LPS (1 mg/kg bodyweight). 4 Hours after injection, animals were sacrificed and epididymal white adipose tissue was isolated.

Cell culture

Human SGBS preadipocytes and primary human adipocytes were cultured as described earlier. (5) After full differentiation, LPS (10 ng/ml) was added for 24 hours. During differentiation, primary human adipocytes were treated with IL-1F6 (100 ng/ml), IL-1F8(100 ng/ml) or IL-1β (10 ng/ml). The concentrations chosen for IL-1F6 and IL-18 were based on study of Towne et al. (6), who demonstrated that 100 ng/ml was the lowest concentration that significantly activated the NF-κB pathway.

Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated, differentiated towards M1 or M2 macrophages and cultured as described earlier. (7) Macrophages were stimulated for 24 hours with LPS (10 ng/ml).

RAW 264.7 mouse monocytic cells were cultured in DMEM containing 10% FCS and treated with LPS during 24 hours.

RT-PCR

Total RNA was reverse transcribed followed by quantitative PCR analysis using the Biorad 7300 Real-Time PCR system (Applied Biosystems) with β2M (human) or 36B4 (mouse) as housekeeping genes.

Statistical analysis

Differences were analyzed by the Student's *t* test. $P \leq 0.05$ was considered to denote significance.

All applicable institutional and governmental regulations concerning the ethical use of human volunteers and animal models were followed during this research.

Results

Expression of IL-1F6 and IL-1F8 in adipose tissue and in cell cultures

IL-1F6 was expressed in adipose tissue, while IL-1F8 was not detected. Although IL-1F6 was primarily expressed in the stromal vascular fraction, expression was also detectable in adipocytes. (*figure 1*).

We used LPS as a model of inflammation to study the effects on IL-1F6 and IL-1F8 gene expression. (8) qPCR analysis revealed that IL-1F6 and IL-1β, but not IL-1F8 were significantly up regulated in white adipose tissue of LPS-treated animals (*figure 1*).

Next, we tested the responses of adipocytes and macrophages to LPS treatment in vitro. IL-1F6 expression was significantly higher in human SGBS adipocytes (*figure 1*), as well as in RAW 264.7 cells (*table 1*) after LPS treatment.

Inasmuch adipose tissue contains differentially polarized macrophages, we compared IL-1F6 and IL-1F8 expression in M1 and M2 macrophages. IL-1F6 was predominantly produced by M2 macrophages (*figure 1*). LPS induced IL-1F6 expression significantly in M2 macrophages (*figure 1*). IL-1F8 was produced mainly by M1 macrophages (*figure 1*) and LPS did not significantly induce IL-1F8 expression (*figure 1*).

IL-1β-like effects of IL-1F6 on adipocyte differentiation

Cytokines of the IL-1 family can affect differentiation and function of adipocytes. (9) We determined the effect of IL-1F6 and IL-1F8 on expression of four adipogenic genes. Expression of PPARgamma was significantly down regulated after treatment with IL-1F6 (*table 1*). FABP4, Adiponectin and glucose transporter 4 (GLUT4) expression showed a non-significant trend to down regulation (*table 1*). IL-1F8 had no significant

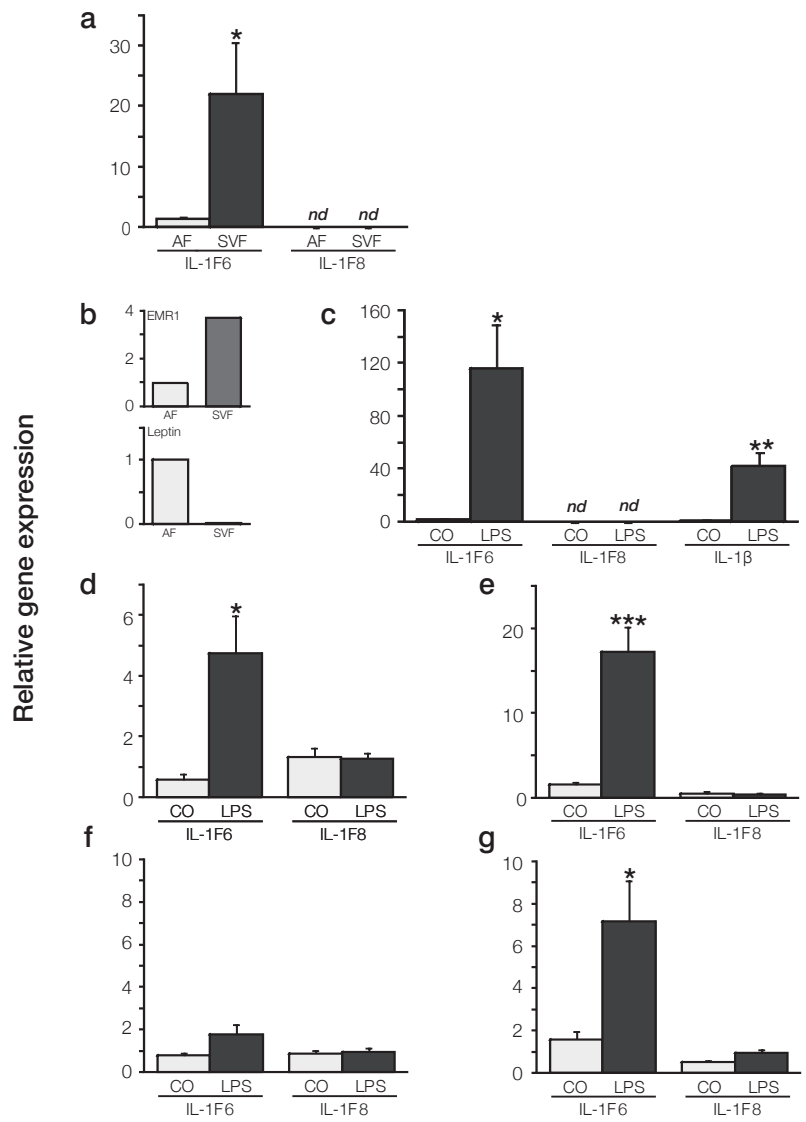

Figure 1 IL-1F6 and IL-1F8 expression.

Expression of IL-1F6 and IL-1F8 in SVF and adipocyte fraction (AF) of human adipose tissue (**a**). Adequacy of fraction separation is shown by EMR1 (macrophage marker) and leptin (adipocyte marker) expression (**b**). Expression of IL-1F6, IL-1F8 and IL-1β with and without LPS stimulation in mice adipose tissue (**c**), SGBS adipocytes (**d**), RAW 264.7 macrophages (**e**), M1 macrophages (**f**), M2 macrophages (**g**). Data are presented as mean ± standard error of the mean for 3 or more experiments combined. *p<0.05, **p<0.01, ***p<0.005.

effect on expression of these adipogenic genes (*table 1*). Finally, IL-1F6 and IL-1F8 were both able to significantly induce the expression of IL-6 and IL-8 in human adipocytes (*table 1*).

Table 1 Expression of FABP4, PPARgamma, Adiponectin, GLUT4, IL-6 and IL-8 in primary human adipocytes treated with or without IL-1F6, IL-1F8 or IL-1β.

	Control	IL-1F6 100ng/ml	IL-1F8 100 ng/ml	IL-1β 10 ng/ml
FABP4	1.00	0.82±0.06	0.88±0.14	0.00±0.00***
PPARgamma	1.00	0.62±0.08*	1.17±0.22	0.02±0.01***
Adiponectin	1.00	0.71±0.07	0.89±0.27	0.01±0.00***
GLUT4	1.00	0.6±0.12	0.75±0.41	0.27±0.20
IL-6	1.00	1.75±0.11***	1.05±0.05	16.94±0.99***
IL-8	1.00	9.00±2.70*	3.69±0.82*	1065±40***

*p<0.05, **p<0.01, ***p<0.005.

Discussion

In this study, we report for the first time that the novel member of the interleukin-1 cytokine family IL-1F6, but not IL-1F8, is present in adipose tissue and may be involved in the inflammatory link between obesity and insulin resistance. Adipose tissue-resident macrophages appear to be the primary source of IL-1F6.

We speculated that IL-1F6 and IL-1F8 may induce an IL-1β like response in adipose tissue, (9) since IL-1F6 and IL-1F8 activate equal signaling pathways as IL-1β, using partly similar cellular receptors. (6) Our results demonstrate that IL-1F6 can inhibit adipocyte differentiation, although less effective than IL-1β. Perhaps higher concentrations of IL-1F6 would lead to more pronounced results since the used dose of IL-1F6 is the lowest dose that induces a significant increase of NF-κB and IL-6 and IL-8 gene expression levels were much higher after stimulation with IL-1β 10 ng/ml compared to IL-1F6 100 ng/ml. (6) Nevertheless, both IL-1-F6 and IL-1F8 are able to induce responses in adipocytes albeit the effectiveness is lower as compared to IL-1β.

We were surprised by the finding that IL-1F6, which seems to have pro-inflammatory effects on adipocyte differentiation, was primarily produced by M2 macrophages, but new insights show that ATMs with an M2 phenotype can also produce high amounts of pro-inflammatory cytokines, in fact even more than M1 macrophages. (10)

The finding that IL-1F6 is present in adipose tissue and inhibits PPARgamma expression and probably adipocyte differentiation adds an additional mediator to the list of cytokines that are involved in the link between obesity and adipose tissue inflammation and probably insulin resistance. These findings may also be relevant to PPARγ-agonists, which are used in the treatment of diabetes mellitus. It is tempting to hypothesize that blockade of IL-1F6 and IL-1F8 may have therapeutic potential in obesity or insulin resistance by relieving a blockade of PPARgamma expression. It has been shown earlier in vivo that IL-1F5 antagonizes the effects of IL-1F6, (11) and the pathophysiologic consequences of this finding for the adipose tissue remain te be investigated.

Further research is necessary to determine whether the effect of IL-1F6 on adipocyte differentiation is a direct or indirect effect.

Acknowledgments

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Treatment with anakinra improves disposition index but not insulin sensitivity in nondiabetic subjects with the metabolic syndrome: a randomized, double-blind, placebo-controlled study

Edwin J.P. van Asseldonk, Rinke Stienstra, Tim, B. Koenen, Leo A.B. Joosten, Mihai G. Netea, Cess J. Tack

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Abstract

Context: Obesity induces chronic low-grade inflammation in adipose tissue that may promote the development of insulin resistance. IL-1 is one of the key inflammatory factors.
Objective: To demonstrate an improvement of total body insulin sensitivity by blocking IL-1. Secondary objectives were adipose tissue-specific changes and effects on inflammation.
Design: randomized double blind cross-over study.
Setting: ambulatory care
Participants: Non-diabetic, obese subjects with the metabolic syndrome.
Intervention: 150 mg of anakinra subcutaneously once daily or matching placebo for 4 weeks.
Main outcome measure: insulin sensitivity as measured by euglycemic hyperinsulinemic clamp.
Results: A total of 13 out of 19 subjects completed the study. Although anakinra treatment resulted in a significantly lower level of inflammation illustrated by a reduction in circulating CRP concentrations and leukocyte numbers, insulin sensitivity was not significantly different after anakinra treatment ($2.8 \times 10^{-2} \pm 0.5 \times 10^{-2}$) compared to placebo treatment ($2.4 \times 10^{-2} \pm 0.3 \times 10^{-2}$ μmol kg^{-1} min^{-1} pmol^{-1}, $P = 0.15$).
Adipose tissue examination, performed to analyse local effects of IL-1Ra, showed an increased influx of macrophages after treatment with anakinra most likely due to an injection site reaction caused by the vehicle (0.28 ± 0.05 *vs.* 0.11 ± 0.01 macrophages per adipocyte, $P = 0.005$). The differences in individual subject insulin sensitivity after anakinra as compared to placebo between subjects was negatively correlated with macrophage infiltration into the adipose tissue ($r^2 = 0.46$, $P = 0.01$).
The disposition index increased significantly after anakinra treatment ($P = 0.04$) reflecting an improvement in Beta-cell function. However, other measurements of Beta-cell function did not show any differences.
Conclusions: Our results suggest that anakinra does not improve insulin sensitivity in obese, insulin-resistant, non-diabetic subjects.

Clinical trial registration number
NCT00928876, Clinicaltrials.gov.

Keywords
inflammation, insulin sensitivity, interleukin-1 receptor antagonist

Introduction

The global prevalence of obesity is rapidly rising and paralleled by an increasing incidence of insulin resistance and type 2 diabetes mellitus. Although the pathophysiological basis underlying obesity-associated insulin resistance has not been fully unravelled, many reports suggest that chronic low grade inflammation originating from adipose tissue represents an important link. (1)

Storage of excess energy results in expansion of adipose tissue mass and an increase in adipocyte size. Concurrent with this expansion, oxygen delivery to the adipocyte is decreased and may eventually lead to microhypoxia and adipocyte death. Together with the enhanced pro-inflammatory profile of enlarged adipocytes, (2) this results in the recruitment of macrophages that further amplify the inflammatory status of the adipose tissue. (3) Pro-inflammatory cytokines as tumor necrosis factor α and IL-6 that are released in increased amounts by adipocytes and activated macrophages of obese individuals can induce insulin resistance. (4, 5)

In addition to its role in immunity, the pro-inflammatory cytokine Interleukin (IL)-1 (both IL-1a and IL-1²) is also involved in the development of diabetes mellitus. By inducing beta-cell destruction, IL-1 contributes to the pathogenesis of type 1 diabetes mellitus. (6) Furthermore, a positive association between IL-1 levels and the presence of obesity has been found. (7) IL-1β induces insulin resistance in cultured human adipocytes (8) and primary rat hepatocytes (9) and IL-1β knockout mice are more insulin sensitive as their wildtype littermates. (10, 11) Based on these observations, one might expect that blockade of IL-1β would improve insulin resistance in humans.

IL-1β exerts its effects by binding to the type 1 IL-1receptor (IL-1R). (12) This results in recruitment of IL-1 receptor accessory protein (IL-1RAcP), necessary for signal transduction. (13) Activation of IL-1R leads to fever, synthesis of acute phase proteins, leukocytosis, thrombocytosis and anemia. (14) IL-1Receptor antagonist (IL-1RA) inhibits IL-1β action by competing with IL-1β for binding to the IL-1R. Binding of IL-1RA to the IL-1R does not result in recruitment of IL-1RAcP and thereby blocks intracellular signalling. (13)

Blocking the IL-1 effects in patients with type 2 diabetes mellitus by anakinra, a recombinant human IL-1RA, has been shown to improve glycemic control. Whether IL-1 blockade has a beneficial effect on insulin sensitivity in non-diabetic subjects characterized by insulin resistance remains to be determined.

In the present study, we investigated whether blocking IL-1 by recombinant IL-1Ra in humans for four weeks can improve insulin sensitivity in non-diabetic, obese, insulin resistant subjects with the metabolic syndrome.

Materials and methods

Study design

Overweight subjects with the metabolic syndrome, yet no type 2 diabetes mellitus, were included in this randomized, double blind, placebo-controlled, two period, crossover study. Subjects were recruited by means of advertisements in a local newspaper and from the outpatient clinic of the Radboud University Nijmegen Medical Centre. We compared recombinant human IL-1Ra (150 mg of anakinra) with matching placebo. Study medication was self-administrated subcutaneously once daily for four weeks. Between both treatment periods there was a four week wash-out. Because prior studies suggest that the conventional dose of 100 mg anakinra once daily is not sufficient in patients with high body weight, (15) the dose was increased to 150 mg daily. This dose has been applied in patients with rheumatoid arthritis. (16) Anakinra was purchased from the regular manufacturer (Biovitrum), the study was performed completely independent from any company. The Pharmacy department of Radboud University Nijmegen Medical Centre prepared all study medication and was responsible for the blinding and randomization procedure. Subjects were randomized in blocks of four, in a 1:1 allocation ratio.

Participants

The study was approved by the institutional ethics review board and assessed by an independent monitor. Written informed consent was obtained from all subjects before randomization.

Inclusion criteria were: age 18 years or older, body mass index > 30 kg/m^2 and 3 or more characteristics of the metabolic syndrome according to the definition of the third report of the National Cholesterol Education Program's Adult Treatment Panel (NCEP-ATP-III). (17) Exclusion criteria were the known presence of diabetes mellitus, a fasting plasma glucose 7.0 mmol/L or higher, HbA1c 6.2 % or higher, immunodeficiency or immunosuppressive treatment, current use of anti-inflammatory medication (100 mg or less of aspirin per day was allowed), signs of current infection or treatment with antibiotics, a history of recurrent infections or tuberculosis, pregnancy or breast feeding, a serum alanine aminotransferase or aspartate aminotransferase level of more than three times the upper limit of the normal range, a serum creatinine level higher than 130 μmol/l, neutropenia (a leukocyte count of less than 2x10^9/l), or the presence of any other medical condition that might interfere with the current study protocol and inability to give informed consent

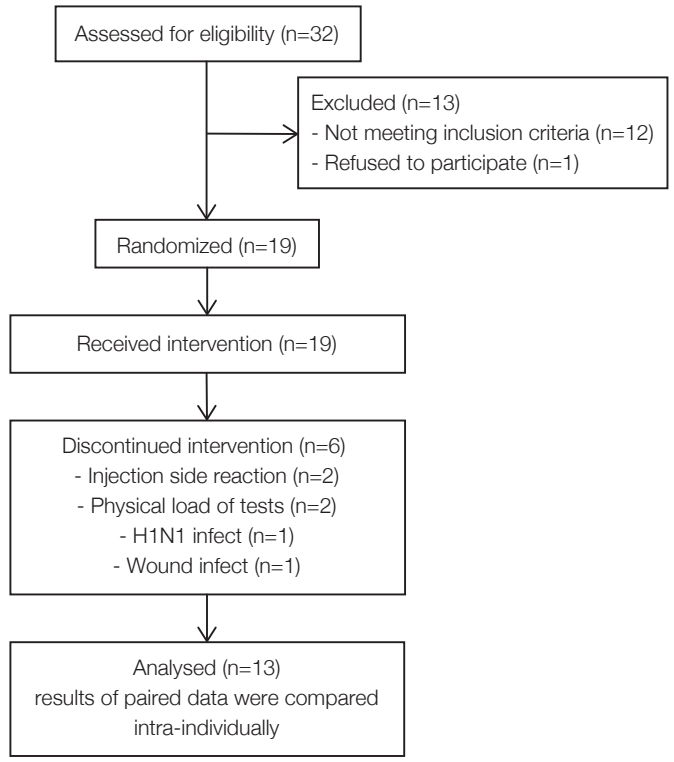

Figure 1 Design, enrollment, withdrawal and completion of the study.

Study procedures

At the end of both treatment periods we performed a euglycemic hyperinsulinemic clamp (insulin infusion rate 360 pmol/m^2/min, (18) and on a separate day a 75 gram oral glucose tolerance test, with measurement of glucose, insulin and pro-insulin at baseline and at 30, 60, 90 and 120 minutes.

A subcutaneous fat biopsy was obtained and analyzed for adipose tissue morphology and macrophage influx. All study procedures were performed after an overnight fast.

Subcutaneous adipose tissue biopsy

A subcutaneous adipose tissue biopsy was taken from the abdominal region just before the clamp, about 30 minutes after a subcutaneous injection with placebo or anakinra to analyze local effects of the active substance (recombinant IL-1Ra) of

anakinra. Biopsies were taken under local anesthesia (2% Lidocaine hcl), from an area that was not affected by a local injection site reaction caused by the vehicle of anakinra, about 10 cm lateral of the umbilicus using a Hepafix Luer lock syringe (Braun, Melsungen, Germany) and a 2.10 x 80 mm Braun medical Sterican needle (Braun). The adipose tissue was washed using a 0.9% normal saline solution. The adipose tissue was snap frozen and stored at -80 ^{o}C until further analysis or fixated in 4% paraformaldehyde for embedding in paraffin.

Immunohistochemistry

An antibody against cluster of differentiation (CD68) (AbD Serotec, Kidlington, UK) was used to stain macrophages. Immunohistochemistry was performed as described earlier. (19) Macrophage influx was quantified by counting the number of adipocytes and macrophages in four representative fields of adipose tissue. A mean number of 609 ± 24 adipocytes were counted per subject for each treatment period.

RNA isolation and real-time PCR analysis

Total RNA was extracted from sc adipose tissue using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA concentration was determined using the NanoDrop (NanoDrop Technologies, Wilmington, MA). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed using Power-SYBR Green master mix and the 7300 real-time PCR system (Applied Biosystem, Warrington, UK). Expression of genes was normalized to *Beta-2 microglobulin (β2M)* gene expression levels. Used primer sequences were: β2M: ATGAGTATGCCTGCCGTGTG (sense), CCAAATGCGGCATCTTCAAAC (antisense), peroxisome proliferator-activated receptor γ *(PPARγ)*: ATTGACCCAGAAAGCGATTCC (sense), TCTTCCATTACGGAGAGATCCAC (antisense), *Fatty acid binding protein 4* (*FABP4)*: AGCACCATAACCTTAGATGGGG (sense), CGTGGAAGTGACGCCTTTCA (antisense), *Adiponectin*: ATCGGTGAAACCGGAGTACC (sense), GCATGTTGGGGATAGTAACGTAA (antisense)

Biochemical analysis

Glucose concentrations were measured using the oxidation method (Glucose Analyser 2; Beckman Instruments Inc., Fullerton, CA). Plasma concentrations of total adiponectin, IL-6 , IL-1Ra, high sensitivity C-reactive protein (hsCRP) and Pro-insulin were determined using ELISAs(R&D Systems, Minneapolis, MN; Pro-insulin: Millipore, Billerica, MA, hsCRP in-house developed ELISA by the University Hospital Maastricht). (20) Insulin levels were determined by a radioimmunoassay. For C-peptide determination, chemiluminescence was used. Inter- and intra-assay CV's were respectively: adiponectin 2.7 % and 1.3 %; IL6 3.3 % and 5.0 %; IL1-ra 5.0 % and 8.0 %; hsCRP 4.7 % and 3.8 %; insulin 9.7 % and 4.7 %; pro-insulin 4.7 % and 5.6 %.

Measurements of other parameters were performed in the clinical laboratory unit of the Radboud University Nijmegen Medical Centre.

Statistical analysis

We considered a 20% improvement of the primary endpoint, insulin sensitivity, as determined by euglycemic hyperinsulinemic clamp, as clinically relevant. Assuming a test-to-test correlation coefficient of 0.5 (paired tests) and a mean glucose infusion rate of 30 ± 7 μmol/kg/min, would require a total of 11 subjects to detect a 20 % change in insulin sensitivity with a power of 80 % at a significance level of 0.05. Dropouts were replaced.

Differences were analyzed by the Student's *t* test. Correlation analysis was performed by regression analysis. Two-tailed $P \leq 0.05$ was considered to denote significance. Data are presented as mean ± SEM.

Results

Nineteen of the 32 initially screened subjects underwent randomization and were enrolled in the study between June and October of 2009. A total of 13 subjects, 9 females and 4 males, completed the study. Nine of the 13 subjects who completed the study used anakinra in the second treatment period. Two of the 6 drop-outs used anakinra in the second treatment period. Two subjects were discontinued from the study due to an infection, one had an H1N1 infection (during anakinra treatment) and one had a wound infection (during placebo treatment in the first treatment period). Two subjects withdrew due to the physical load of the tests and 2 subjects withdrew due to injection site reactions (*figure 1*). Dropouts were significantly younger ($P = 0.008$), had a higher BMI ($P = 0.02$) and had a larger waist circumference ($P = 0.05$) in comparison to study subjects that completed the study. All other parameters were similar between study subjects that withdrew or completed the study.

The trial was terminated after the last subject completed all studies in February 2010. Baseline characteristics of all subjects that completed the study are shown in Table 1. BMI did not change during the study.

Twelve out of 13 subjects experienced local injection site reactions (*figure 2B*) of varying severity. No other adverse events were observed during the study. Sufficiency of the intervention was assessed by measuring serum and adipose tissue levels of IL-1Ra. The serum levels of IL-1Ra 30 minutes after the last injection of anakinra or placebo were 735 ± 86 after anakinra and 0.7 ± 0.1μg/L after placebo, $P < 0.001$. Levels of IL-1Ra in adipose tissue lysates were 29.2 ± 7.5 after anakinra and 1.8 ± 0.4 ng/L after placebo, corrected for protein levels, $P < 0.05$. There were no carry-over effects detected. Insulin sensitivity of subjects treated with placebo in the first

Table 1 Baseline characteristics of the participants.

Characteristic	
Age	53.6 ± 2.8
Sex (male:female)	4 : 9
Body mass index (kg/m^2)	33.2 ± 1.3
Waist circumference (cm)	110.5 ± 3.2
Blood pressure (mmHg)	
Systolic	143.8 ± 2.8
Diastolic	83.3 ± 2.5
Fasting glucose (mmol/L)	5.49 ± 0.15
HbA1c (%)	5.58 ± 0.09
CRP (mg/L)	5.62 ± 0.08
Hemoglobin (mmol/L)	8.48 ± 0.18
Leucocytes (x10^9/L)	6.49 ± 0.54
Cholesterol (mmol/L)	
Total	5.68 ± 0.32
HDL	1.02 ± 0.07
LDL	3.63 ± 0.30
Triglycerides	2.85 ± 0.50
Interleukin-1Ra (μg/L)	0.76 ± 0.14
HOMA-IR (mmol/pmol)	5.67 ± 0.64
Medication with anti-inflammatory effects (number of study subjects)	
simvastatin 20 mg/day	3
atorvastatin 40 mg/day/ lisinopril 20 mg/day/ aspirin 100 mg/day	1

treatment period was not significantly different from insulin sensitivity of subjects receiving placebo in the second treatment period (2.4 x 10^{-2} ± 0.3 x 10^{-2} *vs.* 2.7 x 10^{-2} ± 0.5 x 10^{-2} μmol kg^{-1} min^{-1} pmol^{-1}, $P = 0.59$).

Systemic inflammation

Inasmuch IL-1Ra has been attributed with anti-inflammatory effects, (21) we measured several markers indicative of systemic inflammation. HsCRP levels (anakinra 3.57 ± 1.40 *vs.* placebo 5.37 ± 1.78 mg/L, $P = 0.05$) (*figure 3A*) and leukocyte counts (anakinra 5.32 ± 0.41 *vs.* placebo 6.39 ± 0.57 x 10^9/L, $P = 0.002$) (*figure 3B*) were

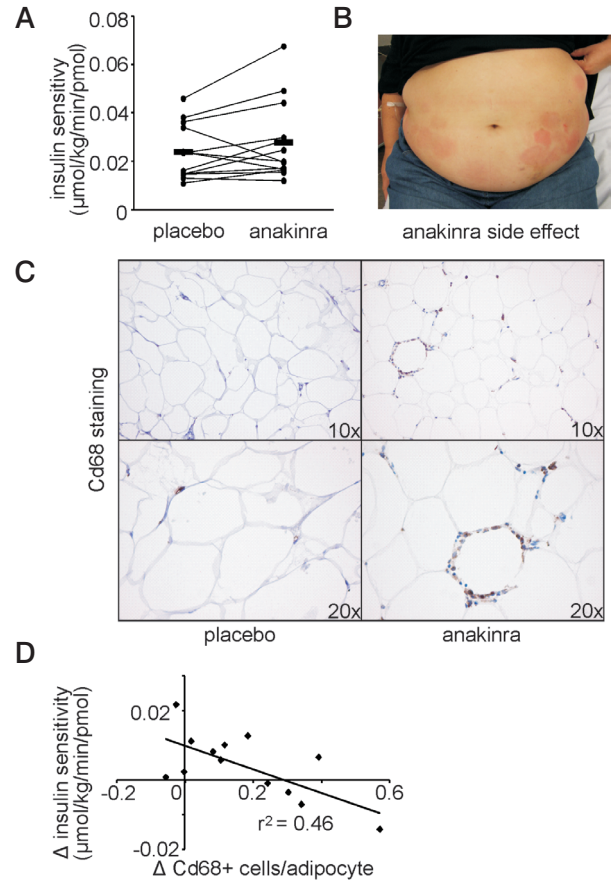

Figure 2 Effects of anakinra on insulin sensitivity and adipose tissue.

Insulin sensitivity index after treatment with placebo and anakinra are represented for each individual participant, black dashes represent mean values (**A**). Injection site reaction as experienced by one of the participants of the study (**B**). CD68 staining images of subcutaneous adipose tissue of an individual participant after placebo and after anakinra treatment (**C**). Correlation of the change in insulin sensitivity index after anakinra treatment compared to placebo with the severity of macrophage infiltration into the adipose tissue after anakinra treatment compared to placebo (**D**).

significantly reduced in study subjects after anakinra treatment. Circulating IL-6 levels were below the detection limit of our assay (3 ng/l) for almost all patients in both treatments.

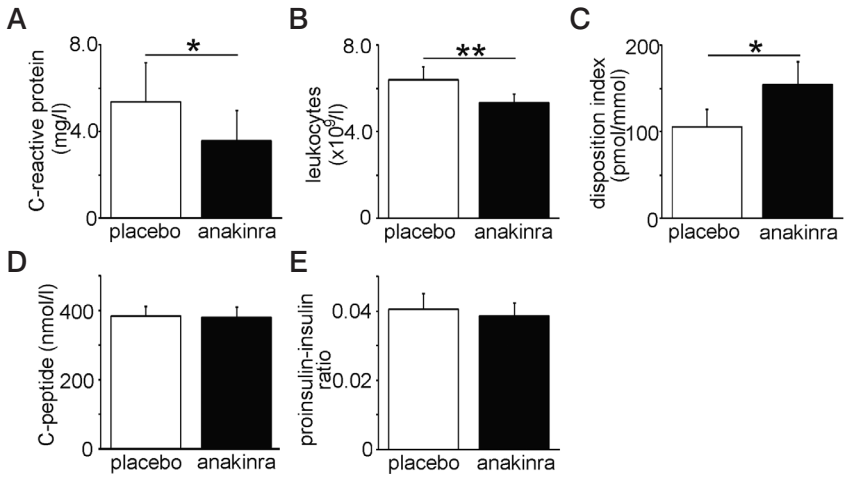

Figure 3 Effect of anakinra on inflammation and beta cell function.

HsCRP (**A**) and leukocyte count (**B**) after treatment with placebo and anakinra. Disposition index, calculated as the change in circulating insulin concentration during the first 30 minutes of the oral glucose tolerance test, divided by change in circulating glucose concentration during the first 30 minutes of the oral glucose tolerance test (**C**). Area under the curve for C-peptide during the oral glucose tolerance test (**D**). Fasting pro-insulin-insulin ratio (**E**). Data are represented as mean ± SEM *P < 0.05 **P < 0.01.

Insulin sensitivity and Beta-cell function

Although participants were clearly insulin resistant as illustrated by an insulin sensitivity index of 2.4 x 10^{-2} ± 0.3 x 10^{-2} μmol kg^{-1} min^{-1} $pmol^{-1}$ insulin after placebo treatment (normal range in lean subjects 4.0-7.0 x 10^{-2} μmol kg^{-1} min^{-1} $pmol^{-1}$ insulin, (22) treatment with anakinra for 4 weeks did not result in an improvement in insulin sensitivity (anakinra 2.8 x 10^{-2} ± 0.5 x 10^{-2} *vs.* placebo 2.4 x 10^{-2} ± 0.3 x 10^{-2} μmol kg^{-1} min^{-1} $pmol^{-1}$ insulin, $P = 0.15$) (*figure 2A*). Average HbA1c (anakinra 5.57 ± 0.11 *vs.* placebo 5.62 ± 0.08 %, $P = 0.35$) and fasting glucose (anakinra 5.34 ± 0.13 *vs.* placebo 5.46 ± 0.15 mmol/L, $P = 0.17$) levels were unchanged after anakinra treatment. Inasmuch no stable isotopes were used during the clamp, we were unable to determine effects of anakinra treatment on liver insulin sensitivity levels. Alternatively, liver insulin sensitivity was estimated by using data obtained during the OGTT. (23) Liver insulin sensitivity was not different between placebo versus anakinra treatment ($P = 0.66$).

The disposition index during the OGTT (change in insulin (0-30 min)/change in glucose (0-30 min)) improved after anakinra treatment, $P = 0.04$ (*figure* 3C) suggestive

for a somewhat enhanced beta-cell function. However, other parameters indicative for beta-cell function including stimulated C-peptide levels (AUC during the OGTT) (*figure 3D*) and fasting pro-insulin-insulin ratio (*figure 3E*) did not differ significantly between placebo and anakinra treatment. Raw OGTT-data are represented in figure 4.

Based on OGTT-criteria, 5 of the study participants had impaired glucose tolerance. In this group no statistically significant differences in insulin sensitivity or Beta-cell function were found, maybe based on the small sample size.

No significant differences in triglycerides, HDL-cholesterol, LDL-cholesterol and total cholesterol were present between placebo and anakinra treatment.

Adipose tissue

The systemic low-grade inflammatory reaction characteristic for obesity is mainly inflicted by inflammation in the adipose tissue. (3) The inhibition of IL-1 by IL-1Ra (active substance of anakinra) is expected to reduce this inflammatory reaction. However the vehicle (citrate), which is used to lower the pH of the solution preventing aggregation of anakinra, is known to induce an injection site reaction during the first weeks of treatment in a majority of patients. (16) We performed fat biopsies to study the local effects of anakinra treatment in abdominal subcutaneous adipose tissue.

Anakinra treatment led to a high number of infiltrating cells into the subcutaneous adipose tissue of various study subjects. Immunohistochemical visualization of macrophages using CD68-staining revealed an increased number of macrophages in the adipose tissue after anakinra treatment (0.28 ± 0.05 *vs.* 0.11 ± 0.01 macrophages per adipocyte, $P = 0.005$) (*figure 2C*). In addition, IL-6 concentrations in adipose tissue tended to be higher after anakinra treatment (anakinra 26.9 ± 12.4 μg/g adipose tissue *vs.* placebo 5.7 ± 1.2 μg/g adipose tissue, $P = 0.07$) suggestive

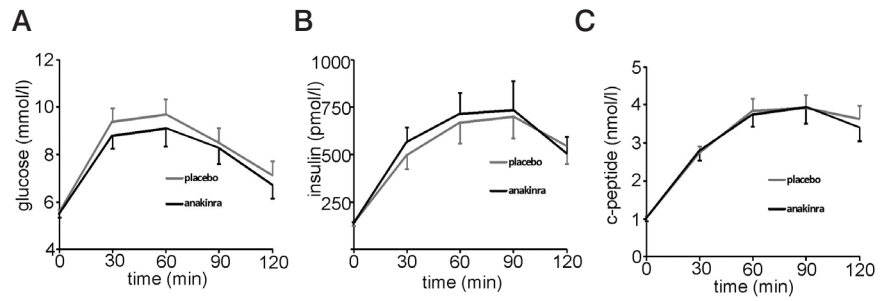

Figure 4 Oral glucose tolerance test data.

Glucose (**A**), Insulin (**B**) and C-peptide (**C**) excursion during the oral glucose tolerance test. Data are represented as mean ± SEM.

of an enhancement in inflammatory properties of the adipose tissue. Moreover, expression levels of the adipogenic genes *PPARγ*, *FABP4* and *Adiponectin* determined by quantitative PCR analysis in subcutaneous adipose tissue revealed significantly decreased levels of *PPARγ* ($P = 0.03$) and *FABP4* ($P = 0.04$), whereas *Adiponectin* tended to decrease ($P = 0.07$) after anakinra treatment.

The analysis of the macrophage influx into the adipose tissue after anakinra treatment revealed a wide distribution in severity among study subjects ranging from no influx up to a 12-fold increase in macrophage content of the adipose tissue. The severity of the inflammatory reaction in the abdominal subcutaneous adipose tissue, reflected by the number of infiltrating macrophages after anakinra treatment, was negatively correlated with the difference in individual subject insulin sensitivity level after anakinra treatment ($R^2 = 0.46$, $P = 0.01$) (*figure 2D*).

Subgroup analysis using an increase in macrophage influx of over 300% after anakinra treatment as a cut-off point, unveiled that in study subjects with low levels of infiltrating macrophages, insulin sensitivity after anakinra treatment improved significantly by 39 ± 9 % (95 % CI = 18 – 61 %, $P = 0.003$) compared to placebo, while in subjects with more than a threefold increase in infiltrating macrophages, insulin sensitivity decreased by -14 ± 12 % (95 % CI = -47 – 19 %, $P = 0.31$). The average insulin sensitivity of subjects with low macrophage influx was 53% (95% CI = 20 – 86 %, $P = 0.006$) better as compared with subjects with high macrophage influx. Levels of circulating hsCRP were comparable in both groups (< 300 % increase in infiltrating macrophages 2.49 ± 1.35 *vs.* > 300 % increase in infiltrating macrophages 5.29 ± 0.71 mg/L, $P = 0.35$). Adipose tissue IL-6 appeared lower in subjects with no or mild infiltration of macrophages compared to the subjects with extensive infiltration although the differences did not attain statistical significance (13.7 ± 5.9 *vs.* 53.2 ± 34.4 μg/g adipose tissue, $P = 0.14$).

Discussion

The present study did not find an improvement in insulin sensitivity after four weeks of treatment with the IL-1 receptor antagonist anakinra in an insulin resistant population. The selected study population enabled us to analyze the effects of an anti-inflammatory intervention on insulin sensitivity without the potential interference of anti-diabetic medication and the confounding effects of changing glycemic control.

In accordance with earlier findings in a population of type 2 diabetic patients, (15) were we unable to detect an improvement in insulin sensitivity after anakinra treatment in our study population. However, an animal study using XOMA 052 (anti-IL-1β antibody) has reported a positive effect both on insulin sensitivity as well as on Beta-cell function. (10) Another animal study using 1400.24.17 (anti-IL-1β antibody) showed reduced HbA1c after 18 weeks of treatment, without consistent improvements in insulin sensitivity and glucose tolerance. (24) An important difference between the animal studies versus the human trial of Larsen *et al.* and our trial is the use of specific anti-IL-1β antibodies in the animal trials. The human trials do use IL-1RA, which has effects on both IL-1α and IL-1β.

There are a number of explanations for the lack of improvement in insulin sensitivity in our study.

First, circulating levels of IL-1Ra in study participants may vary considerably. It has been suggested that IL-1Ra levels at baseline determine the effects of anakinra treatment, with those with the lowest level showing the highest response. (25) Nevertheless, in our study we were unable to find any association between IL-1Ra levels at baseline and changes in insulin sensitivity after anakinra treatment, which largely excludes the possibility that insufficient production of IL-1Ra contributes to the development of insulin resistance in our study subjects. Noticeably, also in our population baseline IL-1Ra levels were quite variable.

Secondly, the dose of IL-1Ra used in our study or infiltration into target tissues may have been insufficient. However, the dosage used in our study was 1.5 times higher than normally used and concentrations of IL-1Ra in the circulation were 1000-fold higher compared to placebo treatment, accompanied by a 16-fold increase in IL-1Ra levels in adipose tissue after anakinra treatment. Moreover, the reduction in inflammatory markers including hsCRP and leucocytes suggest that treatment with anakinra did provoke a systemic anti-inflammatory effect and was effective.

Thirdly, as adipose tissue is a causative factor for systemic low grade inflammation during the development of obesity (3) and is partly responsible for determining systemic insulin sensitivity, we evaluated local effects of anakinra in adipose tissue biopsies. Based on earlier results of in vitro studies that demonstrated lower insulin sensitivity and reduced levels of the adipogenic markers *PPARγ* and *FABP4* after IL-1β treatment of adipocytes, we hypothesized that inhibiting IL-1-signaling by anakinra treatment would enhance adipogenic gene expression levels. (8, 26) In contrast, anakinra treatment led to reduced gene expression levels of *PPARγ* and *FABP4* and increased levels of macrophage infiltration. This finding is surprising since treatment with anakinra reduced systemic levels of inflammation. The influx of macrophages, probably accompanied by other immune cells, is most likely not the direct effect of IL-1Ra but might be the effect of one of the additives present in the commercial preparations of anakinra. Injection site reaction is a well-known side effect of anakinra during the first weeks of treatment and is known to result in the infiltration of

macrophages. (27) Earlier studies have demonstrated the importance of infiltrating macrophages in the subcutaneous adipose tissue in provoking systemic insulin resistance. (28) The lack of positive findings of our study on insulin sensitivity may thus (partly) be explained by negative effects of local adipose tissue inflammatory injection site reactions on insulin sensitivity. In line with this explanation, the severity of the inflammatory reaction caused by the injection site reaction in subcutaneous adipose tissue turned out to be inversely correlated with the improvement in systemic insulin sensitivity after anakinra treatment.

The relation between IL-1, inflammation in adipose tissue and its effect on systemic insulin sensitivity by affecting systemic inflammation is well known. However in this study local inflammation in the abdominal subcutaneous adipose tissue is correlated with systemic insulin sensitivity, without influencing systemic inflammation. This can partly be explained by a direct negative effect of local inflammation on the local adipose tissue. Alternatively, the inflamed adipose tissue might have led to an altered adipokine secretion profile that negatively interfered with insulin sensitivity in peripheral organs including liver and muscle.

It should be stressed that the adipose tissue biopsy was not taken out of an area with macroscopic inflammation. However, because injection sites were varied over time (as advised) over different abdominal areas, the diffuse inflammation observed may reflect tissue responses from earlier injections.

Finally, the hypothesis may be wrong and IL-1 may have no relationship with insulin resistance associated with obesity and the metabolic syndrome. Given the many supporting observations suggestive for a role of IL-1 in the inflammatory response linked to obesity, (29) this explanation seems less likely.

In respect to beta-cell function we found a hint towards improved insulin secretion capacity as reflected by the disposition index, although the other evaluated parameters for beta-cell function did not improve significantly. However disposition index is known to be an independent predictor for the development of type 2 diabetes mellitus. (30) It should also be stressed that the participants in this study were selected for insulin resistance (obesity/metabolic syndrome) but not insulin secretion deficits. All subjects had glucose levels in the non-diabetic range.

This is the first study that has specifically studied the effects of IL-1 blockade on insulin sensitivity in human non-diabetic subjects. A similar study has been performed in subjects with type 2 diabetes. (15) This latter study measured both insulin sensitivity and insulin secretion before and after 13 weeks of treatment. Glycemic control improved after 4 and 13 weeks of treatment and this was attributed to an improvement in beta-cell function while insulin sensitivity appeared to be unchanged. As such, our findings are in line with the results of the study performed by Larsen *et al.* The fact that we did not find a clear improvement in insulin secretion may be explained by the differences in study population. The subjects included in our study were non-diabetic and had normal levels of HbA1c and fasting glucose at baseline and had no indication of defective beta-cell function, whereas Larsen *et al.* included patients with type 2 diabetes mellitus.

The dose and duration of treatment were different in our study as compared to Larsen *et al.* (100 mg sc once daily for 13 weeks by Larsen *et al. vs.* 150 mg sc once daily for 4 weeks in our study). The larger dose and shorter treatment period used in our study may have aggravated the injection site reaction since adverse effects are dose dependently and vanish after prolonged treatment. (16) Theoretically, a prolonged treatment of subjects in our study could have resulted in waning of side effects – since injection site reactions of anakinra do disappear after 4 to 6 weeks of treatment – and may have revealed an improvement in insulin sensitivity. However, a prolonged treatment period would substantially extend the duration of the (cross-over) trial, increasing the risk for carry-over effects, while biological effects of IL-1 blockade were expected to be present within weeks.

In summary, the results of this study do not support the concept that blockade of IL-1 by anakinra treatment improves insulin sensitivity. It cannot be fully excluded, though, that potentially positive effects on insulin sensitivity are (partly) offset by local inflammatory reactions at the injection site of anakinra. Whether treatment with other modalities that block IL-1 but do not produce local injection site effects potentially has a beneficial effect on insulin sensitivity remains to be determined. The present study results support a beneficial effect of blocking IL-1 on beta-cell function.

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5

One week treatment with the IL-1 receptor antagonist anakinra leads to a sustained improvement in insulin sensitivity in insulin resistant patients with type 1 diabetes mellitus

Edwin J.P. van Asseldonk, Pleun C. van Poppel, Duby B. Ballak, Rinke Stienstra, Mihai G. Netea, Cees J. Tack

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Abstract

Inflammation associated with obesity is involved in the development of insulin resistance. We hypothesized that anti-inflammatory treatment with the Interleukin-1 receptor antagonist anakinra would improve insulin sensitivity.

In an *o*pen label proof-of-concept study, we included overweight patients diagnosed with type 1 diabetes with an HbA1c level over 7.5 %. Selecting insulin resistant patients with longstanding type 1 diabetes allowed us to study effects of anakinra on insulin sensitivity. Patients were treated with 100 mg anakinra daily for one week. Insulin sensitivity, insulin need and blood glucose profiles were measured before, after one week and after four weeks of follow-up.

Fourteen patients completed the study. One week of anakinra treatment led to an improvement of insulin sensitivity, an effect that was sustained for four weeks. Similarly, glucose profiles, HbA1c levels and insulin needs improved.

In conclusion, one week of treatment with anakinra improves insulin sensitivity in patients with type 1 diabetes.

Keywords

Type 1 diabetes, anakinra, Interleukin 1, Insulin Sensitivity, Inflammation, Adipose tissue

Introduction

The upsurge in the prevalence of obesity has fuelled a rapid increase in the prevalence of type 2 diabetes mellitus worldwide. This is explained by the fact that insulin resistance associated with obesity leads to a more rapid exhaustion of beta-cell function. There is strong evidence that low-grade inflammation is involved in the pathogenesis of obesity-induced insulin resistance.

One of the key mechanisms involved in obesity-associated inflammation, at least at the level of the adipose tissue, is activation of the inflammasome and release of the potent cytokine IL-1β.(1-4) High levels of glucose are known to promote a pro-inflammatory state.(5-8) Since it is a well-known fact that chronically elevated glucose levels induce insulin resistance, that is reversible upon installation of normoglycemia, an enhanced inflammatory state may underlie this phenomenon.(9, 10)

Low grade, systemic inflammation also appears to play a pivotal role in the pathophysiology of beta-cell toxicity and progressive beta-cell failure associated with diabetes.(11) Similarly, chronically elevated glucose levels further deteriorate beta-cell function.(12-14)

These lines of evidence suggest that interventions aimed at reducing inflammation in general and IL-1-activation in particular may both improve insulin sensitivity and insulin secretion. Indeed, blocking IL-1 in patients with type 2 diabetes mellitus using recombinant human IL-1 receptor antagonist (IL-1Ra) anakinra has been shown to improve glycemic control.(15) A study using an anti-IL-1β antibody to block IL-1β action reported an improvement in glycemic control in animals, paralleled by an enhancement in beta-cell function and insulin sensitivity.(16) However, other intervention trials using specific anti-IL-1b antibodies were not consistently reporting positive effects on glycemic control.(17-19) In obese, insulin-resistant individuals, we found evidence for an improved beta-cell function after anakinra treatment, but could not confirm an effect on insulin sensitivity after blockade of IL-1 signaling.

It should be noted that any change in glycemic control in patients with type 2 diabetes will affect glucose toxicity and hence both insulin resistance and beta-cell function. In this regard, any change in insulin sensitivity leading to lower glucose levels could result in improved beta-cell function.

We hypothesized that the involvement of IL-1β in obesity-associated insulin resistance may be mostly pronounced under conditions of chronic hyperglycemia. To assess this hypothesis, we studied the effect of the interleukin-1 receptor antagonist anakinra on insulin sensitivity in subjects with longstanding type 1 diabetes that displayed a “type 2 phenotype”. Given the fact that beta-cell function is absent in these patients, this experimental approach has the advantage that any change in insulin resistance will immediately translate to lower glucose levels and lower insulin need.

Our results provide in vivo evidence in humans for an insulin sensitizing effect of IL-1 blockade in the presence of hyperglycemia, inflammation and insulin resistance.

Table 1 Baseline characteristics of the participants.

Characteristic	
Age (years)	47 ± 9
Sex (male:female)	(9:5)
Diabetes duration (years)	30.9 ± 13.1
Body mass index (kg/m^2)	31.7 ± 4.6
Blood pressure (mmHg)	133 ± 13 / 76 ± 9
Fasting glucose (mmol/L)	9.45 ± 2.81
HbA1c (% (mmol/mol))	8.61 ± 0.70 (70)
Insulin regimen (basal-bolus:continuous subcutaneous insulin infusion)	6:8
Insulin dose (U/day)	78.6 ± 32.9
C-reactive protein (mg/L)	1.97 ± 2.85
Leucocytes (x10^9/L)	5.96 ± 2.20
Neutrophiles (x10^9/L)	3.76 ± 1.72
Interleukin-1Ra (ng/L)	359 ± 221
Cholesterol (mmol/)	
Total	4.37 ± 0.65
HDL	1.25 ± 0.37
LDL	2.69 ± 0.53
Triglycerides	0.96 ± 0.10
Diabetes complications	
Macrovascular	1
Nephropathy	2
Polyneuropathy	7
Retinopathy	9
Medication with potential anti-inflammatory effects (number of patients)	
Acetylsalicylic acid 38 mg/day	1
Acetylsalicylic acid 100 mg/day	2
Perindopril 8 mg/day	1
Enalapril 20 mg/day	2
Enalapril 40mg/day	1
Fosinopril 20 mg/day	1
Irbesartan 300 mg/day	1
Atorvastatin 40 mg/day	1
Rosuvastatin 5 mg/day	1
Simvastatin 20 mg/day	1
Simvastatin 40 mg/day	1

Baseline characteristics are reported as mean ± standard deviation.

Materials and methods

2.1 Study design

Overweight patients with type 1 diabetes mellitus were included in this open-label proof-of-concept study. Patients were recruited from the outpatient clinic of the Radboud University Nijmegen Medical Centre. We treated patients with anakinra at 100 mg subcutaneous for eight consecutive days. Study medication was self-administered once daily. Anakinra was purchased from the regular manufacturer (Swedish Orphan Biovitrum). Notably, the study was performed completely independent from any company.

2.2 Participants

The institutional ethics review board approved the study. Written informed consent was obtained from all subjects.

Inclusion criteria were type 1 diabetes mellitus for more than five years, body mass index higher than 25 kg/m^2, insulin requirement > 0.5 U/kg bodyweight per day, HbA1c > 7.5% (58 mmol/mol), and age between 18 and 65 years.

Exclusion criteria were immunodeficiency or immunosuppressive treatment, current use of anti-inflammatory medication (100 mg or less of aspirin per day was allowed), signs of current infection or treatment with antibiotics, a history of recurrent infections or tuberculosis, pregnancy or breast feeding, a serum alanine aminotransferase or aspartate aminotransferase level of more than three times the upper limit of the normal range, a serum creatinine level higher than 130 μmol/l, neutropenia (a leukocyte count of less than 2x10^9/l), or the presence of any other medical condition that might interfere with the current study protocol. Participants unable to give informed consent were also excluded.

2.3 Study procedures

Immediately before the first injection, directly after the last injection and four weeks after the last injection of anakinra we performed an euglycemic hyperinsulinemic clamp (insulin infusion rate 360 pmol/m^2/min)(20) and obtained a subcutaneous fat biopsy. All study procedures were performed after an overnight fast. Patients were requested to record glucose levels and insulin use as usual, but as a minimum the two days before start of study medication, during the intervention, and four weeks after the last treatment with anakinra.

2.4 Subcutaneous adipose tissue biopsy

A subcutaneous adipose tissue biopsy was taken from the abdominal region just before the clamp, about 30 minutes after a subcutaneous injection with anakinra. Biopsies were taken under local anesthesia (2% Lidocaine hcl), from an area about

10 cm lateral of the umbilicus using a Hepafix Luer lock syringe (Braun, Melsungen, Germany) and a 2·10 x 80 mm Braun medical Sterican needle (Braun). Adipose tissue was washed using a 0.9% normal saline solution. The adipose tissue was snap frozen and stored at -80 ^{o}C until further analysis, or it was fixed in 4 % paraformaldehyde for embedding in paraffin.

2.5 Immunohistochemistry

An antibody against CD68 (AbD Serotec, Kidlington, UK) was used to stain macrophages. Immunohistochemistry was performed as described earlier.(21) Macrophage influx was quantified by counting the number of adipocytes and macrophages in ten representative fields of adipose tissue. A mean number of 205 ± 6 adipocytes were counted per subject for each biopsy.

2.6 Biochemical analysis

Glucose concentrations were measured using the oxidation method (Biosen C-Line; EKF-diagnostic GmbH, Barleden, Germany). Concentrations of IL-1Ra, IL-8, serum amyloid A and adiponectin were determined using ELISAs (R&D Systems, Minneapolis, MN and serum amyloid A: Hycult Biotech, Plymouth Meeting, PA). HsCRP was measured by in-house developed ELISA by University Hospital Maastricht (22). IL18 was determined using magpix (Bio-Rad laboratories Inc, Hercules, Ca). Insulin levels were determined by a radioimmunoassay. Inter- and intra-assay CV's were respectively: IL1-ra 5.0% and 8.0%; IL-8 7.4 % and 5.6%; hsCRP 4.7% and 3.8%, adiponectin 4.3% and 2.4%; IL-18 7% and 5%; insulin 9.7% and 4.7%.
Measurements of other parameters were performed in the clinical laboratory unit of the Radboud University Nijmegen Medical Centre.

2.7 Statistical analysis

We considered a 15 % improvement of the primary endpoint, insulin sensitivity, as determined by euglycemic hyperinsulinemic clamp, as clinically relevant. Assuming a test-to-test correlation coefficient of 0.5 (paired tests) and a mean glucose infusion rate of 45.7 ± 8.5 μmol/kg/min(10), would require a total of 12 subjects to detect a 15 % change in insulin sensitivity with a power of 80 % at a significance level of 0.05. Taking into account the technical difficulties that are frequently experienced when performing euglycemic clamps, we included 14 subjects in our study. Dropouts were replaced. Differences were analysed by the Student's *t* test. Correlation analysis was performed by regression analysis. Two-tailed $P \leq 0.05$ was considered to denote significance. Data are presented as mean ± SEM.

Results

3.1 Study population

A total of 14 patients were recruited from the outpatient clinic and enrolled in the trial. All patients completed the trial. Clinical characteristics of the study population are provided in Table 1. Patients had longstanding type 1 diabetes mellitus, were mostly obese, had suboptimal diabetes control, and relatively high insulin needs. Fasting C-peptide levels were below the detection limit of the assay (0.03 nmol/l) in all patients except for one patient who had a C-peptide level of 0.04 nmol/l, both before and after the intervention. Medication, other than insulin, was continued during the study.

No serious adverse events were observed during the trial. One patient reported painful injections of the study medication. Adipose tissue IL-1Ra concentrations were significantly higher directly after the intervention, and had returned to baseline values 4 weeks after the intervention (baseline 1339 ± 347 μg/l *vs.* directly after 3980 ± 416 μg/l, $P < 0.01$ and *vs.* four weeks after the intervention 1942 ± 476 μg/l, $P = 0.54$) (*figure 1*).

3.2 Systemic inflammation

As the insulin sensitizing action of anakinra is mediated by a reduction of the inflammatory status, several inflammation markers were measured. Leukocyte and neutrophil counts were significantly reduced during the intervention, an effect that was sustained for up to 4 weeks (*figure 2a and b*). No differences were found in systemic levels of C-reactive protein (*figure 2c*), IL-1α, IL-6, IL-8, IL-18, TNFα and serum amyloid A. IL-1β was not detectable systemically. Serum adiponectin levels did not change during anakinra treatment.

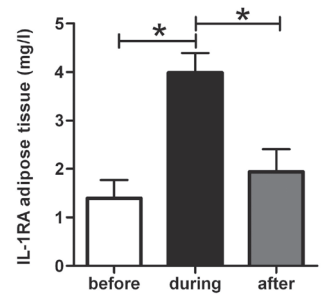

Figure 1 Interleukin-1 Receptor Antagonist concentrations.

IL-1Ra levels in adipose tissue before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment.

3.3 Fat biopsies

To quantify local inflammation induced by anakinra injection (a well-known side effect of one of the solvents) fat biopsies were taken. Macrophage numbers in fat tissue were counted using an immuno-histochemical approach. The number of macrophages did not significantly change during or after the intervention. (*figure 2d and e*).

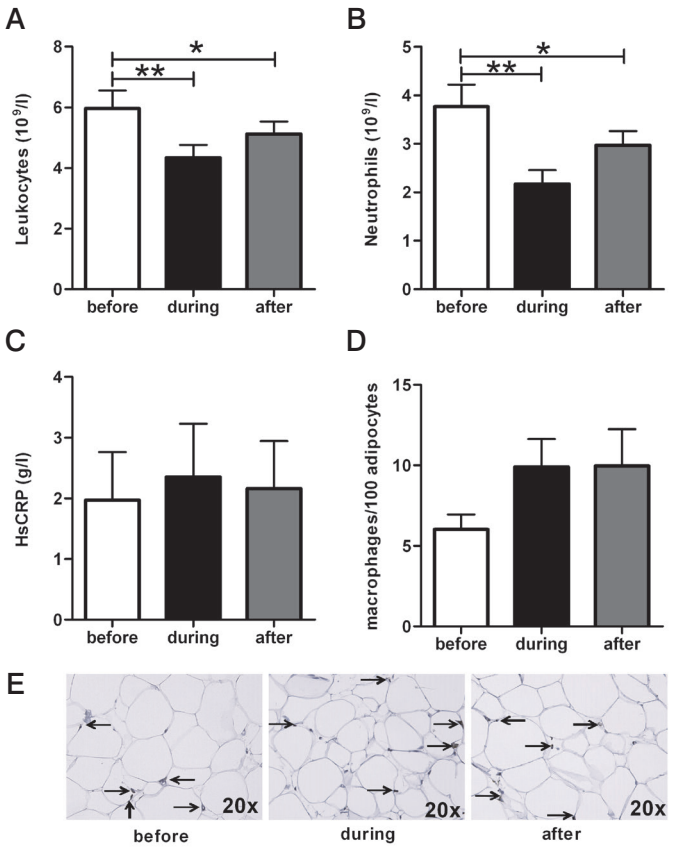

Figure 2 The effects of anakinra on inflammation.

Serum levels of leukocytes (**A**), neutrophils (**B**) and HsCRP (**C**), before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment. Number of macrophages per adipocyte in subcutaneous adipose tissue biopsy before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment (**D** and **E**).

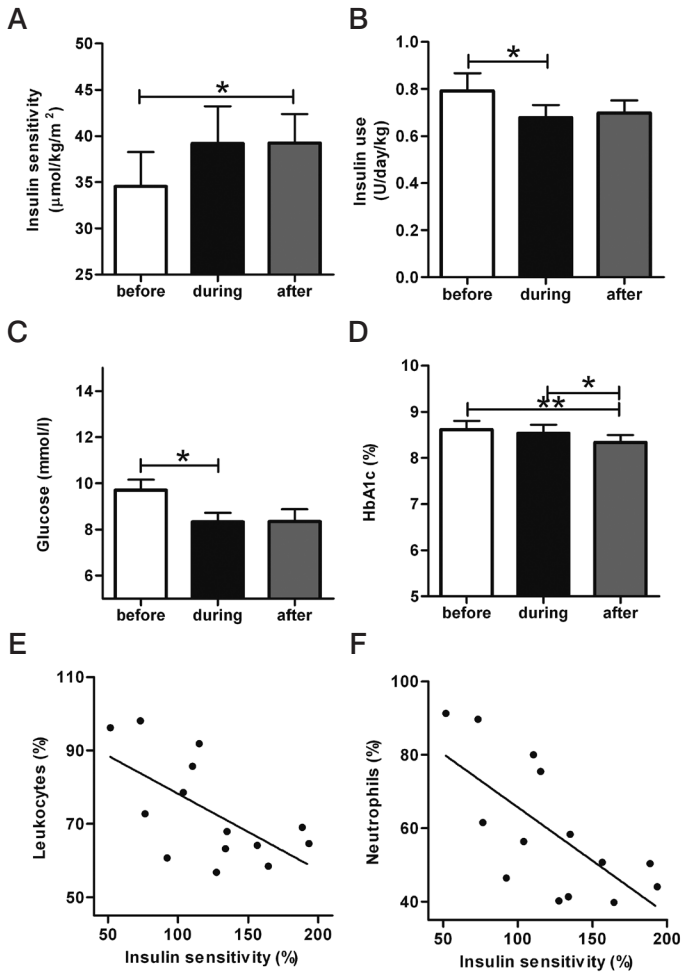

Figure 3 The effects of anakinra on insulin sensitivity and glycemic control.

Insulin sensitivity as determined by euglycemic hyperinsulinemic clamp before, during and after anakinra treatment (A). HbA1c before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment (B). Glucose levels during the day as reported by patients before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment (C). Amount of insulin per kilogram bodyweight used by patients before, during (directly after the last injection) and after (4 weeks after the last injection) anakinra treatment (D). Change in systemic leukocyte counts (E) and systemic neutrophil counts (F) correlated with change in insulin sensitivity, black lines represent linear regression analysis. Data are reported as mean ± standard error of the mean, * $P < 0.05$, ** $P < 0.01$.

3.4 Insulin sensitivity and glycemic control

At baseline, subjects were moderately insulin resistant. Insulin sensitivity as determined by the glucose infusion rate (euglycemic hyperinsulinemic clamp), was 34.5 ± 3.7 μmol kg^{-1} min^{-1} (reference value for lean individuals is 53.9 μmol kg^{-1} min^{-1}). (23) During treatment with anakinra, insulin sensitivity numerically increased to 39.2 ± 4.0 μmol kg^{-1} min^{-1}, although this change did not attain statistical significance, but the improvement was persistent and significant four weeks after the last administration of anakinra (insulin sensitivity 39.2 ± 3.2 μmol kg^{-1} min^{-1}, $P = 0.02$) (figure 3a). HbA1c levels four weeks after termination of anakinra treatment were in accordance with the results of the clamps and dropped from baseline 8.61 ± 0.19% (71 mmol/mol) to 8.34 ± 0.16% (68 mmol/mol), $P < 0.01$) (*figure 3b*).

Improved glycemic control was also shown by a decrease in mean glucose levels from 9.71 ± 0.45 mmol/l at baseline, to 8.33 ± 0.40 mmol/l during anakinra usage ($P < 0{\cdot}01$) and to 8.34 ± 0.55 mmol/l after termination of the anakinra intervention ($P = 0.04$) (*figure 3c*). The improved glycemic control was achieved with a lower requirement of insulin. At baseline, average insulin usage was 78.6 ± 8.8 U/day, during anakinra treatment the patients used 66.9 ± 6.5 U/day and remained lower four weeks after the last injection of anakinra (69.7 ± 7.2 U/day) (*figure 3d*).

The improvement in insulin sensitivity appeared to be associated with the anti-inflammatory efficacy of anakinra treatment since the enhancement in insulin sensitivity levels correlated with the reduction in leukocyte ($R^2 = 0.39$, $P = 0.02$) (*figure 3e*) and neutrophil counts ($R^2 = 0.47$, $P < 0.01$) (*figure 3f*) directly after the treatment.

3.5 Cholesterol

Lipid levels (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) were not significantly different after the intervention compared to baseline.

Discussion

The main finding of our study is that a one week treatment with the IL-1 receptor anakinra decreases systemic inflammation and improves insulin sensitivity in insulin resistant patients with type 1 diabetes. This change in insulin sensitivity is mirrored by better glucose control and decreased insulin needs.

The demonstration of improved glycemic control with anakinra treatment is in line with an earlier study demonstrating a beneficial effect of anakinra in patients with type 2 diabetes after 13 weeks of treatment.(15) However, there are also important differences between the two studies. Larsen *et al.* studied patients with type 2 diabetes and found a beneficial effect on beta-cell function, explaining the improved glucose control. However, this direct effect on the beta-cell cannot be the explanation in our study performed in patients with type 1 diabetes, as by definition these patients have no residual beta-cell function.

Earlier trials using anakinra in humans did not detect improvements in insulin sensitivity. In the trial of Larsen et al. that used euglycemic-hyperinsulinemic clamp studies performed in a subgroup of patients and an analysis of the insulin-sensitivity index by the homeostasis model assessment (HOMA), did not reveal any difference after anakinra as compared to placebo treatment.(15) The same holds true for our previous trial in which glucose disposal rates were not significantly different between anakinra and placebo treatment.(24) The lack of effect of anakinra on insulin sensitivity in these earlier studies may partially be explained by injection site reactions that are a well-known adverse event affecting a large percentage of treated patients. The reaction is transient and is caused by one of the solvents for solution for injection of anakinra.(25) Indeed, in our previous trial that included four weeks of treatment with anakinra, an inverse correlation between the number of macrophages in the subcutaneous adipose tissue caused by injection site reactions and the improvement in systemic insulin sensitivity levels was observed.(24) A recent study in mice showed a direct link between adipose tissue macrophages and bone marrow myeloid progenitor proliferation. Treatment with anakinra resulted in decreased leukocyte production, fewer adipose tissue macrophages and improved glucose tolerance.(26)

Because of the design of the current trial, in which the dose of anakinra was reduced by 33% and the treatment period was reduced by 75% in comparison to our earlier trial, results were less likely to be influenced by a pro-inflammatory reaction in the subcutaneous adipose tissue. Indeed, patients in this trial did not suffer from an enhancement in local inflammatory status, as the number of infiltrating macrophages in the subcutaneous abdominal adipose tissue did not significantly change after the intervention period. Furthermore no injection site reactions were recorded. Although we are not informed about the effect of anakinra on the inflammatory status of the visceral adipose tissue it appears reasonable to expect that an increase in inflammatory status of the subcutaneous abdominal adipose tissue blurs a potential effect of long term (more than one week) anakinra treatment on insulin sensitivity, as 50% of patients treated with anakinra reported such an adverse event.(15)

A second reason for the difference in findings is that our previous trial consisted of non-diabetic subjects, and hence hyperglycemia was not involved. Since glucose toxicity induces insulin resistance, anakinra may especially reduce the insulin resistance associated with high glucose levels, which is in agreement with earlier results showing that glucose toxicity is partly mediated by IL-1β.(27-29)

Finally, our study population existed of patients diagnosed with type 1 diabetes mellitus. Endogenous levels of IL-1Ra in patients with type 1 diabetes mellitus are reported to be lower in comparison to patients with type 2 diabetes mellitus.(30) Hence,

treatment with anakinra may be more effective in patients that suffer from low circulating levels as compared to patients with higher basal plasma concentrations of IL-1ra.

Studies testing IL-1β antibodies in humans report positive effects on glycemic control.(17, 19) The clinical trial using Gevokizumab does show improvement in beta-cell function, but no effects on insulin sensitivity measured by the insulin sensitivity index were seen. Canakinumab did not result in improvement in homeostatic model of assessment of insulin resistance. These antibodies are specifically blocking IL-1β, while anakinra blocks both IL-1α and IL-1β. Furthermore, the bioavailability of these antibodies in the adipose tissue is unknown. In the present study we show a clearly increased level of anakinra in adipose tissue during treatment. Besides, the studies testing IL-1β antibodies did not focus on insulin sensitivity. The outcome measures used are less sensitive in recording changes in insulin sensitivity, as compared to the clamp technique used in this study.

The therapeutic efficacy of anakinra treatment observed in our study may largely be the result of a decrease in systemic inflammatory levels, as represented by lower leukocyte and neutrophil counts. This is supported by our observation that a decrease in leukocyte counts correlates with an improvement in insulin sensitivity. One would expect reduced levels in other inflammation parameters like hsCRP, serum amyloid A and IL-6 as well. However this was not seen in the current trial. An explanation could be the already low inflammatory levels before initiation of the trial. Other explanations are the short treatment interval, or an assay failure.

Our study has important potential clinical implications. While the current, once daily treatment with anakinra is inconvenient and might be complicated with injection site reactions, a short period of treatment with IL-1ra, either subcutaneously or i.v., may be effective in patients with extreme insulin resistance due to longstanding high glucose levels. Here, optimal treatment duration and dose need to be determined.

Our study has limitations. First of all, this was an open trial and hence cannot exclude a study effect. The relatively strong and sustained effect and the correlation between improvements in insulin sensitivity and reduction in systemic inflammation argue against this possibility. Another possibility that cannot be excluded is that patients have changed their dietary or exercise habits that may promote improvements in insulin sensitivity. Noticeably, no significant changes in bodyweight were reported in any of the study participants during the intervention. Finally, caution should be taken when extrapolating the validity of our data in patients with type 1 diabetes and obesity to patients with the metabolic syndrome/type 2 diabetes as differences in etiology may exist.

The strength of our study lies in the precisely characterized treatment in a carefully selected group of patients. By testing the effects of anakinra in patients without residual beta-cell function we were able to rule out secondary effects on insulin sensitivity mediated by reduced glucose toxicity.

In conclusion, this study proves that one week of treatment with the IL-1 receptor antagonist anakinra improves insulin sensitivity, an effect that is sustained for at least 4 weeks. The change in insulin sensitivity is mirrored by lower insulin needs and better glucose control. This study strongly suggests involvement of IL-1 in the insulin resistance associated with the combination of obesity and hyperglycemia. Although some lack of consistency in the outcome of previous studies focused on effects of anakinra treatment in patients with diabetes exists, our results argue for beneficial effects of IL-1 inhibition in specific groups of patients. Hence, future studies should focus on the identification of patients that may for example have low endogenous levels of IL-1ra, high circulating levels of pro-inflammatory markers or diabetes for more than five years and therefore particularly benefit from anakinra treatment to improve glycaemic control. Whether this translates to a chronic treatment approach in some subsets of patients (those with pronounced hyperglycemia-associated insulin resistance) remains to be determined.

Acknowledgments

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6

The Interleukin-1 receptor antagonist anakinra improves first-phase insulin secretion and insulinogenic index in subjects with impaired glucose tolerance

Pleun C.M. van Poppel, Edwin J.P. van Asseldonk, Jens J. Holst, Tina Visbøll, Mihai G. Netea, Cees J. Tack

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Abstract

Inflammation at the level of the beta cell seems to be involved in progressive beta cell dysfunction in type 2 diabetes mellitus. We assessed the effect of blocking Interleukin-1 (IL-1) by anakinra (recombinant human IL-1 receptor antagonist) on beta cell function.

Sixteen participants with impaired glucose tolerance were treated with anakinra 150 mg daily for 4 weeks in a double blind, randomized, placebo controlled, cross-over study with a wash-out period of 4 weeks. At the end of each treatment period, oral glucose tolerance tests (OGTT) and hyperglycaemic clamps were performed.

First phase insulin secretion improved after anakinra treatment compared to placebo, 148±20 vs 123±14 mU/l respectively ($p = 0.03$), and the insulinogenic index was higher after anakinra treatment.

These results support the concept of involvement of IL-1β in the (progressive) decrease of insulin secretion capacity associated with type 2 diabetes mellitus.

Clinical trials.gov number NCT01285232

Introduction

Type 2 diabetes mellitus occurs when beta cells fail to appropriately increase insulin secretion in response to insulin resistance. (1) Beta cell function progressively deteriorates over time (2, 3) and inflammation at the level of the beta cell seems to be involved. (4)

The proinflammatory cytokine interleukin-1 (IL-1) appears to be an important mediator in the inflammatory process in the beta cell. In vitro, high glucose levels increase beta cell production of IL-1β followed by functional impairment and apoptosis of beta cells. Beta cells can be rescued from hyperglycaemia-induced apoptosis by addition of the interleukin-1 receptor antagonist (IL-1Ra), a naturally occurring inhibitor of IL-1β. (5)

Blocking the effects of IL-1 in patients with type 2 diabetes mellitus by anakinra, a recombinant human IL-1RA, has been shown to improve glycaemic control through enhanced beta cell function. (6) However, in patients with diabetes, improvement of beta cell function can be either direct or indirect due to improvement in glucose levels, since hyperglycaemia itself impairs both insulin secretion and insulin sensitivity (concept of glucotoxicity). (7)

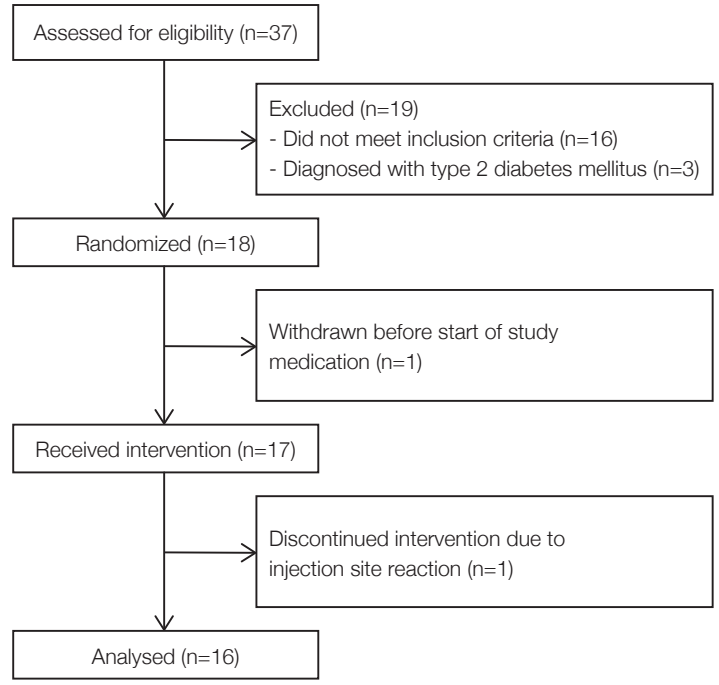

Figure 1 Design, enrollment, withdrawal and completion of the trial.

We hypothesized that treatment with the IL-1Ra anakinra can improve beta cell function subjects with impaired glucose tolerance (IGT).

Materials and methods

Study population

Included were participants with IGT defined as either impaired fasting glucose (IFG) (fasting glucose 5.6-6.9 mmol/l) and/or IGT (2 hour plasma glucose 7.8-11.0 mmol/l) by 75 g oral glucose tolerance test (OGTT) and/ or hemoglobin A_{1c} (HbA_{1c}) levels of 5.7-6.4%. (8) Other inclusion criteria were body mass index > 25 kg/m^2 and age 40-70 years.

Study design

This was a randomized, double-blind, placebo controlled crossover study. Participants were randomly assigned to treatment with anakinra 150 mg subcutaneous (sc) once daily for 4 consecutive weeks or placebo sc once daily for 4 consecutive weeks. After a wash-out period of 4 weeks, participants crossed over to the other treatment arm. At the end of each treatment, a hyperglycaemic clamp and a 75 g OGTT were performed. The Pharmacy Department provided anakinra and matching placebo injections and was responsible for blinding and randomization.

Hyperglycaemic clamp

The hyperglycaemic clamp procedure was performed after an overnight fast. After a 30 minute equilibration period, the clamp was started (t=0) by an intravenous bolus of 0.8 ml glucose 20% solution per kg body weight followed by a variable glucose 20 % infusion in order to maintain a blood glucose level of 10 mmol/l for a total duration of 120 minutes. At 120 minutes, an arginine bolus of 5 grams was administered to measure maximum insulin secretory capacity.

Blood glucose levels were measured in whole blood using the oxidation method (Biosen C-line, EKF diagnostics, GmbH) every 5 minutes to allow precise adjustment of the glucose infusion rate. Blood samples were taken at t= 0, 2.5, 5, 7.5, 10, 20, 40, 60, 80, 100, 120, 122.5, 125, 127.5, 130 and 150 minutes and for assessment of insulin concentration. After 150 minutes of hyperglycaemia, glucose infusion was discontinued.

The OGTT was performed in the morning after an overnight fast, at least 24 hours before or after the hyperglycaemic clamp. After collection of the fasting blood samples, the subject drank 75 g of anhydrous glucose in 250-300 ml water over the course of 5 minutes. Blood samples were collected every 30 minutes for 2 hours after the test load. Insulin measurements were performed (Magpix, Luminex) and samples were assayed for total glucagon-like peptide-1 (GLP-1) and glucagon. (9)

Statistical analysis

Statistical analyses were performed using Graphpad 5.0. Differences in means were tested by paired Student's t-test for normally distributed and Wilcoxon signed rank test for non-normally distributed data. Results were expressed as mean ±SEM unless otherwise indicated. Significance was set at a p-value of less than 0.05.

Table 1 Baseline characteristics (mean±SD).

Characteristic	
Age (years)	54.6±8.5
Sex (male:female)	9:7
Weight (kg)	99.0±24.1
BMI (kg/m^2)	31.7±4.8
Blood pressure systolic (mmHg)	131±15
Blood pressure diastolic (mmHg)	79±9
Fasting glucose (mmol/l)	5.3±0.7
HbA1c (%)	5.8±0.5
Hemoglobin (mmol/l)	8.6±0.8
Leukocytes (x10^9/l)	6.4±1.4
CRP (ug/ml)	3.1±3.7
IL-1Ra (pg/ml)	774±445
Use of antihypertensives	31%
Use of statins	19%

Results

A total of 16 participants (7 females and 9 males) completed the trial. (*figure 1*) Baseline characteristics of the participants were (mean±SD): age 55±9 years, BMI 32±5 kg/m^2, HbA_{1c} 5.8±0.5 % and fasting glucose level 5.3±0.71 mmol/l. (*table 1*)

Leukocyte (anakinra 5.4±0.3 x10^9/l vs placebo 6.2±0.4 x10^9/l, $p = 0.017$) and neutrophil counts (anakinra 2.9±0.3 x10^9/l vs placebo 3.7±0.3 x10^9/l, $p = 0.001$) were significantly reduced after anakinra treatment, as were CRP levels (anakinra 0.9±0.2 ug/ml versus placebo 2.7±0.6 ug/ml, $p < 0.001$).

First phase insulin secretion improved after anakinra treatment compared to placebo (148±20 vs 123±14 mU/l respectively, p=0.03). Second phase insulin secretion, insulin response after arginine stimulus and maximal insulin secretion, did not differ between the two treatment arms (*figure 2*).

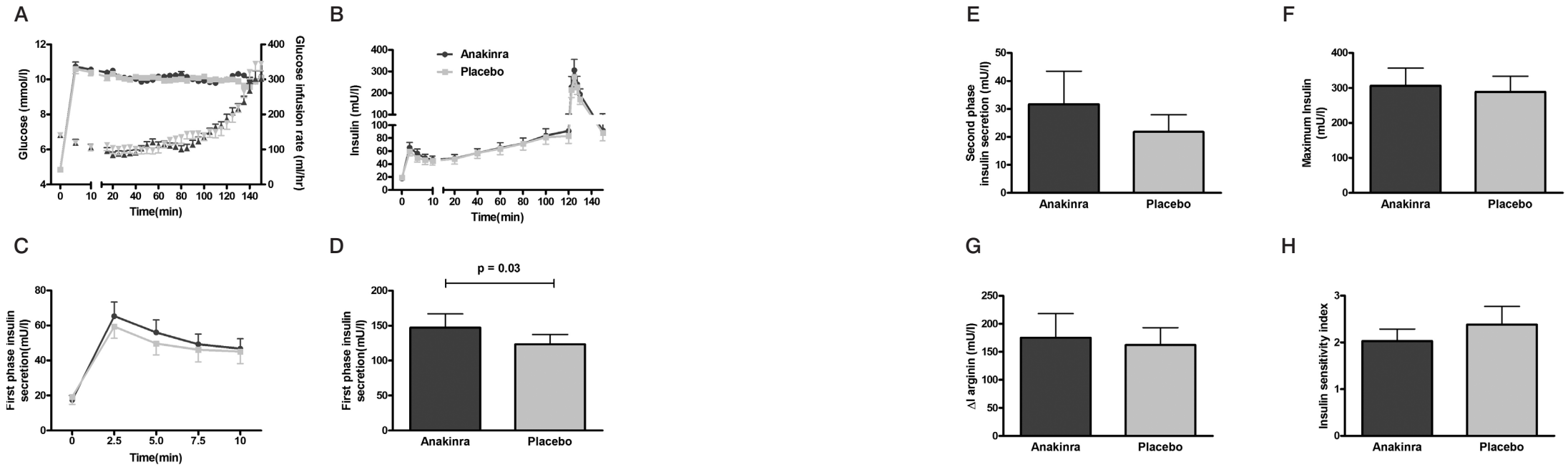

Figure 2 Hyperglycaemic clamp.

Glucose (**A**) levels and glucose infusion rate (GIR) during the hyperglycaemic clamp after treatment with anakinra (dark grey) and placebo treatment (light grey). The glucose levels are depicted as symbols with connecting line and the GIR as symbols only. During the hyperglycaemic clamp, glucose levels were nearly identical in both treatment periods (anakinra 9.98± 0.04 mmol/l vs placebo 10.02±0.04 mmol/l respectively, p = 0.43). The mean CV of the hyperglycaemic clamp was below 4 % in both treatment periods. Insulin (**B**) levels during the hyperglycaemic clamp after treatment with anakinra and placebo. First phase insulin secretion was calculated as the sum of increments of plasma insulin levels from 2.5 to 10 minutes of the clamp. First phase insulin secretion after treatment with anakinra (dark grey) and placebo treatment (light grey) are shown as insulin levels (**C**) and as the mean sum of increments (**D**). Second phase insulin secretion (**E**) was taken as the average increment in plasma insulin levels from 80 to 120 minutes of the clamp. The maximal insulin concentration (**F**) was the single highest post arginine insulin level. The acute insulin response to arginine (ΔI arginine) (**G**) was calculated by subtracting as the insulin level at 120 minutes from the mean insulin level of 122.5 and 125 minutes. Insulin sensitivity was assessed as an insulin sensitivity index (ISI) (**H**), defined as glucose infusion rate (GIR) to maintain hyperglycaemia from 80 to 120 minutes divided by the mean plasma insulin level during the same interval. Data are expressed as mean±SEM.

Figure 2 Continued.

Anakinra had no effect on the insulin sensitivity index (2.0±0.3 mmol/mU anakinra and 2.4±0.43 mmol/mU placebo treatment, $p = 0.29$)

In line with the first phase clamp results, the insulinogenic index derived from the OGTT improved after anakinra compared to placebo treatment (14.5±1.7 vs 11.4±1.1 mU/mmol respectively, $p = 0.036$). (*figure 3*)

Anakinra treatment had no effect on GLP-1 and glucagon levels during OGTT.

Anakinra treatment did not change fasting glucose levels (5.5±0.2 vs 5.5±0.3 mmol/l) or HbA_{1c} levels compared to placebo (5.6±0.1 % vs 5.7±0.1%, $p=0.068$).

Fourteen participants experienced injection site reactions during anakinra treatment. One subject withdrew because of the local reaction. All reactions disappeared after the cessation of anakinra treatment. One participant developed erysipelas and one participant developed a thrombophlebitis of the antecubital vein, which was used for infusion of glucose 20% during the clamp, both after anakinra treatment. There were no other infections or serious adverse events.

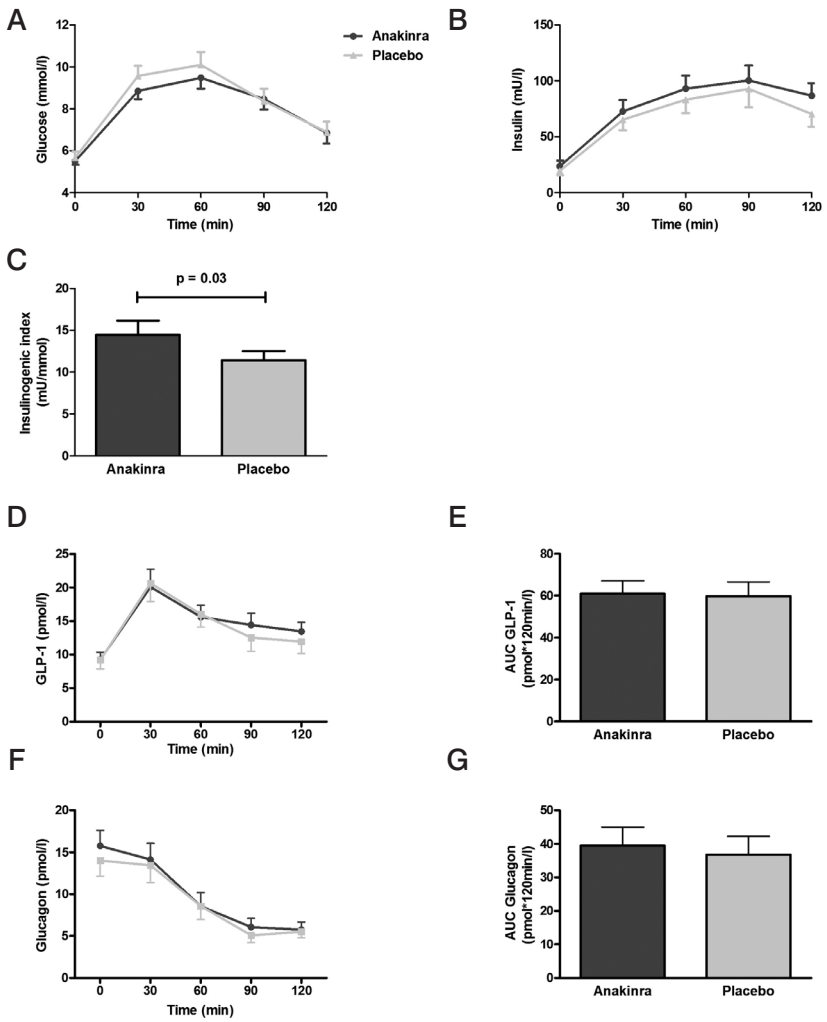

Figure 3 Oral glucose tolerance test.

Glucose (**A**) and insulin (**B**) levels during OGTT for anakinra (dark grey) and placebo (light grey) treatment. The area under the curve (AUC) for glucose during the OGTT did not differ between anakinra and placebo treatment, while the AUC for insulin tended to be higher after anakinra treatment (321±40 vs 286±41mU*120 min/l, p=0.09). The insulinogenic index (**C**) was calculated as the increase in insulin level from 0 to 30 minutes divided by the increase in plasma glucose level from 0 to 30 minutes. The insulinogenic index was higher after treatment with anakinra compared to placebo treatment. The GLP-levels during OGTT (**D**) and area under the curve for GLP-1 (**E**) are shown for both anakinra (dark grey) and placebo treatment (light grey). Glucagon levels during OGTT (**F**) and area under the curve for glucagon (**G**) are also depicted.
Data are expressed as mean±SEM.

Discussion

The main finding of the present study is that four weeks of anakinra treatment improves the insulinogenic index and augments the first phase insulin secretion. These observations suggest that anakinra can improve beta cell function. However, second phase insulin secretion, insulin response after arginine, and the maximal insulin concentration were not influenced by anakinra. The improved first phase insulin response was not mediated by an enhanced incretin response.

These findings are in line with Larsen *et al.* (6) and with our earlier study in non-diabetic subjects with the metabolic syndrome, where anakinra improved the disposition index. (10) A recent study using canakinumab, a human monoclonal anti-IL-1β antibody, found a trend towards improving insulin secretion rates in patients with type 2 diabetes mellitus and an improved insulin secretion in subjects with IGT. (11)

A number of mechanisms may underlie these results. First, anakinra might inhibit intra-islet inflammation and thereby preserve beta cell function. In vitro studies show that high glucose levels induce IL-1β production by beta cells followed by beta cell dysfunction and apoptosis and that these deleterious effects can be prevented by IL-1Ra. Second, anakinra may improve beta cell function by decreasing systemic inflammation. Elevated levels of IL-1β, CRP and IL-6 are predictive of type 2 diabetes mellitus. (12) The reduction in inflammatory markers in our study suggest that anakinra treatment induces a systemic anti-inflammatory effect. Third, an improvement in beta cell function could be explained by a compensatory response to a decline in insulin sensitivity. However, we did not find significant changes in the insulin sensitivity index.

In summary, our study supports the concept of involvement of IL-1β in the decrease of insulin secretion capacity associated with type 2 diabetes mellitus. These findings are relevant since improvement in beta cell function can delay and/or prevent progression to frank diabetes.

Acknowledgements

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7

Summary
Discussion and future perspectives

Summary

The overall objective of this thesis was to gain more insight in the role of inflammation on insulin sensitivity and beta cell function, both key elements in the development of type 2 diabetes. Given the complexity of immune responses and the heterogeneity of the interaction between the immune system and the host's metabolism, research was focused on several individual components of the immune system. This chapter describes the results of the research projects included in the thesis and puts them into perspective.

Although, the link between obesity and type 2 diabetes mellitus is evident, it is unclear which exact mechanisms make some individuals more susceptible for developing type 2 diabetes mellitus than others. Obesity is characterized by a chronic low grade inflammatory state, as reflected by relatively high circulating levels of pro-inflammatory parameters. Furthermore, adipose tissue of insulin resistant individuals is characterised by large adipocytes and relatively high numbers of pro-inflammatory macrophages together with the presence of other pro-inflammatory immune cells from both the adaptive and innate immune system. To gain more insight in the mechanisms causing obesity induced inflammation it is important to decipher the type of receptors that are expressed by the adipose tissue that can function as potential instigators of pro-inflammatory responses.

Possible candidates include the Pathogen recognition receptors (PRRs) that are indispensable for recognizing danger and pathogen associated molecular patterns (DAMPs and PAMPs). When a DAMP or PAMP is recognized by a PRR, an immune response is initiated, leading to clearance of the potentially dangerous pathogen or dysfunctional cell(s).

In **chapter 2** we describe the expression of different TLRs in adipose tissue. The TLR-family is the best-known group of PRRs. TLR-2 and TLR-4 have earlier been described to translate the presence of saturated free fatty acids into pro-inflammatory cytokine production, similar to LPS recognition. First, we observed that many TLRs are expressed in adipose tissue. TLR-3 and TLR-5 were remarkable as their expression was significantly higher in the mature adipocyte fraction as compared to the non-adipocyte fraction. This pattern was found in subcutaneous as well as in visceral adipose tissue. Second TLR-3 expression was induced during differentiation of adipocytes. When adipocytes were treated with poly (I:C), an agonist of TLR-3, the expression of IL-8 and MCP-1 increased, while adiponectin and PPAR-γ expression decreased. All these findings suggested that TLR-3 is functionally active in adipocytes. In vivo TLR-3 knock-out mice that were exposed to a high-fat diet (HFD) displayed

unchanged body weight and insulin sensitivity as compared to wildtype animals. Inflammation in the adipose tissue was similar as compared to HFD-fed wildtype mice. Additionally, TLR-3 expression in adipose tissue was not affected by the HFD intervention of 16 weeks.

In human adipose tissue, TLR-3 expression tended to be negatively correlated with IL-8 expression and positively with FABP-4 expression, but not with body weight, insulin sensitivity or number of crown-like structures. TLR-3 expression in adipose tissue was not correlated with systemic levels of C-reactive protein.

Altogether, these findings show that TLR-3 is abundantly present in adipose tissue and especially in adipocytes. However, absence of TLR-3 in vivo has no, or only very modest effects on metabolic functions including insulin sensitivity after 16 weeks of HFD-feeding.

IL-1β plays an important role in adipose tissue inflammation and insulin sensitivity in general. The IL-1 family member IL-18 has also been shown to have consequences on insulin sensitivity. IL-1F6 and IL-1F8 are two novel IL-1 family members. Both are pro-inflammatory cytokines, involved in inflammatory processes in skin and lung. In **chapter 3** we studied whether IL-1F6 and IL-1F8 were present in adipose tissue and whether exposure to IL-1F6 and IL-1F8 had effects on adipocytes. IL-1F6 is primarily present in the stromal vascular fraction, but was also detected in the mature adipocyte fraction. Treatment with LPS induced IL-1F6 expression. IL-1F8 was not detected in adipose tissue. Treatment of adipocytes with IL-1F6 significantly reduced PPAR-γ expression, while other adipogenic gene expression tended to be lower as well, suggestive for induction of insulin resistance of the cell. IL-1F8 did not affect expression of adipogenic genes. Both, IL-1F6 and IL-1F8 treatment induced IL-6 and IL-8 gene expression in adipocytes suggestive of a pro-inflammatory effect.

These findings show that IL-1F6 is present in adipose tissue, in particular in the stromal vascular fraction, and that its expression is induced by LPS. Adipocyte treatment with IL-1F6 and IL-1F8 has a modest, IL-1-like effect on metabolic and inflammatory gene expression. IL-1F8 is not present in adipose tissue.

As IL-1 is involved in adipose tissue and beta-cell inflammation it may be anticipated that IL-1 blockade may improve insulin sensitivity and insulin secretion. This hypothesis was tested in three studies, described in Chapter 4, 5 and 6. The effects of treatment with the IL-1receptor antagonist (IL-1ra) anakinra were examined in subjects with unique clinical characteristics, which made it possible to focus specifically on insulin sensitivity and insulin secretion and allowed us to exclude most important confounding factors.

The combined results of these three trials clarify the pathophysiological effects of IL-1 mediated inflammation on glycaemic control.

First we describe in **chapter 4** the results of anakinra treatment in obese individuals with the metabolic syndrome, but without diabetes mellitus. This group of subjects was chosen, as these subjects are likely to be insulin resistant, but do not have profound hyperglycemia and do not use glucose lowering medication. In a double-blind, placebo-controlled cross-over study, we treated subjects with anakinra or matching placebo subcutaneously once daily for 4 weeks.

Anakinra significantly decreased systemic inflammation and increased IL-1RA levels, but did not affect insulin sensitivity. Anakinra did significantly improve the disposition index as calculated from the results of the oral glucose tolerance test, compatible with a beneficial effect on beta-cell function. We noted injection site reactions in several individuals. Differences in individual insulin sensitivity after anakinra and placebo treatment appeared to be inversely correlated with macrophage influx in subcutaneous adipose tissue biopsies.

This study suggests that IL-1receptor blockade with anakinra probably has a positive effect on beta-cell function but does not have a clear beneficial effect on insulin resistance in obese subjects with the metabolic syndrome.

Following the results of the first study described in chapter 4 we hypothesized that the potential effect of anakinra treatment on insulin sensitivity may have been blurred by a local injection site reaction. Alternatively, the effects may be less pronounced in the absence of high glucose levels.

Therefore we designed a second study described in **chapter 5**. Here we focussed specifically on insulin sensitivity in patients with diabetes. Overweight individuals with longstanding type 1 diabetes mellitus were treated with anakinra. As these patients have no residual beta-cell function, any improvement in glycemic control has to be the result of improvement in insulin sensitivity. Moreover, this group of patients was chosen to determine whether anakinra might have an effect on hyperglycemia-induced insulin resistance.

The study design included one week of treatment with a lower dose of anakinra. This approach was chosen to reduce the possibly negative effect of injection site reactions on insulin sensitivity. The study showed that treatment with anakinra reduced systemic leukocyte counts. Insulin sensitivity increased after one week treatment with anakinra, which effect persisted in the following four weeks off treatment. Glucose profiles, insulin need and HbA1c levels improved similarly. As this study did not include a control arm, a study / placebo effect on the primary outcomes cannot be excluded, but the fact that the improvement of insulin sensitivity was strongly correlated to the decrease in leukocyte and neutrophil count argues against this explanation.

In **chapter 6** we determined effects of anakinra on beta-cell function in more detail. Here, we recruited subjects with impaired glucose tolerance, but without diabetes mellitus. As these patients do not have diabetes mellitus yet, is it less likely that an effect on beta cell function is secondary to decreased glucose toxicity (which in turn, could be a consequence of improved insulin sensitivity).

In a double-blind, placebo-controlled, cross-over trial, participants were treated with anakinra or matching placebo subcutaneously, once daily for four weeks. Anakinra significantly decreased leukocyte counts and c-reactive protein levels. Treatment increased first phase insulin secretion as measured with a hyperglycemic clamp and improved the insulinogenic index. No effects were observed on insulin sensitivity, GLP-1 and glucagon.

Together, the results of the last three chapters suggest that IL-1 blockade with anakinra has a modest effect on beta-cell function in patients with (pre-) diabetes. The effect on insulin sensitivity seems minimal in subjects with the metabolic syndrome, but appears promising in patients with chronic hyperglycemia-associated insulin resistance. Effects of anakinra on insulin sensitivity might partly be offset by local injection site reactions.

Discussion and future perspectives

The prevalence of diabetes mellitus will increase over the next decades with an estimated global prevalence of 9% in 2014 and 592 million people with diabetes in 2035. (1) Worldwide health care expenditure for diabetes was 548 billion US dollars in 2013, 11 % of the total expenditure. (1) The increasing prevalence of diabetes is the consequence of the obesity epidemic. The best way to counteract a further increasing prevalence of diabetes is to prevent people to become obese. While this sounds simple, reality is complex, as food is everywhere and always available and healthy food is often relatively expensive. Further, modern society is characterized by decreased need for physical activity. Still, the primary goal for (non-) governmental organisations aiming to increase public health should be to make healthy food more widely available and to promote physical activity.

Besides this primary goal it is important to unravel the association between obesity and insulin resistance. Understanding the pathophysiological basis of diabetes mellitus will help us to treat and hopefully prevent the disease. Obesity-induced inflammation may be an important link between obesity and diabetes. Obese individuals are characterized by elevated levels of inflammatory markers, (2, 3) probably caused by an inflammatory response in the adipose tissue. Immune cells, like macrophages and T-cells infiltrate adipose tissue of obese individuals. (4, 5) However much remains to be learned about the factors that initiate and propagate this inflammatory reaction. In this respect we have focussed on toll-like receptors, more specifically TLR-3, and the IL-1 cytokine family, which will be discussed in more detail below.

Toll like receptor 3

In this thesis have we demonstrated that many toll-like receptors are expressed in both mature adipocytes and in the stromal vascular fraction of adipose tissue. It was already known that TLR-2 and TLR-4 can be activated by saturated fatty acids and induce a pro-inflammatory response. Given the abundant expression of TLR-3 in mature adipocytes, TLR-3 may also be of importance in determining the link between obesity and inflammation. We showed that TLR-3 activation in adipocytes indeed results in an inflammatory response, reduced adipocyte differentiation and inhibition of insulin signalling. (6, 7) However, in vivo did we neither find an effect on insulin sensitivity, nor on obesity-induced inflammation. These findings are in contrast to a study, which tested a more extreme high-fat diet, over a longer time-interval in a TLR-3 knockout model and showed improved glucose tolerance and protection against liver steatose. (8) This disconcordance may imply that TLR-3 exerts different effects between liver and adipose tissue. The expression of TLR-3 in mature adipocytes in comparison to stromal vascular fraction is less prominent in mice than

in humans. While this suggests that TLR-3 activation may have more profound effects in humans, we found in human fat tissue, that TLR-3 expression was neither correlated with BMI or HOMA-IR, nor with markers for adipocyte differentiation or adipose tissue inflammation. Noticeably, these results were generated using subcutaneous adipose tissue. It is not known whether the same holds true for the metabolically more active visceral depot.

Altogether, the function of TLR-3 in adipose tissue appears relatively small, but we cannot completely rule out a modest effect of TLR-3 on adipose tissue inflammation or insulin sensitivity. Based on the results of this study, it appears that TLR3 is present and active in adipose tissue yet its precise function remains unknown.

Another interesting question is whether TLR-3 is involved in beta-cell destruction in (type-1) diabetes mellitus. (9) It has been hypothesized that viruses are involved in the pathogenesis of beta-cell destruction in type-1 diabetes mellitus. TLR-3 expression in pancreatic islets is described in some cases of diabetes mellitus characterised by fulminant destruction of pancreatic beta-cell function. (10) In this thesis, we have not studied the role of TLR-3 in relation to beta-cell function.

Interleukin-1 cytokine family

In the last years, the function of the many IL-1 cytokine family members have become more clear, mechanistic pathways have been unravelled and effects of many of the IL-1 family members on metabolic functions have been elucidated. Just before the start of this research project it was revealed that IL-18 had significant effects on metabolism. Blocking IL-18 in mice resulted in hyperphagia, obesity and insulin resistance. (11) Prompted by this finding, other IL-1 family members have been studied extensively for their effects on metabolism. This thesis described the effects of IL-1F6 and IL-1F8 on adipocyte differentiation. (12) These cytokines are nowadays termed IL-36α and IL-36β respectively. (13)

IL-36 cytokines are present in keratinocytes and other epithelial cell types and appear to be involved in the regulation of inflammatory responses in human psoriatic disease. (14-16) IL-36Ra is associated with a form of pustular psoriasis. (17) IL-36 is produced by epithelial and dendritic cells and activates dendritic cells to produce IL-12 and promotes survival, proliferation and IL-2 secretion of CD4+ T0-cells. This results in polarization of these naïve T-cells in an IL-2 dependent way to CD4+T1-cells resulting in interferon gamma mediated immune responses. (18, 19) Altogether, IL36 appears to function as an early danger signal that activates and modulates the immune response. A recent study evaluated the effects of IL-36 on host defence in mycobacterial infection but found no difference in survival between IL-36 knockout mice and wild-type control mice. (20) It will be of interest to determine whether IL-36 blocking strategies can have positive effects on severe inflammatory conditions, such as psoriasis, without affecting the immune response to mycobacterial infections.

While research on IL-1F6 and IL-1F8 has focussed on psoriasis and mycobacterial infections, it has been demonstrated that other IL-1 cytokine members do have profound effects on metabolism and future investigations may reveal whether the same accounts for, especially, IL-1F6. While these are still early days for the new members of the IL-1 cytokine family, they seem to be important in the regulation of obesity induced inflammation hence modification of these cytokines may yield new avenues to target obesity induced inflammation and improve glycaemic control. For example has it recently been showed that IL-37 protects against obesity induced inflammation and insulin resistance. (21)

Interleukin-1

After the 2007 landmark trial showing positive effects of anakinra treatment on glycaemic control (22) many subsequent trials have tested IL-1(β) blockade in diabetes mellitus.

While we applied the IL-1 receptor antagonist anakinra, the actions of IL-1β can also be blocked by monoclonal antibodies, and this has been studied in large clinical trials. The results have been rather disappointing. Effects on glycaemic control have been modestly positive at best, (23-25) while others found no effect. (26, 27) Two trials that showed an improvement in glycemic control, reported a (non-significant) beneficial effect on insulin secretion. (23, 24)

One of the trials reporting no effect on glycaemic control, did report a non-significant improvement in HbA1c and postprandial glycaemia and a significant improvement in insulin secretion. (27) In one trial, anakinra or canakinumab were tested versus placebo in patients with recent onset type 1 diabetes mellitus, and did not report an improvement in C-peptide levels. (26) However, this trial was intended as an immune intervention trial in type 1 diabetes, with pathophysiological mechanisms likely to be different from the inflammatory activation in type 2 diabetes.

Of all trials with IL-1β antibodies, two trials have fully focussed on patients with advanced diabetes mellitus and high HbA1c levels. (22, 23) It is remarkably that these trial report relatively large improvement in diabetes-related measurements.

All other trials have included patients with well-controlled type 2 diabetes mellitus or patients with recent onset type 1 diabetes mellitus.

Thus, the effectiveness to improve glucose control in type 2 diabetes of IL-1β blockade strategies seems very limited and the initially reported positive findings have not been reproduced. This may be explained by a number of factors. First, it may be that the intervention does not work in relatively well-controlled patients. We found a clear, sustained effect in type 1 diabetes patients with marked chronic hyperglycemia. Hence, it would be of interest to perform a trial focussing on patients with high HbA1c levels or marked insulin resistance to elucidate potential effects of IL-1(β) blockade in this particular group of patients.

Table 1 Overview of trials testing IL-1β antibodies in diabetes mellitus.

Auteur (ref)	Drug	Subjects	Main effects
Larsen (22)	Anakinra	70 T2DM patients	Glycemic control ↑ Insulin secretion ↑ Insulin sensitivity ≈
Cavelti-Weder (23)	Gevokizumab	98 T2DM patients	Glycemic control ↑ Insulin secretion ≈(↑ n.s.) Insulin sensitivity ≈
Sloan-Lancaster (24)	LY2189102	106 T2DM patients	Glycemic control ↑ Insulin secretion ≈ (↑ n.s.) Insulin sensitivity ≈
Hensen (25)	Canakinumab	551 T2DM patients	Glycemic control ≈ (↑ n.s.) Insulin secretion ≈ Insulin sensitivity ≈
Rissanen (27)	Canakinumab	190 T2DM patients 54 Impaired glucose tolerance	Glycemic control ≈ Insulin secretion ↑ Insulin sensitivity ≈
Moran (26)	Canakinumab/ Anakinra	69 Canakinumab/ placebo 69 Anakinra/placebo recent onset T1DM patients	Glycemic control ≈ Insulin secretion ≈

T1DM= type 1 diabetes mellitus, T2DM= type 2 diabetes mellitus, n.s.= non-significant. In grey trials testing IL-1β antagonism in type 1 diabetes mellitus.

Second, anti-inflammatory interventions may not work in all patients, as enhanced inflammation and / or IL-1β may not underlie insulin resistance in all patients. Hence, another strategy would be to focus on individuals with a pro-inflammatory status that might be identified using circulating concentrations of C-reactive protein. This approach has been applied in the CANTOS-trial, which is using Canakinumab to reduce IL-1 mediated inflammation. (28) First reports on the effects of Canakinumab treatment in patients with diabetes and elevated CRP levels, however, did not yield clear beneficial effects on glucose control. (25) Similarly, it has been suggested that patients with lower IL-1RA levels, and thus a potentially higher pro-inflammatory status, would benefit more from anakinra treatment. (29) In our trials, we could not correlate the effectiveness of anakinra treatment with circulating IL-1RA levels, but the number of patients was relatively low.

Alternatively, other "host characteristics" such as genetic polymorphisms relevant for variations in the immune systems, might be important in determining the effectiveness of anti-IL1 therapy. While this possibility is likely given the complexity of the immune system, more research is needed to determine a phenotype/genotype most suited for anti-inflammatory therapy and to identify those patients that would benefit most from IL-1ra, or other anti-inflammatory treatment. Finally, the role of IL-1β in human (patho-) physiology might be less important then we think it is, although many in vitro and in vivo findings argue against this possibility.

It is important to note that treatment with anakinra differs significantly from treatment with IL-1β blocking antibodies. Anakinra has a relatively low bioavailability necessitating daily injections. On the other side does anakinra block not only IL-1β but also IL-1α. The role of IL-1α on glycaemic control is completely unknown. The fact that IL-1α is primarily present intracellularly and IL-1 blocking drugs act extracellularly, suggests that an IL-1α mediated effect is less likely. On the other hand, IL-1α is known to reduce adipocyte differentiation and IL-1α levels are higher in obese mice, although mRNA-levels of IL-1α are lower. (30) Furthermore, IL-1α reduces insulin signalling in murine adipocytes. (31) Recently it has been shown that endogenous oil derived of human adipocytes recruits immune cells in an IL-1α dependent way. (32) Altogether it would it be interesting to test specific IL-1α antibodies or therapies effectively blocking both IL-1α and IL-1β, for example by applying the soluble decoy IL-1 receptor protein Rilonacept.

Alternative approaches to block excessive inflammatory responses by TNFα-antibodies, salsalates or a combination of anti-inflammatory strategies represent potentially interesting interventions, yet will need further study.

Taken together, blocking IL-1β appears to have a positive, but small effect on glycaemic control in a subset of patients. Most likely, this effect is mediated by a moderate improvement in insulin secretion. Effects on insulin sensitivity are less clear, but, as we show in this thesis, may exist in subgroups. Future research efforts should focus on ways to identify the right intervention for the right patient. Clearly, further work is needed before anti-inflammatory therapies in general and IL-1β-blocking strategies in particular can be applied successfully in daily clinical practice.

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Nederlandse samenvatting

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Nederlandse samenvatting

Diabetes mellitus (suikerziekte) komt wereldwijd heel veel voor: in 2014 had 9% van de wereldbevolking diabetes mellitus. De verwachting is dat het aantal mensen met diabetes de komende jaren fors gaat toenemen. Dit is een groot probleem, onder andere omdat diabetes mellitus het risico op hart- en vaatziekten sterk verhoogt.

Diabetes mellitus wordt gekenmerkt door chronisch verhoogde glucosewaarden (bloedsuikerwaarden). Er bestaan verschillende vormen van diabetes, het meest bekend zijn type 1 en type 2 diabetes. Type 1 diabetes ontstaat vaak op jonge leeftijd, wordt veroorzaakt door destructie van insulineproducerende cellen in de eilandjes van Langerhans en moet altijd worden behandeld met insuline. Insuline is essentieel voor normale glucosewaarden. Insuline zorgt ervoor dat glucose de lichaamscel in wordt getransporteerd.

Diabetes mellitus type 2 is veruit het meest voorkomend. Bij diabetes mellitus type 2 (ook wel ouderdomsdiabetes genoemd) is het belangrijkste probleem dat de gevoeligheid van de lichaamscellen voor de werking van insuline afneemt. Als de lichaamscellen minder gevoelig worden voor insuline, gaat de alvleesklier meer insuline produceren, zodat het bloedsuikergehalte in eerste instantie normaal blijft. In veel gevallen raakt de alvleesklier na verloop van tijd uitgeput, gelijktijdig neemt de insulinegevoeligheid verder af. Op dat moment stijgt de bloedsuikerwaarde en spreken we van diabetes mellitus.

We spreken van gestoorde glucosetolerantie als de bloedsuikerspiegel na een maaltijd meer dan normaal stijgt, maar niet zodanig dat er sprake is van diabetes mellitus. Gestoorde glucosetolerantie is vaak een voorstadium van diabetes mellitus.

Diabetes mellitus type 2 komt vaker voor bij overgewicht. Als het aantal mensen met overgewicht stijgt zal ook de prevalentie van diabetes mellitus stijgen. Om de prevalentie van diabetes mellitus te verminderen, is het meest voor de hand liggend om te streven naar het behandelen, of – nog beter – naar het voorkómen van overgewicht. Dit zou bijvoorbeeld bereikt kunnen worden door goede voorlichting, door ongezonde voeding duurder te maken, gezonde voeding te subsidiëren en door lichamelijke activiteit te bevorderen. Helaas is dit makkelijker gezegd dan gedaan. We leven immers in een maatschappij waarin (ongezond) voedsel altijd en overal beschikbaar is en waarin lichaamsbeweging steeds minder noodzakelijk is. Naast bovengenoemde maatregelen is het daarom ook belangrijk om de interactie tussen overgewicht en diabetes mellitus beter te begrijpen om zo mogelijk een meer gerichte behandeling te kunnen ontwikkelen.

In dit proefschrift hebben we geprobeerd meer inzicht te krijgen in de relatie tussen overgewicht en diabetes mellitus. Het is een interessant gegeven dat de ene persoon met sterk overgewicht (obesitas) geen diabetes mellitus ontwikkelt, terwijl een ander zonder overgewicht juist wel diabetes mellitus type 2 krijgt. De relatie tussen overgewicht en diabetes mellitus type 2 is erg complex. Daarom hebben we hier gekeken naar de invloed van één specifieke factor, namelijk ontsteking, op het ontstaan van insulinetekort en insulineresistentie.

Het was reeds bekend dat mensen met overgewicht gemiddeld hogere ontstekingswaarden in het bloed hebben dan slanke mensen. Verondersteld wordt dat dit het gevolg is van een chronische, milde ontstekingsreactie in het vetweefsel. Waarom deze ontstekingsreactie ontstaat, is niet geheel duidelijk. Eén hypothese is dat groei van de hoeveelheid en grootte van vetcellen leidt tot lokaal zuurstoftekort in het vetweefsel. Hierdoor gaan er vetcellen dood. Deze dode cellen brengen een ontstekingsreactie op gang die ervoor zorgt dat de dode cellen worden opgeruimd. Als we nu verder redeneren, zou het zo kunnen zijn dan deze ontsteking leidt tot verminderd functioneren van de overige vetcellen. Dit zou dan weer kunnen leiden tot een verminderde gevoeligheid van de vetcellen voor insuline. We spreken dan van insulineresistentie en dat kan vervolgens leiden tot diabetes mellitus type 2.

In het eerste deel van dit proefschrift hebben we onderzoek gedaan naar het effect van verschillende componenten van de ontstekingsreactie op de vetcellen en op de werking van insuline. In de tweede helft van dit proefschrift hebben we geprobeerd om de ontstekingsreactie te remmen om vervolgens te kijken wat het gevolg hiervan is voor zowel insulinegevoeligheid als insulineproductie.

Een ontstekingsreactie ontstaat als het afweersysteem van ons lichaam een moleculair patroon herkent als mogelijk gevaarlijk. Dit kan zowel een lichaamsvreemde stof (bijvoorbeeld een bacterie) als een lichaamseigen stof (die zich op een ongebruikelijk plek bevindt) zijn.

Receptoren die potentieel gevaarlijke moleculen kunnen binden worden 'Pathogen recognition receptors' (PRR's) genoemd. De meest bekende groep van PRR's zijn de Toll-Like Receptors (TLR's). In **hoofdstuk 2** hebben we gekeken naar het voorkomen van TLR's in het vetweefsel en hun aanwezigheid in de verschillende componenten van het vetweefsel. TLR-3 en TLR-5 hadden een opvallend verspreidingspatroon. Ze bleken met name voor te komen in de vetcelfractie, dit in tegenstelling tot de overige TLR's die met name voorkwamen in de niet-vetcelfractie. Vervolgens hebben we vastgesteld dat TLR-3 functioneel actief is. Door TLR-3 te stimuleren bleken de vetcellen meer pro-inflammatoire genen (bijvoorbeeld IL-8 en MCP-1) en minder vetcelstimulerende genen (PPAR-gamma en adiponectine) tot expressie te brengen.

Toen we vervolgens muizen die geen TLR-3 tot expressie konden brengen, vergeleken met normale muizen bleek er echter geen verschil te zijn in lichaamsgewicht, insulinegevoeligheid en ontstekingsniveau in het bloed. Ook bij mensen bleek er geen relatie tussen de expressie van TLR-3 in vetweefsel en het lichaamsgewicht, ontsteking of insulinegevoeligheid te bestaan. Al deze resultaten bij elkaar genomen moeten we concluderen dat TLR-3 veel tot expressie komt in het vetweefsel en ook functioneel actief is, maar nauwelijks effect lijkt te hebben op de stofwisseling.

In het vervolg van dit proefschrift hebben we ons gefocust op de Interleukine (IL)-1 familie. IL-1 is een boodschappermolecuul dat een belangrijke rol speelt in de lichaamsreactie op ontsteking. In geval van een ontsteking wordt IL-1 geproduceerd door afweercellen zoals macrofagen. Door de productie van IL-1 treden er in het lichaam verschillende reacties op die leiden tot een adequate afweerreactie. In de afgelopen decennia zijn er verschillende op IL-1 lijkende moleculen ontdekt. Samen vormen deze de IL-1 cytokinefamilie. Van verschillende leden van de IL-1 cytokinefamilie is bekend dat ze effect hebben op de stofwisseling.

In **hoofdstuk 3** hebben we onderzocht of twee relatief nieuw ontdekte leden van de IL-1 familie, IL-1F6 en IL-1F8, aanwezig zijn in het vetweefsel en wat hun effect op vetcellen is.

IL-1F6 konden we, in tegenstelling tot IL-1F8, inderdaad aantonen in vetweefsel. Blootstelling van vetcellen aan IL-1F6 had tot gevolg dat de aanwezigheid van vetcel-stimulerende genen afnam, terwijl de aanwezigheid van pro-inflammatoire genen juist toenam. IL-1F8 stimuleerde de expressie van pro-inflammatoire genen. Met name IL-1F6 heeft een soort IL-1-achtig effect op vetcellen.

In de tweede helft van dit proefschrift hebben we getest of blokkade van IL-1 effect heeft op insulinegevoeligheid en insulineproductie (beta-celfunctie). Anakinra is een in Nederland geregistreerd geneesmiddel dat wordt toegepast bij de behandeling van onder andere reumatoïde artritis. Het blokkeert de IL-1 receptor. In drie onderzoeken hebben we verschillende patiëntengroepen, met nauwkeurig vastgestelde kenmerken op het gebied van insulinegevoeligheid en insulineproductie, behandeld met anakinra. Hierdoor waren we in staat om te bepalen wat het effect van IL-1 blokkade is op insulineproductie en -gevoeligheid.

In **hoofdstuk 4** beschrijven we de resultaten van het onderzoek met anakinra bij personen met overgewicht en risicofactoren voor hart- en vaatziekten (metabool syndroom), maar zonder diabetes mellitus. Deze onderzoekspopulatie is insulineresistent, maar heeft geen verhoogde bloedsuikerwaarde en gebruikt dus ook geen medicijnen voor diabetes mellitus. Hierdoor is het makkelijker om te kijken naar de effecten van anakinra op insulinegevoeligheid en insulineproductie. We beoordeelden

de insulinegevoeligheid met behulp van een euglycemische hyperinsulinemisch clamp, de meest gevoelige onderzoeksmethode voor het meten van insulinegevoeligheid. Hiernaast deden we een orale glucose tolerantietest, met deze test kan een indruk worden gekregen van de insulineproductie.

De proefpersonen werden gedurende vier weken behandeld met anakinra of placebo (nepmedicijn), daarna volgde een tweede behandelperiode met nu de andere vorm van behandeling. Vóór de eerste en na iedere onderzoeksperiode deden we bovengenoemde onderzoeken, en we namen een klein beetje weefsel af uit het onderhuids buikvet. Anakinra bleek geen invloed te hebben op de insulinegevoeligheid, maar verbeterde wel de insulineproductie.

Veel proefpersonen hadden last van een ontstekingsreactie op de plaats waar anakinra werd ingespoten. Dit is een bekende bijwerking. Het bleek dat de sterkte van de ontstekingsreactie in het vetweefsel correleerde met de verandering in insulinegevoeligheid. Het zou dus zo kunnen zijn dat een mogelijk positief effect van anakinra op de insulinegevoeligheid te niet wordt gedaan door de lokale ontstekingsreactie. Een andere mogelijkheid is dat een effect van anakinra op insulinegevoeligheid minder sterk is bij normale bloedsuikerwaarden.

In **hoofdstuk 5** is vervolgonderzoek gedaan naar de effecten van anakinra op insulinegevoeligheid bij mensen met hoge glucosewaarden. Voor dit onderzoek hebben we patiënten met lang bestaande diabetes mellitus type 1, overgewicht en relatief matige diabetesregulatie geselecteerd. Deze patiënten hebben geen resterende insulineproductie, zodat iedere verbetering van de bloedsuikerregulatie het gevolg moet zijn van betere insulinegevoeligheid. Deze patiënten kregen een relatief lage dosering anakinra gedurende een week. Hierdoor waren er minder problemen met lokale ontstekingsreacties.

Na een week behandeling met anakinra verbeterde de insulinegevoeligheid, bloedsuikerwaarden en verminderde de insulinebehoefte. Dit effect was nog steeds zichtbaar vier weken na het einde van de behandeling met anakinra. Deze resultaten suggereren dat het blokkeren van IL-1 bij sommige patiënten nuttig kan zijn. Aangezien dit onderzoek niet geblindeerd was en geen controlegroep bevatte kan een studie-effect (effect puur door het feit dat patiënten vaker worden gecontroleerd en zichzelf controleren doordat ze deelnemen aan een onderzoek) als verklaring voor de verbetering niet geheel worden uitgesloten. Het feit dat de verbetering in insulinegevoeligheid sterk gecorreleerd was met de verbetering in ontstekingsgraad maakt dit minder waarschijnlijk.

Ten slotte hebben we in **hoofdstuk 6** de effecten van anakinra op de insulineproductie onderzocht. Voor dit onderzoek werden proefpersonen geselecteerd met een verminderde glucosetolerantie. Bij deze patiënten stijgt de bloedsuikerwaarde na

een maaltijd meer dan normaal. Dit is het gevolg van verminderde insulineproductie. Deze studie had dezelfde opzet als het eerder in hoofdstuk 4 beschreven onderzoek. Alle proefpersonen ondergingen een hyperglycemische clamp vóór de eerste en na iedere onderzoeksperiode. Een hyperglycemische clamp is de meest nauwkeurige methode voor het bepalen van de insulineproductiecapaciteit. Anakinra verlaagde opnieuw de ontstekingswaarden in het bloed. De insulineproductie tijdens de eerste fase van de clamp verbeterde. Bij dit onderzoek werd geen verbetering gezien in insulinegevoeligheid en ook niet in andere parameters voor diabetes regulatie.

De drie onderzoeken naar het effect van IL-1 blokkade met anakinra laten zien dat anakinra enige verbetering van de beta-celfunctie geeft. De effecten op insulinegevoeligheid zijn minimaal bij patiënten met het metabool syndroom, maar meer uitgesproken bij patiënten met chronisch verhoogde bloedsuikerwaarden. De effecten van anakinra op insulinegevoeligheid worden mogelijk negatief beïnvloed door lokale ontstekingsreacties ten gevolge van anakinra injecties.

Alles bijeen genomen zou het remmen van ontsteking in het algemeen en het blokkeren van de werking van IL-1 in het bijzonder bij sommige patiënten nuttig kunnen zijn, maar het effect is beperkt en er zijn ook patiënten bij wie geen enkel effect optreedt.

Toekomstige onderzoeken moeten er daarom op gericht zijn om de juiste (anti-inflammatoire) behandeling te vinden voor de juiste patiënt. Voor het overige is het van belang om te onderzoeken wat het effect van anti-inflammatoire behandeling op hart- en vaatziekten is. Er zijn namelijk ook sterke aanwijzingen dat verhoogde ontstekingswaarden gerelateerd zijn aan een verhoogd risico op hart- en vaatziekten. Kortom de relatie tussen diabetes mellitus en ontsteking is ingewikkeld, maar biedt zeker aanknopingspunten voor toekomstige behandelingen.

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Curriculum vitae

Edwin Josef Petrus van Asseldonk werd geboren op 18 maart 1980 in Zijtaart (gemeente Veghel). Hij voltooide zijn vwo-opleiding aan het Zwijssen College te Veghel. In 1998 begon hij met zijn studie geneeskunde aan de Radboud Universiteit Nijmegen (destijds Katholieke Universiteit Nijmegen). Tijdens zijn studie was hij twee jaar als bestuurslid actief binnen de Medische Faculteits Vereniging Nijmegen (MFVN). Edwin behaalde zijn doctoraalexamen in 2003. Tijdens zijn coschappen verrichtte hij wetenschappelijk onderzoek bij de afdeling Nefrologie van het Brigham and Women's hospital in Boston, USA (Prof. dr. J.V. Bonventre). Hier deed hij onderzoek naar de functie van het eiwit Kidney Injury Molecule 1.

In 2005 behaalde hij zijn artsexamen en begon hij zijn opleiding tot internist aan de afdeling Interne Geneeskunde van het Radboud UMC te Nijmegen (opleiders Prof. dr. J.W.M. van der Meer, Prof. dr. J. de Graaf en Prof. dr. J.W.A. Smit). Tussen 2005 en 2008 was hij voor het perifere deel van zijn opleiding werkzaam in het Canisius Wilhelmina Ziekenhuis te Nijmegen (opleiders Dr. A.S.M. Dofferhoff en Dr. M.J.T.M. Mol). Tussen 2008 en 2012 onderbrak hij zijn opleiding tot internist voor het doen van onderzoek naar de effecten van obesitas-geïnduceerde ontsteking op diabetes mellitus, wat uiteindelijk heeft geleid tot dit proefschrift. In 2012 vervolgde Edwin zijn opleiding tot internist in het Radboud UMC en startte hij de differentiatie tot endocrinoloog (opleider Prof. dr. A.R.M.M. Hermus), welke hij afrondde in september 2014.

Vanaf januari 2015 is hij werkzaam als internist-endocrinoloog in Oskarshamn, Zweden.

Edwin woont samen met Maaike en samen hebben zij drie kinderen; Helle, Tobias en Voske.