



Anti-inflammatory role of GLP-1 and the effect of gastric bypass on diabetes- and obesity-associated inflammation

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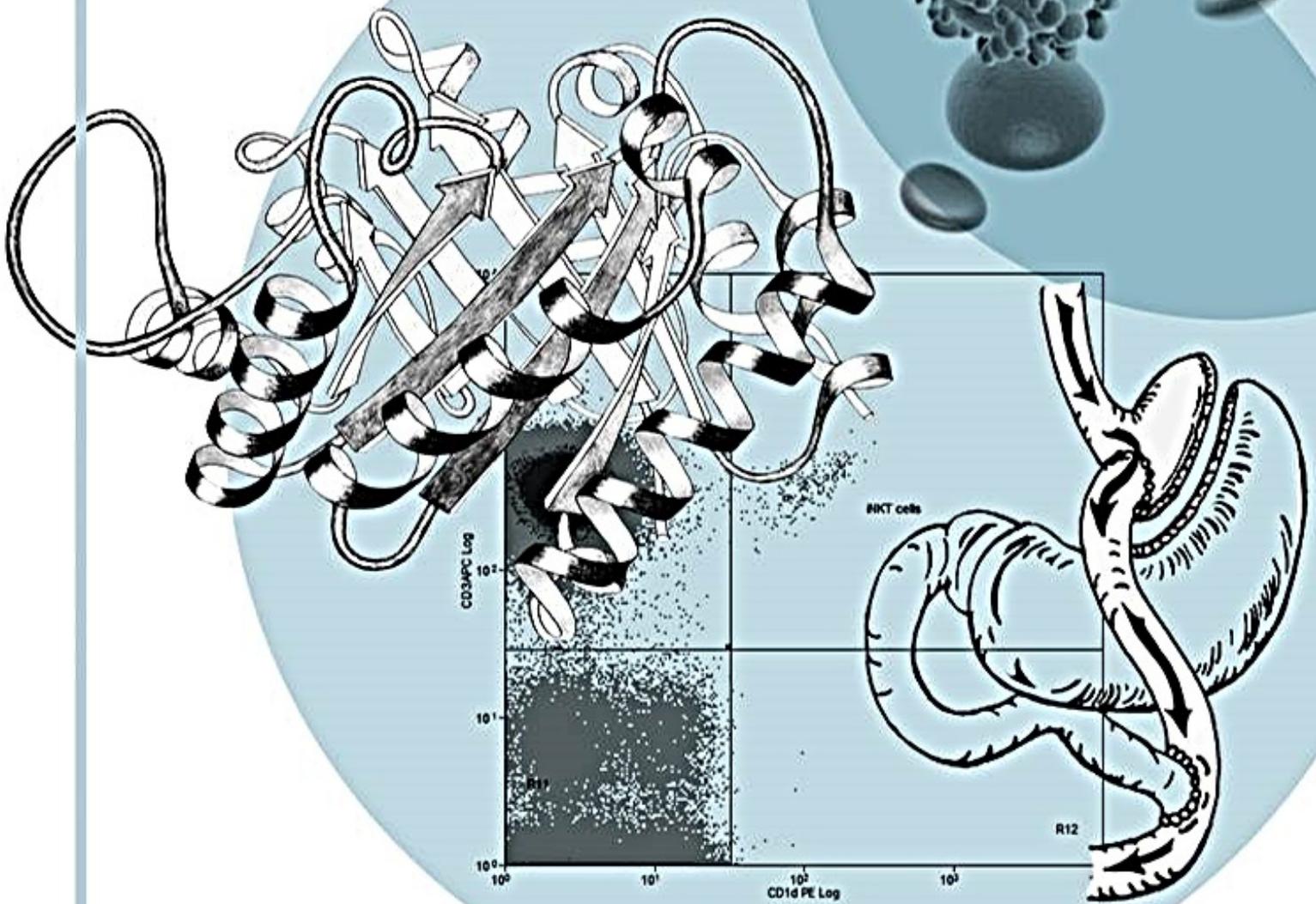
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Anti-inflammatory Role of GLP-1 and the Effect of Gastric Bypass on Diabetes- and Obesity-associated Inflammation

PhD Thesis 2014

Kirsten Katrine Lindegaard Bovbjerg



Anti-inflammatory role of GLP-1 and the effect of gastric bypass on diabetes- and obesity-associated inflammation

Ph.D. thesis 2014

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To Clara

Anti-inflammatory role of GLP-1 and the effect of gastric bypass on diabetes- and obesity-associated inflammation

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PREFACE

The present thesis is submitted to the Technical University of Copenhagen to obtain the *Philosophiæ Doctor*, Ph.D. degree. The studies have been performed in the period from 2011 to 2014 at the Department of Molecular Pharmacology, Zealand Pharma A/S, Glostrup, Denmark; Department of National Veterinary Institute, Technical University of Denmark; Copenhagen, Denmark; Bioneer A/S, Hørsholm, Denmark; and Department of Endocrinology at Hvidovre Hospital, Copenhagen, Denmark under supervision of my external supervisors Vice President, MD, Adam Steensberg, and Senior scientist, Ph.D., Jesper Mosolff Mathiesen, and internal supervisor Professor, Peter M.H. Heegaard.

The thesis contains an *Introduction* which gives an overview of the subjects presented in the thesis, followed by the *Background* section consisting of a review of the literature relevant for the work presented in the three manuscripts in addition to the two small experimental studies. Next, the *Rationale*, *Aim* and *Hypothesis* are presented. The *Result* section covers two experimental studies, including a discussion of the obtained results and provides a short summary of the results stated in the manuscripts. The *Discussion* section discusses and evaluates our findings in relation to our hypotheses and to the literature, and *Conclusion and Perspectives* briefly summarizes the main results and their implications and future perspectives. The *Methods* section consists of details and information on the methods and materials applied in the experimental *in vitro* and *in vivo* work and in the clinical studies.

The following manuscripts are included in the thesis:

Manuscript 1 – submitted to Journal of Clinical Immunology

Effects of Roux-en-Y gastric bypass on fasting and postprandial levels of inflammatory markers in obese subjects with normal glucose tolerance or type 2 diabetes

Manuscript 2 – in preparation for submission

Effect of Roux-en Y gastric bypass on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects

Manuscript 3 – submitted to Scandinavian Journal of Immunology

Effect of 12-week treatment with glucagon-like peptide 1 receptor agonist liraglutide on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects with type 2 diabetes

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This PhD has been a challenging task in so many ways. Luckily, my time as a Ph.D.-student has been filled with people who have helped me along, and to whom I owe a debt of gratitude.

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When I began work on this PhD, the plan was to go abroad and do gastric bypass research in pigs in Indianapolis. Instead I ended up at the Dept. of Endocrinology at Hvidovre Hospital. And what luck. I have enjoyed every visit to the hospital, and very much appreciated the unique openness and knowledge-sharing attitude I have been met with. Thank you! Thanks to technician Alis Andersen who volunteered as control subject and collected blood samples, Carsten Dirksen for trying to convince patients to take Victoza, Kirstine Bojsen-Møller for helping out, and Siv Hesse Jacobsen for her open-mindedness and friendship. Last but not least – Sten Madsbad. It has been a true pleasure to get to know Sten and I am forever grateful for his contributions to my PhD, for his engagement in my work and his pleasant personality.

Thanks to Kåre Engkilde from the Bartholin Institute– for always helping out whenever possible and for being my favorite iNKT research partner. Thanks to colleagues and friends at Zealand Pharma, in particular the Dept. of Molecular Pharmacology. Special thanks to Frosty – for trust and letting me loose in lab, Evelyn – for fighting the battle of worldwide animal welfare, Jacob – for keeping an eye on me, Jens-Peter – for being the reason for loving flow cytometry, Jolanta – partner in crime, Betina – for solicitude and support, and Rasmus – for turning frustration into everlasting optimism.

My family and friends for supporting me throughout and for providing me with all sources of ingestible caffeine during the writing process. Lotte for late night calls, Julie for creativity, Susanne for fashionable grammar, and Stine for helping out at the last minute.

Finally – Thanks to my beautiful, funny and clever daughter Clara for all her smiles during the hard times.

Kirsten Lindegaard Bovbjerg, July 2014

ENGLISH SUMMARY

Obesity-associated type 2 diabetes (T2D) is characterized by a state of chronic, low-grade inflammation with an excessive secretion of pro-inflammatory mediators, such as IL-6, TNF- α , and leptin from the adipose tissue and decrease in the anti-inflammatory adipokine adiponectin. T2D is accompanied with a set of metabolic abnormalities comprising the metabolic syndrome, such as hypertension, dyslipidemia, and insulin resistance. Although the exact causes for the onset of clinical disease remain largely unknown, emerging evidence seems to suggest that obesity-induced inflammation, especially in the adipose tissue, is involved in the metabolic dysregulation and therefore plays an important role in the pathogenesis of this deteriorating disease.

Bariatric surgery, including the Roux-en Y gastric bypass (RYGB), is one of the most effective treatments for severe obesity. In addition to weight loss, the RYGB procedure is associated with immediate improvement in glycemic control and insulin secretion. The exact mechanisms for the immediate and long-term positive effect of RYGB on glucose metabolism and obesity related co-morbidities remain unclear. Changes in inflammatory cellular and molecular mediators have been found following RYGB, suggesting one potential mechanism by which bariatric surgery re-establishes insulin sensitivity.

RYGB also enhances circulating levels of certain gastrointestinal-derived hormones, particularly the postprandial secretion of glucagon-like peptide 1 (GLP-1). A growing body of literature reports anti-inflammatory and other immunological effects of GLP-1 in animals and in humans suggesting that GLP-1 acts beyond purely glucoregulatory mechanisms. The exaggerated postprandial GLP-1 secretion following RYGB may thus be involved in the beneficial metabolic effects both directly via the classical glucoregulatory pathways and indirectly via anti-inflammatory and immune-regulatory mechanisms.

The findings of a direct GLP-1-mediated effect on lymphocyte subpopulations *in vitro* and *in vivo* and the few but interesting case reports on clinical improvements in psoriasis patients treated with GLP-1 receptor agonists have supported immune-regulatory actions of GLP-1.

The main aim of this thesis was to explore potential anti-inflammatory and immunoregulatory effects of GLP-1 on immune system parameters to increase the mechanistic understanding of the effects observed in the clinic and to provide new aspect on the interplay between metabolism, obesity and inflammation.

The *in vitro* work and animal study, and the three manuscripts constituting this thesis describe different approaches in studying GLP-1 effects on immune system parameters. In **Study I**, the functionality of human expanded Tregs in the presence of GLP-1 was examined *in vitro*. **Study II** investigated anti-inflammatory effect of GLP-1 *in vivo* in the PMA-induced ear inflammation model. Three human studies

were conducted that generated the three manuscripts in the thesis; two RYGB studies (**Study III, Manuscript I and Study IV, Manuscript II**) and one study in obese T2D subjects commencing liraglutide therapy (**Study V, Manuscript III**), to evaluate immunological changes either after surgery (1 week, 3 months, and 1 year) or after 12 weeks of GLP-1 therapy.

DANISH SUMMARY (DANSK RESUMÉ)

Fedme-associeret type 2 diabetes (T2D) er karakteriseret ved en tilstand af lav kronisk inflammation med overdreven sekretion af pro-inflammatoriske mediatorer, så som IL-6, TNF- α og leptin fra fedtvævet og en reduktion i anti-inflammatorisk adipokine adiponectin. Med T2D følger et sæt metaboliske abnormaliteter, bestående af det metaboliske syndrom, så som forhøjet blodtryk, dyslipidemi og insulin resistens. Selvom de eksakte årsager til påbegyndelsen af klinisk sygdom er stort set uvisse, tyder nye beviser på at fedme-induceret inflammation, specielt i fedtvævet, er involveret i den metaboliske dysregulering og derfor spiller en vigtig rolle i patogenesen af denne forværrede sygdom.

Fedme kirurgi, herunder Roux-en Y gastrisk bypass (RYGB), er en af de mest effektive behandlinger af svær overvægt. Ud over vægttab er RYGB proceduren associeret med øjeblikkelig forbedring i glykæmisk kontrol og insulin sekretion. Den eksakte mekanisme bag den øjeblikkelige og langsigtede positive effekt af RYGB på glukose metabolisme og fedme relaterede co-morbiditet forbliver uklar. Efter RYGB er der fundet ændringer i inflammatoriske cellulære og molekulære mediatorer, hvilket indikerer én potentiel mekanisme med hvilken bariatric kirurgi genetablerer insulin sensitivitet.

RYGB øger også cirkulerende niveauer af visse mavetarm-hormoner, især postprandial sekretion af glucagon-like peptide-1 (GLP-1). En voksende mængde af litteratur rapporterer om anti-inflammatoriske og andre immunologiske effekter af GLP-1 i dyr og i mennesker, hvilket tyder på at GLP-1 virker ud over glucoregulatoriske mekanismer. Den øgede postprandiale GLP-1 sekretion, som opstår efter RYGB, er derfor muligvis involveret i de gavnlige metaboliske effekter, både direkte via den glucoregulatoriske pathway og indirekte via anti-inflammatoriske og immunregulatoriske mekanismer.

Fund af direkte GLP-1-medieret effekt på lymfocyt subpopulationer *in vitro* og *in vivo* og de få, men interessante case reports om kliniske forbedringer i psoriasis patienter behandlet med GLP-1 receptor agonister, understøtter GLP-1s immunregulatoriske handlinger.

Hovedformålet med denne afhandling var at undersøge potentielle anti-inflammatoriske og immunregulatoriske effekter af GLP-1 på parametre relateret til immunsystemet, for at øge den mekanistiske forståelse for de effekter der er observeret klinisk og for at tilføje et nyt aspekt på samspillet mellem metabolisme, fedme og inflammation.

In vitro studiet, et dyreforsøg og de tre manuskripter, der udgør denne afhandling beskriver forskellige vinkler på at studere GLP-1s effekter på immunsystemet. I Studie I blev funktionaliteten af humane ekspanderede Tregs, under tilstedeværelse af GLP-1, evalueret *in vitro*. I Studie II blev de anti-inflammatoriske effekter af GLP-1 *in vivo* undersøgt i en PMA-induceret øre-inflammations model. Tre

humane studier blev gennemført, og danner grundlaget for de tre manuskripter i denne afhandling; to RYGB studier (**Studie III, Manuskript I og Studie IV, Manuskript II**) og et studie i overvægtige T2D patienter tilskrevet liraglutid behandling (**Studie V, Manuskript III**) for samlet set at evaluere immunologiske ændringer efter operation (1 uge, 3 måneder, og 1 år) eller efter 12 ugers GLP-1 behandling.

ABBREVIATIONS

AMPK	5'-Adenosine Monophosphate-activated Protein Kinase
ANOVA	Analysis of Variance
AP	Activator Protein
APC	Antigen Presenting Cell
aPKC	Atypical Protein Kinase C
BALB/c mice	Bagg Albino (inbred research mouse strain)
BMI	Body Mass Index
BM	Betamethasone
cAMP	3'-5'-Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
CD	Classification Determinant
cPLA2	Cytosolic Phospholipase A2
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
DC	Dendritic Cell
DDR	Danger Recognition Receptors
DIO	Diet Induced Obese
DPP-4	Dipeptidyl Peptidase-4
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
FFA	Free Fatty Acids
FITC	Fluorescein IsoThioCyanate
α -GalCer	α -galactosylceramide
GI	Gastrointestinal
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like Peptide 1
GLP-1 R	Glucagon-like Peptide 1 Receptor
GLUT	Glucose Transporter
GM-CSF	Granulocyte Monocyte-Colony Stimulating Factor
GPCR	G-Protein-Coupled Receptor
HEPES	(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
HFD	High Fat Diet
HOMA	Homeostasis Model Assessment
HP	Haptoglobin

IFN	Interferon
I κ B α	Inhibitor of nuclear transcription factor NF- κ B
IKK	Inhibitor of KappaB Kinase
IL	Interleukin
iNKTs	Invariant Natural Killer T-cell
IP3	Inositol Trisphosphate
IRF	Interferon Regulatory Factor
IRS	Insulin Receptor Substrate
JNK	Jun N-terminal Kinase
KC	Keratinocyte Chemoattractant
LPS	Lipopolysaccharide
M1	Type 1 Macrophages
M2	Type 2 Macrophages
MCP	Monocyte Chemoattractant Protein
MEM	Minimum Essential Medium
MIP	Macrophage Inflammatory Protein
MR	Mineralcorticoid Receptor,
MSD	Meso Scale Discovery
mTOR	Mammalian Target of Rapamycin
NAFLD	Non-Alcoholic Fatty Liver Disease
NEAA	Non-Essential Amino Acids
NF- κ B	Nuclear Factor Kappa Beta
NGT	Normal Glucose Tolerant
NKT	Natural Killer T-Cell
NOD	Non-Obese Diabetic
nTreg	Natural regulatory T-cells
ob/ob	Obese mice
PAI-1	Plasminogen Activator Inhibitor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate

PPR	Pattern Recognition Receptor
PYY	Peptide YY
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RYGB	Roux-en Y Gastric Bypass
SAA	Serum Amyloid A
SEM	Standard Error of the MEAN
Shc	Src Homology 2 Domain Containing
SPF	Specific Pathogen-Free
SVF	Stromal Vascular Fraction
T1D	Type 1 Diabetes Mellitus
T2D	Type 2 Diabetes Mellitus
TCR	T-Cell Receptor
Teff	Effector T-lymphocytes
TGF	Transforming Growth Factor
Th1	T-Helper Cell Type 1
Th17	T-Helper Cell Type 17
Th2	T-Helper Cell Type 2
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
Tregs	Regulatory T-cells
Tresp	Responder T-cells
WHO	World Health Organization

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INTRODUCTION

Type 2 Diabetes (T2D) and obesity are associated with a state of chronic low-grade inflammation, which is involved in metabolic dysregulation both in animals and in humans (Hotamisligil, 2006; Gregor and Hotamisligil, 2011). The adipose tissue of obese individuals is characterized by an altered production of secretory compounds, including free fatty acids (FFAs), cytokines and chemokines (e.g. IL-6, TNF- α , and IL-8), and adipokines (leptin and adiponectin). Inflammatory responses in the adipose tissue are regulated by an altered composition of immune cells characterized by depletion of cells with anti-inflammatory and immunoregulatory phenotype (e.g. alternatively activated macrophages and CD4⁺ Th2 cells) and accumulation of pro-inflammatory immune cells (e.g. classically activated macrophages and Th1 cells) (Kwon and Pessin, 2013). The inflamed adipose tissue is believed to be the prime organ in disseminating systemic low-grade inflammation, which affects a range of tissues and organs. Chronic activation of the immune system may eventually contribute to the long-term complications of obesity, such as insulin resistance, T2D, fatty liver disease, and cardiovascular disease (Osborn and Olefsky, 2012).

Bariatric surgery, including the Roux-en Y gastric bypass (RYGB) procedure, is one of the most effective treatments for severe obesity. In addition to weight loss, the Roux-en Y gastric bypass (RYGB) procedure is associated with immediate improvement in glycemic control and insulin secretion (Buchwald et al., 2009). The exact mechanisms for the immediate and long-term positive effect of RYGB on glucose metabolism and obesity related co-morbidities remain unclear. Changes in inflammatory cellular and molecular mediators have been found following RYGB (Swarbrick et al., 2008; Miller et al., 2011), suggesting one potential mechanism by which bariatric surgery re-establishes insulin sensitivity. RYGB also enhances circulating levels of certain gastrointestinally derived hormones, particularly the postprandial secretion of glucagon-like peptide 1 (GLP-1) (Madsbad 2014X). GLP-1 is an incretin hormone secreted in response to nutrients by L-cells located in the intestine and among other affects, GLP-1 increases glucose-dependent insulin secretion and inhibits glucagon release from the pancreas, delays gastric emptying and decreases food intake (Holst, 2007).

Native GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP-4), resulting in a half-life of only 1-2 min, which limits its therapeutic usefulness (Baggio and Drucker, 2007; Vilsboll and Holst, 2004). This has led to the development of DPP-4 resistant GLP-1 analogues with a longer half-life and DPP-4 inhibitors which slows down the breakdown of endogenous secreted GLP-1 by inhibition of the DPP-4 enzyme. GLP-1 receptor agonists, which are structurally related to GLP-1, make it possible to pharmacologically increase the plasma GLP-1 level five to ten-fold (Ahren, 2007; Baggio and Drucker, 2007; Deacon and Ahren, 2011; Madsbad et al., 2008). Both groups of compounds are approved for

treatment of type 2 diabetes, and at present the GLP-1 receptor agonist liraglutide (Novo Nordisk) has entered into registration in Europe and in the US for treatment of obesity.

A growing body of literature reports anti-inflammatory and other immunological effects of GLP-1 in animals (Hadjiyanni et al., 2008; Dozier et al., 2009; Arakawa et al., 2010; Koderá et al., 2011; Hadjiyanni et al., 2010; Lee et al., 2012; Lee et al., 2012; Noyan-Ashraf et al., 2013) and in humans (Hogan et al., 2011; Chaudhuri et al., 2012; Hogan et al., 2014; Wu et al., 2011; Marx et al., 2010; Ishibashi et al., 2011; Hattori et al., 2010; Shiraki et al., 2012; Chen et al., 2012; Faurischou et al., 2011; Wu et al., 2011; Pugazhenthí et al., 2010) suggesting that GLP-1 acts beyond purely glucoregulatory mechanisms, thus *in vitro* work has shown that incubation with the GLP-1 analogues, liraglutide or exendin-4, reduced TNF- α -induced oxidative stress and inflammation in endothelial cells (Shiraki et al., 2012) and inhibited LPS-induced inflammatory responses in cardiomyoblasts (Chen et al., 2012), in adipose tissue macrophages (Lee et al., 2012; Hogan et al., 2014), and in human peripheral mononuclear cells (PBMCs) (Hogan et al., 2014). Data from murine and human studies have demonstrated GLP-1 receptors (GLP-1R) on several lymphocyte subpopulations and have suggested that GLP-1R activation may regulate proliferation and maintenance of T-cell subsets, including regulatory T-cells (Tregs) (Hadjiyanni et al., 2008; Hadjiyanni et al., 2010) and invariant natural killer T-cells (iNKTs) (Hogan et al., 2011). GLP-1 seems to both increase the frequency and improve the function of these cells in recently diagnosed diabetic NOD mice (Xue et al., 2008) and in obese diabetic subjects (Hogan et al., 2011).

Finally, clinical case studies of GLP-1 therapy with liraglutide or exendin-4 in psoriasis patients have shown an immediate and unexpected reduction in the emergence and size of psoriatic plaques independent of changes in glucoregulation and weight loss (Hogan et al., 2011; Faurischou et al., 2011; Ahern et al., 2013; Buysschaert et al., 2012). Psoriasis is a common autoimmune-mediated inflammatory skin disorder involving cells from the adaptive and innate immune system ((Sweeney et al., 2011; Kagen et al., 2006) and an enhanced production of inflammatory cytokines which can be detected locally and in circulation (Davidovici et al., 2010). In two case studies reported by the O'Shea group, the improvement of clinical symptoms in obese T2D psoriasis patients was associated with changes in number and function of iNKTs within 6 and 10 weeks, respectively, after commencing liraglutide therapy (Hogan et al., 2011; Ahern et al., 2013). Expression of the GLP-1R on iNKTs and modulation of functionality (Hogan et al., 2011) suggest a mechanistic link for how GLP-1 might improve disease activity by specific immune-regulatory action on this innate immune subpopulation. Intriguingly, gastric bypass seems to also improve psoriasis symptoms (Higa-Sansone et al., 2004; Hossler et al., 2011; Faurischou et al., 2011) as well as causing increases in the number of iNKT cells (Lynch et al., 2012).

The approximately 10-fold increases in postprandial GLP-1 secretion following gastric bypass procedures may be involved in the beneficial metabolic effects both directly via the classical glucoregulatory pathways and indirectly via anti-inflammatory and immune-regulatory mechanisms. Thus gastric bypass patients offer an interesting look into the possible effects of increased GLP-1 levels on the immune system and its links to the observed increase in insulin sensitivity. A further elucidation of the cellular and molecular mechanisms involved in potential GLP-1-mediated anti-inflammatory effects may provide new aspect on the interplay between metabolism, obesity and inflammation. Ultimately, GLP-1-based therapeutics may offer an opportunity for not only targeting glucose metabolism but also the immune system in the treatment of diabetes and/or other obesity-related diseases.

BACKGROUND

Obesity and type 2 diabetes

Obesity, defined as a body mass index (BMI) ≥ 30 kg/m², has for many years been associated with the lifestyle of the Western societies. However, more recently, developing countries undergoing rapid changes in lifestyle with reduced physical activity and nutrition transition to a western diet are increasing the prevalence of obesity to nearly 500 million people worldwide. It is well established that obesity is accompanied with a set of metabolic abnormalities comprising the metabolic syndrome, such as hypertension, dyslipidemia, insulin resistance. When occurring together, they confer significantly elevated risk for developing diseases related to metabolic dysfunction, such as cardiovascular diseases (CVD), stroke, fatty liver disease, and diabetes mellitus.

Diabetes is a common chronic endocrine disorder characterized by failure to produce or respond to insulin and consequently hyperglycemia. Chronic hyperglycemia causes generation of advanced glycation products that are implicated in secondary complications such as heart disease, eye diseases including retinopathy and cataract, kidney failure and neuropathy (Negre-Salvayre et al., 2009). Similar to obesity, diabetes is a worldwide epidemic affecting approximately 350 million people and WHO projects that diabetes will be the 7th leading cause of death in 2030. Accordingly, diabetes is a global health care problem and a serious, chronic, and non-curable disease that causes irreversible and detrimental effects on a wide range of tissues and organs. Understanding how to prevent and treat diabetes is therefore highly important.

The two forms of diabetes with the highest incidence are Type 1 diabetes mellitus (T1D) and T2D, with T2D being responsible for over 90% of the disease prevalence (Malecki and Klupa, 2005). T1D is an autoimmune disease comprising a permanent destruction of insulin-producing beta-cells of the pancreas

triggered by environmental factors in genetically predisposed individuals (Atkinson and Eisenbarth, 2001). T1D patients produce little or no insulin. T2D results from a decreased sensitivity to insulin of insulin-responsive cells decreasing their ability to metabolize glucose. This, in turn also leads to failure of pancreatic beta-cells to respond to the increasing demands for insulin caused by insulin resistance in tissues. T2D is due primarily to lifestyle factors and genetics (Ali, 2013), but as in T1D, T2D also has some elements of immune-mediated beta-cell destruction caused by a chronically enhanced production of inflammatory mediators (Donath et al., 2003; Odegaard and Chawla, 2012). The incidence of type 2 diabetes is increasing and is closely associated with the amount of obesity and body composition, especially abdominal obesity. Type 2 diabetes and obesity is considered as a “twin epidemic”. Implementation of novel anti-inflammatory therapeutic approaches could be relevant in both T1D and T2D.

Insulin action and signaling

The major role of insulin signaling is to increase glucose uptake in fat and muscle and inhibit glucose production. When insulin binds to the insulin receptor on insulin-responsive cells (i.e. adipocytes, myocytes, hepatocytes, skeletal muscles and beta-cells) it induces a conformational change resulting in autophosphorylation of the transmembrane subunits of the insulin receptor, which is a tyrosine kinase. Activation of the insulin receptor subsequently increases kinase activity against intracellular substrates, such as the Shc adaptor proteins and the insulin receptor substrates (Eller et al., 2011). The IRS proteins serve as docking proteins for the phosphatidylinositol 3-kinase (PI3K). Simplified, this lead to activation of several downstream effector molecules, of which atypical protein kinase C (aPKC) and Akt (otherwise known as protein kinase B) mediates a number of metabolic actions including translocation of GLUT-4 vesicles from the intracellular pool to the plasma membrane to allow uptake of glucose into the cell (Figure I). Moreover, Akt activates glycogen synthesis promoting glucose storage as glycogen. Akt also leads to mTOR-mediated activation of protein synthesis (Saltiel and Kahn, 2001; Johnson et al., 2012).

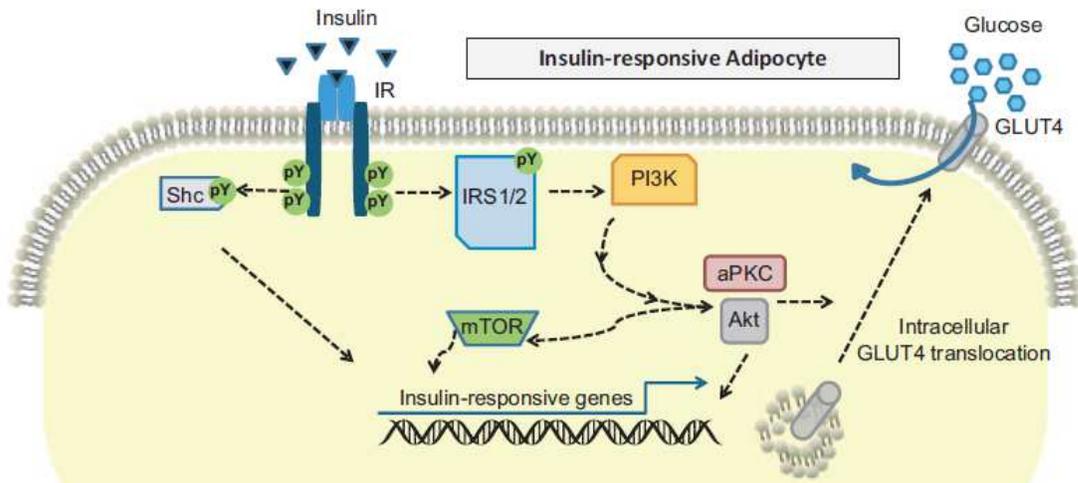


Figure I. Insulin signaling in the insulin-responsive adipocyte. Figure adapted from (Johnson et al., 2012).

Obesity-induced inflammation

Over-eating causes a repetitive or chronic metabolic state of stress that in turn triggers host inflammation and activates the immune system. The abundance of food and resulting obesity pandemic has therefore challenged the classical perception of the immune system as a defense mechanism for host protection against infection and other acute insults (Nijhuis et al., 2009). While acute inflammation is a necessary, naturally occurring immune response to damage; a localized, protective response following trauma or infection, chronic inflammation occurs when the inflammatory agent persists for longer periods of time (Libby, 2007). This chronic activation of the immune system may negatively impact metabolic pathways contribution to the development of obesity-associated complications representative of the metabolic syndrome (Hotamisligil, 2006; Osborn and Olefsky, 2012).

Inflammation in the adipose tissue

The adipose tissue is no longer perceived merely as a depot of energy storage, but as an endocrine organ comprising both adipocytes and non-fat cells, such as endothelial cells, fibroblasts, macrophages, mast cells, neutrophils and other cells from the stromal vascular fraction (SVF). These cells produce and release diverse signaling molecules locally and into circulation depending on the metabolic status (Halberg et al., 2008).

The innate immune cells in the SVF express pattern-recognition receptors (PPRs)—the most well-known being toll-like receptors (TLRs)—or danger recognition receptors (DRRs) that are activated upon the

encounter with various exogenous or endogenous “danger signals” i.e. foreign pathogens or cellular stress-related components. Activation through these receptors induces inflammatory responses, such as recruitment of more immune cells into the adipose tissue, induction of apoptosis and phagocytosis, and activation of pro-inflammatory signaling pathways (Janeway, Jr. and Medzhitov, 2002).

Adipocytes increase their triglyceride storage capacity through hypertrophy and/or hyperplasia when nutrients are in excess. The hypertrophy induces several cell-intrinsic and extrinsic stress signaling pathways which collectively act to promote inflammation and insulin resistance (Veilleux et al., 2011).

The initial triggers of adipose tissue inflammation are most likely a combination of the classical inflammatory responses and metabolically-related disturbances causing dysregulation of the adipose tissue and deterioration of glucose metabolism (Kammoun et al., 2014). Some of these triggering processes are listed in the following and depicted in Figure II:

- Hypoxia which causes neovascularization, endoplasmic reticulum and oxidative stress with enhanced production of reactive oxygen species (ROS)
- Adipocyte cell rupture and death leading to the formation of crown-like structures
- Activation of intracellular stress- and inflammation-related kinases and signaling pathways (e.g. Jun N-terminal kinase JNK, inhibitor of nuclear factor κ B kinase (IKK β), and NF- κ B)
- Enhancement of pro-inflammatory cytokine/chemokine secretion (e.g. IL-1 β , TNF- α , IL-6, IL-8, and leptin) causing recruitment of more immune cells.
- Accumulation of pro-inflammatory M1 macrophages (classically activated) and effector T-lymphocytes (Teff), such as CD8⁺ T-cells and CD4⁺ T-cells, and depletion of M2 macrophages (alternatively activated), Th2 cells and Tregs
- Decrease of anti-inflammatory mediators (e.g. adiponectin and IL-1ra) and
- Dysregulation in free fatty acids (FFAs) fluxes with an overflow of FFA acting as ligands for the TLRs thus activating innate immune pathways further contributing to the systemic propagation of inflammation

These various processes trigger and perpetuate stress responses that eventually promote obesity-induced chronic inflammation and impairment of insulin action.

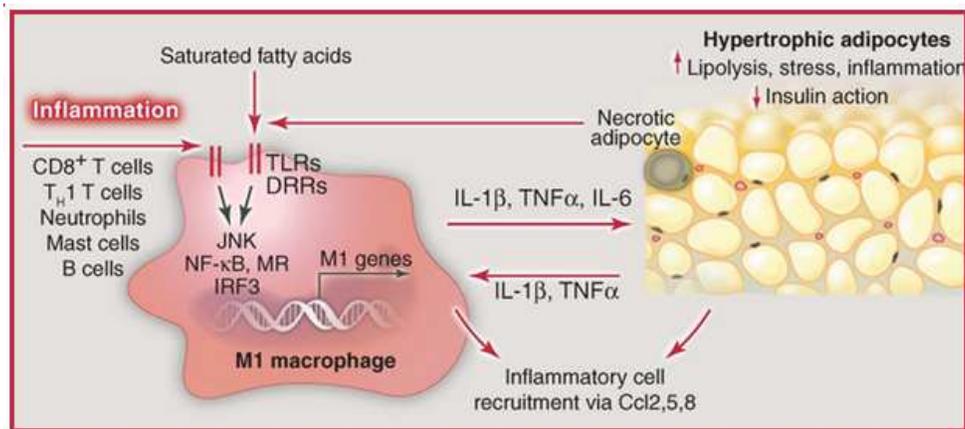


Figure II. Inflammatory responses in the obese state caused by chronic nutrients in excess. There are production of pro-inflammatory mediators, up-regulation of inflammatory gene expression, recruitment of additional leukocytes, cellular stress, all of which reinforce the inflammatory environment leading to impairment of insulin signaling and action in the adipose tissue. Abbreviations not explained in the text: IRF=interferon regulatory factor, MR=mineralcorticoid receptor, Ccl2,5,8=the chemokines MCP-1/2 and RANTES. Figure adapted from Odegaard & Chawla, Science 2013 (Odegaard and Chawla, 2013).

The systemic inflammatory state

The altered secretory profile of the adipose tissue towards production of pro-inflammatory cytokines likely contributes to the low-level systemic inflammation characteristic of obesity and T2D and other metabolic syndrome-associated pathologies. Positive correlations between BMI and systemic inflammatory mediators, such as IL-6, C-reactive protein (CRP), and PAI-1 support that many of the circulating inflammatory mediators could originate directly from the adipose tissue (Weisberg et al., 2003; Xu et al., 2003; Hauner, 2005; Lee and Lee, 2014). It may therefore be speculated that the adipose tissue is a prime organ for augmenting and sustaining systemic inflammation with immune cells from the SVF and adipose-derived secretory factors as important drivers in the cross-talk between adipose tissue inflammation and other metabolic important tissues including the liver, skeletal muscle, pancreas, brain and the vascular wall (Trayhurn et al., 2011). For example, IL-6 secreted in the skeletal muscle may stimulate lipolysis in adipose tissue (Pedersen and Febbraio, 2008) and improve insulin secretion by stimulating pancreatic α -cells to secrete GLP-1 (Ellingsgaard et al., 2011), while adipocyte-derived IL-6 may induce muscle insulin resistance (Kim et al., 2004). Moreover, IL-6 induces hepatic acute-phase reactants such as CRP, serum amyloid A (SAA) and haptoglobin (Heinrich et al., 1990) also implicated in

deterioration of insulin signaling and development of insulin resistance (Scheja et al., 2008; Festa et al., 2002).

Besides the impact of the obesity-induced changes in cellular and molecular inflammatory mediators on deterioration of whole-body homeostasis, the nutrient excess may have direct systemic consequences. When the storage capacity of the adipose tissue is exceeded, the spillover of secretory factors into circulation has important detrimental effects on many organs, ultimately promoting accumulation of lipid metabolites in liver (leading to nonalcoholic fatty liver disease (NAFLD)) and skeletal muscles, impairing insulin signaling in these tissues causing hepatic (central) and whole-body (peripheral) insulin resistance (Guilherme et al., 2008).

Inflammation and insulin resistance

At the cellular level, the molecular mechanism in the development of insulin resistance is impairment or disruption of the insulin signaling cascade in insulin responsive cells, such as myocytes, hepatocytes and adipocytes (Shulman, 2000a; Lee and Lee, 2014). Most studies have focused on the intracellular IRS proteins. The before mentioned obesity-induced activation of cell-intrinsic and extrinsic stress-signaling pathways interferes directly or indirectly with insulin signaling through serine phosphorylation (inactivation) of the IRS-1/2 proteins, leading to abrogation of the association between IRS and the insulin receptor. Stress-related kinases, such as JNK and IKK β , which are affected by several mechanisms, are essential mediators in this process in addition to augmentation of the inflammatory response within the metabolic tissue through their activation of genes involved in inflammation and insulin resistance (Figure III). Activation of inflammatory pathways and the successive disruption of insulin action have been observed in various insulin-responsive tissues. In pancreatic beta-cells, the same and also other cytokine-activated pathways cause cellular stress and ultimately contribute to the development of diabetes by causing beta-cell apoptosis (Donath, 2013).

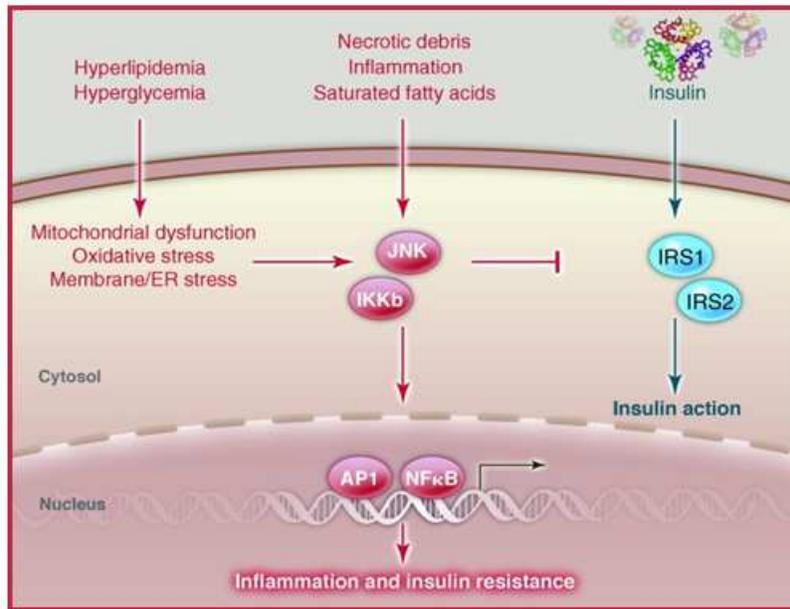


Figure III. Inflammatory signaling pathways that link nutrient excess and associated induction of cellular stress responses to insulin resistance. Abbreviations not explained in the text: AP1=activator protein 1. Figure from Odegaard & Chawla, Science 2013 (Odegaard and Chawla, 2013).

Emerging evidence suggests that obesity-induced inflammation, primarily originating in the adipose tissue, is an important player or even key cause of insulin resistance (Osborn and Olefsky, 2012). The infiltrating macrophages become pro-inflammatory M1 macrophages (classically activated) and effector T-lymphocytes, such as CD8⁺ T-cells and CD4⁺ T-cells accumulates in the adipose tissue, while macrophages of the M2 phenotype (alternatively activated), Th2 cells and Tregs are depleted.

Several pro-inflammatory mediators which are enhanced in obesity have been shown to directly impair insulin action and strong correlations between the degree of infiltrating adipose tissue macrophages (and other pro-inflammatory immune cells from the SVF and insulin resistance has been reported (Koppaka et al., 2013).

The first evidence of this link was established with the finding that increased levels of TNF- α was found in the adipose tissue and circulation of rodents and humans and pharmacological neutralization of TNF- α improved insulin-stimulated glucose uptake in obese rats (Hotamisligil et al., 1993; Hotamisligil et al., 1995). The recent TINSAL-T2D study using salsalate (a pro-drug of salicylate, also known as aspirin), as a broad-specificity anti-inflammatory strategy to target inflammation in T2D patients, reduced circulating leukocytes and lymphocytes and increased adiponectin in addition to an improvement of glycemic control (Goldfine et al., 2013; Goldfine et al., 2010). Clinical trial studying the effect of blocking IL-1 β by

anakinra (recombinant human IL-1 receptor antagonist) on beta-cell function was recently carried out in subjects with impaired glucose tolerance. Treatment for 4 weeks improved insulin secretion compared to placebo. These results supported the concept of blocking inflammatory mediators as part of improving insulin secretion (van Poppel et al., 2014).

Considering the evidence of inflammation as an important common factor in the pathogenesis of obesity-induced comorbidities, could suggest that targeting inflammation may be a viable therapeutic strategy for treatment of these disorders.

Inflammatory markers in obesity and type 2 diabetes

Important mediators in both the innate and adaptive immune response are cytokines, of which a balanced expression of pro- and anti-inflammatory signaling is essential to sustain whole body homeostasis. Cytokines are not only produced by immune cells such as dendritic cells (DCs), macrophages, and lymphocytes, but also non-immune cells such as fibroblasts, adipocytes, endothelial and epithelial cells. They can signal in an autocrine, paracrine or endocrine manner on multiple cell types through interaction with specific receptors.

During infection or inflammation in the tissues, the coordinated migration, activation and recruitment of immune cells, such as macrophages, T-cells and NKT cells results in a sequential production of cytokines that facilitate the proliferation, differentiation, and activation of other immune cells. Each subtype of immune cells can be defined by its cytokine profile. Naïve CD4⁺ T-cells, also known as T helper cells, can be subdivided into: Type 1 T helper (Th1) producing IFN- γ , Type 2 T helper (Th2) producing IL-4, (and IL-5 and IL-13), and regulatory T cells (Tregs), and Type 17 T helper cells (Th17) producing IL-10 (and TGF- β), and IL-17 (and IL-21 and IL-22), respectively (Zhu et al., 2010) (Figure IV).

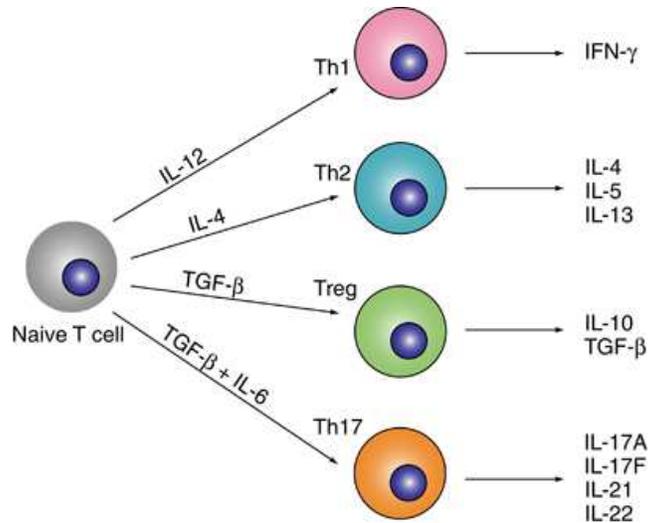


Figure IV: Differentiation of naïve $CD4^+$ T cells. Upon activation by APCs, naïve $CD4^+$ T-cells differentiate into different subpopulations, such as Th1, Th2, Th17, and Treg cells influenced by the local inflammatory milieu (Maniati et al., 2010).

Cytokines interact in a complex network to exert their effects. They have pleiotropic, overlapping, and/or counter regulatory actions, facilitating enhancement or suppression of other cytokines and immune cells. T-cells for example secrete cytokines important for their own differentiation, but IL-12 released from macrophages also contributes in this process. Conversely, IFN- γ - a major Th1 and macrophage-activating cytokine, and IL-4, a classical Th2 cytokine, can polarize macrophages into M1 and M2 phenotypes, respectively (Lumeng et al., 2007). Tregs suppress Th1 responses and macrophage activation by secreting IL-10 and TGF- β . Upon stimulation, macrophages release a large array of cytokines, the most prototypical being IL-1, IL-6, and TNF- α in addition to chemokines, such as MCP-1 and IL-8 that contribute to the recruitment of other immune cells.

In the following section, the most important adipose-derived cytokines in the context of this thesis and the corresponding manuscripts will be described with a focus on the role they play in relation to obesity-induced inflammation and their systemic effects on metabolism.

IL-1 β , TNF- α , and IL-6

IL-1 β is considered a classical pro-inflammatory cytokine playing a fundamental role in the inflammatory cascade and with well-documented detrimental effects on beta-cell function (Maedler et al., 2002; Dinarello, 2009). The contribution of IL-1 β to obesity-induced inflammation and peripheral insulin resistance has only recently gained interest (Boni-Schnetzler and Donath, 2013). Obesity does not appear to influence circulating IL-1 β levels compared to lean controls, however is a risk factor when elevated in

conjunction with enhanced circulating IL-6 (Spranger et al., 2003). Studies in adipocyte cell lines suggest that IL-1 β inhibits insulin action by reducing tyrosine phosphorylation of IRS1 and inhibition of translocation of GLUT-4 to the plasma membrane (Jager et al., 2007). The most direct clinical evidence for the involvement of IL-1 β in insulin resistance has been obtained by blocking the IL-1 β receptor with IL-1 receptor antagonist (IL-1ra), which has been applied as therapeutic in T2D preclinical and clinical trials with subsequent improvement of insulin secretion, glycaemia, and systemic inflammation (van Poppel et al., 2014; Larsen et al., 2007a; Larsen et al., 2007b).

TNF- α is a prototypical inflammatory cytokine with important functions in activation and controlling the adaptive immune system. The role of TNF- α in promoting insulin resistance has been suggested to occur through serine phosphorylation of IRS1 (Hotamisligil et al., 1996). Experiments in obese mice with a targeted null mutation in the gene encoding TNF- α and the two receptors for TNF- α have shown promising results on insulin resistance (Uysal et al., 1997), while studies in humans have been disappointing (Ofei et al., 1996). Circulating TNF- α are increased in obese and/or obese diabetic subjects (Zahorska-Markiewicz et al., 2000; Spranger et al., 2003) (Popko et al., 2010), although studies have also shown no difference from lean controls (Carey et al., 2004).

IL-6 is generally described as a pleiotropic cytokine regulating innate immunity and the acute-phase response. IL-6 is produced mainly by immune cells but under conditions of obesity the source of IL-6 comprises adipocytes and the recruited immune cells in the SVF, predominantly macrophages (Fried et al., 1998; Weisberg et al., 2003). In addition, IL-6 is produced by skeletal muscles during exercise (Steensberg et al., 2000).

IL-6 functions in a tissue-specific and physiological context-dependent manner, and may play both a harmful and a protective role in the development of insulin resistance (Kim et al., 2004; Pedersen and Febbraio, 2007; Mauer et al., 2014). The observations of increased circulating IL-6 in patients with the metabolic syndrome (Weiss et al., 2004; Bastard et al., 2000) and murine clamp experiments showing that infusion of IL-6 results in reduced insulin-stimulated glucose uptake into skeletal muscles (Kim et al., 2004) and spontaneous insulin resistance (Hong et al., 2009) has led to the prevailing dogma of IL-6 as a pro-inflammatory cytokine linked to the development of insulin resistance and T2D. On the other hand, clamp experiments in healthy humans have shown that infusion of IL-6 increased insulin-stimulated glucose disposal and uptake, possibly by increasing GLUT4 translocation to the plasma membrane (Carey et al., 2006; Carey et al., 2004). A recent study reported that IL-6-signaling in murine myeloid cells augmented the responsiveness of macrophages towards an M2 state induced by IL-4 (Mauer et al., 2014). Finally, Ellingsgaard *et al.* recently demonstrated that enhancing circulating IL-6, either through high fat

diet (HFD) or exercise increased production and secretion of GLP-1 leading to improved beta cell insulin secretion and glucose tolerance (Ellingsgaard et al., 2011).

IL-8 is a chemokine secreted by immune cells, but is also produced and released from human adipocytes (Bruun et al., 2001). *IL-8* plays a role in a number of inflammatory processes, such as atherosclerotic processes (Moreau et al., 1999; Boekholdt et al., 2004). Circulating levels of *IL-8* are enhanced in obesity (Bruun et al., 2003; Tateya et al., 2010), in T1D and T2D patients compared to lean healthy subjects (Zozulinska et al., 1999) and to obese subjects with normal glucose tolerance (Strackowski et al., 2003; Hardy et al., 2011). *In vitro* experiments have shown that *IL-8* impairs adipocyte insulin signaling by inhibiting phosphorylation of the Akt kinase (Kobashi et al., 2009).

IL-10 and TGF- β

IL-10 is a classical anti-inflammatory and immune-modulatory cytokine that can be produced by different cell types in humans, including monocytes, macrophages, and lymphocytes, especially Tregs (Moore et al., 1993). Enhanced circulating concentrations of *IL-10* have been found in obese human subjects (Esposito et al., 2003) and in the adipose tissue (Juge-Aubry et al., 2005) compared to lean subjects. However, reduced levels of *IL-10* in patients with metabolic syndrome has also been found (Strackowski et al., 2005). Overexpression of *IL-10* in diet-induced obese (DIO) mice induced an increase in M2 macrophages in the adipose tissue (Fujisaka et al., 2009).

TGF- β is a pleiotropic immunoregulatory cytokine involved in maintaining immune homeostasis under steady-state conditions and with responses depending on the cellular and environmental context characterized by the presence of various immune cells and cytokines (Li et al., 2006). In the presence of *IL-6*, *TGF- β* promotes an immune response by inducing differentiation of pro-inflammatory Th17 cells (Bertola et al., 2012), while the ability to suppress an immune responses occurs by a *TGF- β* -mediated generation of Tregs (Wan and Flavell, 2007a; Wan and Flavell, 2007b). The roles of *TGF- β* in the pathogenesis of T2D are conflicting. Circulating *TGF- β* has been found to correlate with increased adiposity and increased risk of developing T2D (Herder et al., 2009; Yadav et al., 2011; Alessi et al., 2000). In contrast, *TGFB1* expression in skeletal muscles of obese T2D subjects have shown to correlate inversely with HbA1c and fasting plasma glucose, and therefore suggest that *TGF- β* is linked to improved glucose metabolism (Fink et al., 2013).

Adiponectin and leptin

Adiponectin was originally identified as a protein exclusively secreted by the adipocyte (Scherer et al., 1995), i.e. a prototypic adipokine. Newer studies have established that also non-fat cells can secrete the

protein (Fain, 2006). Systemic adiponectin concentrations are high in lean individuals (Fain, 2006), but during obesity, adiponectin concentrations are decreased (Turer et al., 2011). Beneficial properties of adiponectin have been linked to different conditions of the metabolic syndrome including insulin resistance, T2D, and CVD (Weyer et al., 2001; Stefan et al., 2002; Lim et al., 2014). Circulating adiponectins are among the most potent predictors of insulin sensitivity in obese individuals (Li et al., 2009; Kloting et al., 2010).

Adiponectin regulates lipid and glucose metabolism and holds insulin-sensitizing, anti-apoptotic, and anti-inflammatory properties (Turer and Scherer, 2012). In the context of an inflammation-induced impairment of insulin sensitivity, adiponectin have shown beneficial effects by promoting a shift of adipose-resident macrophages into the M2 phenotype and reduce the production of IL-6, TNF- α , and ROS from adipocytes and immune cells in the SVF (Ohashi et al., 2010; Wolf et al., 2004; Dietze-Schroeder et al., 2005), increase FFA oxidation by up-regulating AMPK, and inhibit JNK and NF- κ B activation (Wolf et al., 2004; Ohashi et al., 2010; Folco et al., 2009; Kumada et al., 2004).

Leptin is secreted by adipocytes with a primary physiological role as appetite regulator acting upon the hypothalamus to restrain food intake and increase energy expenditure (Pellemounter et al., 1995). Circulation leptin concentrations are positively correlated to fat mass, but enhanced levels in obese subjects are not suppressive of food intake possibly due to leptin resistance (Jung and Kim, 2013). Leptin-deficiency is associated with increased obesity and insulin resistance and exogenous administration of leptin into leptin-deficient mice restores metabolic dysfunction and induces weight loss.

The actions of leptin are primarily related to the metabolism; however, studies have indicated an immunological role of leptin in the Th1 immune response by stimulating the production of pro-inflammatory cytokines, such as IL-6, and suppressing anti-inflammatory cytokines, such as IL-4 in states of immune dysfunction (Bullo et al., 2003; Brennan and Mantzoros, 2006). Studies in adipose tissue-infiltrating Tregs have suggested a direct effect of leptin on suppressing proliferation of Tregs (Matarese et al., 2001; De, V et al., 2007), although controversy on this matter exist (Feuerer et al., 2009).

Immune cells in obesity and type 2 diabetes

Cells of the immune system are classically divided into the innate (e.g. macrophages, neutrophils, mast cells, NK cells) and the adaptive (B- and T-lymphocytes) arm of the immune system. Innate immune responses are characterized by a rapid sensing an elicitation of immune cell functions following activation

of their PRRs, while the adaptive immune response involves a tightly regulated response to self- and non-self-antigens and development of immunological memory (Janeway, Jr. and Medzhitov, 2002).

For many years, macrophages have gained attention as important key players in adipose tissue inflammation and insulin resistance (Weisberg et al., 2003; Lee, 2013). In recent years attention has also involved T-cells, as these can modify macrophage activation, and there is therefore currently a growing interest in the role of T-cell in obesity-related adipose tissue inflammation and insulin resistance (Kintscher et al., 2008; Nishimura et al., 2009; Winer et al., 2009; Zeyda et al., 2011).

Regulatory T-cells

Natural CD4⁺ Tregs develop in the thymus and play a crucial role in maintaining peripheral tolerance, inhibiting autoimmunity, and restraining chronic inflammatory diseases (Sakaguchi et al., 2008). Circulating Tregs represent 1 – 10% of thymic and peripheral CD4⁺ T-cells. The most important subsets of Tregs are the naturally occurring thymus-derived CD4⁺CD25⁺Foxp3 Tregs, referred to as nTregs (Sakaguchi et al., 1995) and the induced Tregs (also known as adaptive Tregs) that develop in the periphery from conventional T-cells in the presence of TGF- β (Sakaguchi et al., 1995; Curotto de Lafaille and Lafaille, 2009).

Tregs functional characteristics are their ability to suppress activation marker expression, proliferation, as well as pro-inflammatory responses in a wide variety of immune cells from both the innate and the adaptive immune system. They require specific T-cell receptor (TCR)-mediated activation and the presence of IL-2 to elicit specific suppressive activity on their target cells (de la Rosa et al., 2004; Fontenot et al., 2005). T-effector cell (Teff) suppression by Tregs can occur directly, i.e. in the absence of antigen presenting cells (APCs), involving a combination of cell-cell contact-dependent mechanisms (e.g. CD40–CD40L) and production of immune-regulatory cytokines such as IL-10 (and TGF- β and IL-35) (von Boehmer H., 2005; Sakaguchi et al., 2009) (Figure V). In addition to a direct effect on Teff cells, Tregs modulates the function of APCs inhibiting the differentiation of naïve T-cells into Teff cells (Tadokoro et al., 2006).

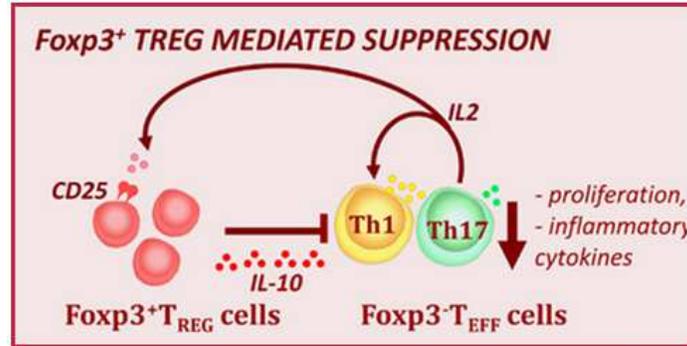


Figure V. Tregs are dependent on Teff-derived IL-2 for mediating suppressive function. Tregs produce the immunosuppressive cytokine IL-10 and mediates suppression of Teff cells and downregulation of pro-inflammatory cytokines and Teff proliferation. Figure from Kornete *et al.* (Kornete et al., 2013).

While Tregs have been intensively investigated with respect to autoimmunity and tolerance in T1D (Kornete et al., 2013), the role of Tregs in diet-induced obesity (DIO) and in obesity-induced insulin resistance have only recently been reported (Winer et al., 2009; Ilan et al., 2010; Feuerer et al., 2009; Deiluiis et al., 2011; Eller et al., 2011). Data from experimental studies in DIO mice and obese humans on the potential role of Tregs in adipose tissue inflammation and insulin resistance are conflicting. While some studies report reduction of Tregs residing in adipose tissue (Feuerer et al., 2009; Deiluiis et al., 2011), other studies have reported enhanced proportions of Tregs in the adipose tissue (Zeyda et al., 2011; Winer et al., 2009). Differences in the applied analytical methods for detection and enumeration of Tregs might be a possible explanation for the inconsistencies. Indeed, adoptive transfer of Tregs in an animal model has been shown to improve insulin resistance and inhibit the abundance of cytotoxic CD8⁺ T-cells in the obese adipose tissue (Eller et al., 2011). Ilan and co-workers reported that injecting *ex vivo* expanded Tregs into *ob/ob* mice protected against weight gain and alleviated metabolic abnormalities (Ilan et al., 2010).

Studies on whether and how human obesity affects circulating Treg cells are limited. Reduced numbers and impaired function of circulating Tregs have been observed in patients with acute coronary syndrome (de Boer et al., 2007), and cutaneous immune-mediated skin diseases, such as psoriasis and atopic dermatitis, are also associated with down-regulation of circulating Treg cells (Quaglino et al., 2013; Kagen et al., 2006). Recent studies in obese subjects have reported decreased number of circulating Tregs compared to lean controls, with a negative correlation to markers of adiposity (body weight, BMI and leptin), systemic inflammation, and glucose intolerance (Yun et al., 2010; Wagner et al., 2013).

Invariant Natural Killer T-cells

Natural Killer T-cells (NKTs) are a subset of T-lymphocytes that, as the name imply, co-express markers for both NK and T-cell lineages. Like conventional T-cells, NKTs express a TCR that, unlike conventional T-cells, reacts with a limited repertoire of lipid or glycolipid antigens presented in context with the MHC-class I-like glycoprotein CD1d. CD1d is expressed on APCs including macrophages, B-cells and non-hematopoietic cells such as hepatocytes and adipocytes (Porcelli, 1995). There are two different subsets of NKTs; NKT type I and NKT type II, of which the type I NKT cells, also known as iNKTs is the most widely studied of the two types. It is characterized by an invariant TCR α chain consisting of V α 14J α 18 gene segments in mice and V α 24J α 18 in humans (Bendelac et al., 2007).

A major function of activated iNKTs is to exert immunoregulatory control by transactivating other immune cells, such as macrophages and DCs. iNKTs are activated either by the synthetic marine sponge-derived glycolipid, α -galactosylceramide (α -GalCer) (Kawano et al., 1997) or with endogenous or bacterial derived lipid ligands (Facciotti et al., 2012). Upon activation, the iNKTs respond rapidly by secreting various cytokines. These include IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF- α , IFN- γ , and granulocyte monocyte-colony stimulating factor (GM-CSF), and chemokines such as RANTES and MIP-1 α that regulate immune responses (Coquet et al., 2008). The activation of iNKTs involves two pathways: i) directly through engagement of the TCR with glycolipid binding to the CD1d complex (Figure VIa), or ii) indirectly by exposure to various cytokines, such as IL-2 and IL-18 or IL-12 and interferons, through activated APCs and the constitutive expression of cytokine receptors by the iNKT (Figure VIb) (Van et al., 2011). Depending on the strength of the TCR signal or the mode of activation (directly or indirectly), the response may be more or less Th1 (e.g. IFN- γ) or Th2 (e.g. IL-4 and IL-13) influenced. Differences in cytokine production can also be ascribed to the distinct CD4⁺ or CD4⁻ iNKT subtypes, with CD4⁺ iNKTs inducing Th1 cytokine secretion, while the CD4⁻ subtype results in a mixture of Th1 and Th2 cytokines (Watarai et al., 2012; Brennan et al., 2013). Lastly, there seems to be organ- or tissue-dependent variations in the responses of iNKT activation (Ji et al., 2012a).

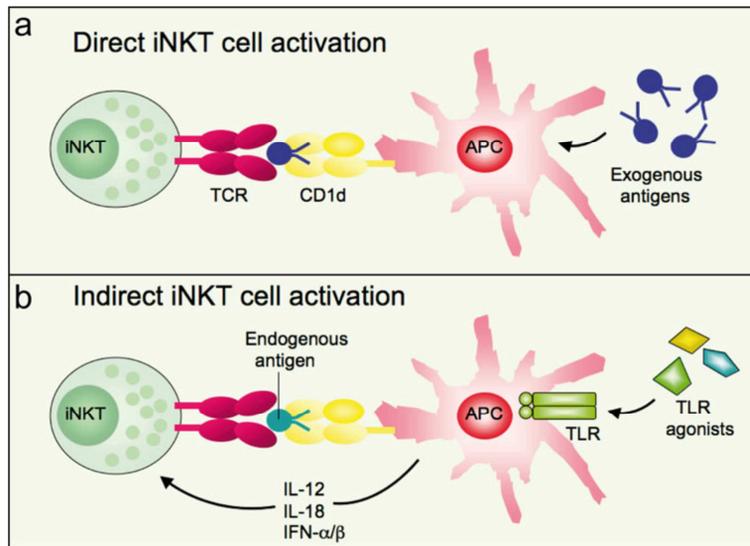


Figure VIa-b. Direct and indirect iNKT cell activation. Figure from Van *et al*, 2011 (Van et al., 2011).

After iNKT maturation in the thymus, a substantial number of cells remain in the thymus, while the remaining iNKTs migrate to peripheral tissues, and are represented in blood, spleen, liver, and bone marrow and as recently discovered in adipose tissue (Lynch et al., 2012). Evaluation of iNKTs in human blood is complicated because of: i) their low frequency ranging from 0.01 – 1% and the largest individual genetic based differences in frequency among the peripheral T-lymphocytes (Bendelac et al., 2007) and ii) the lack of standardized reagents specific for iNKT identification (Godfrey et al., 2010). The adipose tissue of lean rodents and humans contains large numbers of iNKTs, while the numbers are reduced in obese adipose tissue (Schipper et al., 2012; Lynch et al., 2012; Lynch et al., 2009; Ji et al., 2012b). Similar observations have been made of circulating iNKTs in obese patients compared to lean, healthy individuals, and interestingly their numbers increase following weight loss interventions either through surgery or diet (Lynch et al., 2012).

Modulation of iNKT number and function in mice, either by α -GalCer injections or adoptive transfer of iNKTs, induced an increase in iNKT-mediated secretion of IL-4 and IL-10 and decrease in IFN- γ , coupled with a polarization of M1 macrophages into anti-inflammatory M2 macrophages. In addition, weight loss, normalization of glucose homeostasis, and an increase in insulin sensitivity was observed (Hams et al., 2013; Ji et al., 2012a; Ji et al., 2012b; Lynch et al., 2012).

The role of iNKTs in obesity-induced inflammation and insulin resistance in humans is, however, controversial. While some studies suggest a beneficial role of iNKTs in metabolic diseases (Ji et al.,

2012a; Ji et al., 2012b; Lynch et al., 2012; Schipper et al., 2012; Wu and Van, 2013; Wu et al., 2012; Hams et al., 2013), others have not found iNKTs to be protective of obesity-induced inflammation (Mantell et al., 2011; Wu et al., 2012). Some of these discordant observations could, however, be due to different analyses using tetramers or strictly anti-TCR and anti-CD56 or analyzing different subpopulations

Reduced numbers and impaired function of circulating iNKTs have been found in other immune-mediated and auto-immune diseases (van der Vliet et al., 2001), such as rheumatoid arthritis and T1D (Hammond and Kronenberg, 2003; Novak and Lehen, 2011), and recently in obese diabetic psoriasis patients (Ahern et al., 2013; Hogan et al., 2011).

Roux-en-Y gastric bypass

Weight loss surgery (bariatric surgery), which includes a range of procedures that alter food transit through the gastrointestinal (GI) tract, has proven to be a much more effective treatment for obesity than non-surgical interventions. The procedure is recommended for patients with severe obesity (BMI ≥ 40 kg/m² or in patients with co-morbidities BMI ≥ 35 kg/m²) (Sjostrom, 2013). Roux-en-Y gastric bypass (RYGB) (Figure VII) is the most common bariatric operation and accounts for > 95% of the operations in Denmark, followed by vertical sleeve gastrectomy, gastric banding, and the less common biliopancreatic diversion (Buchwald and Oien, 2013).

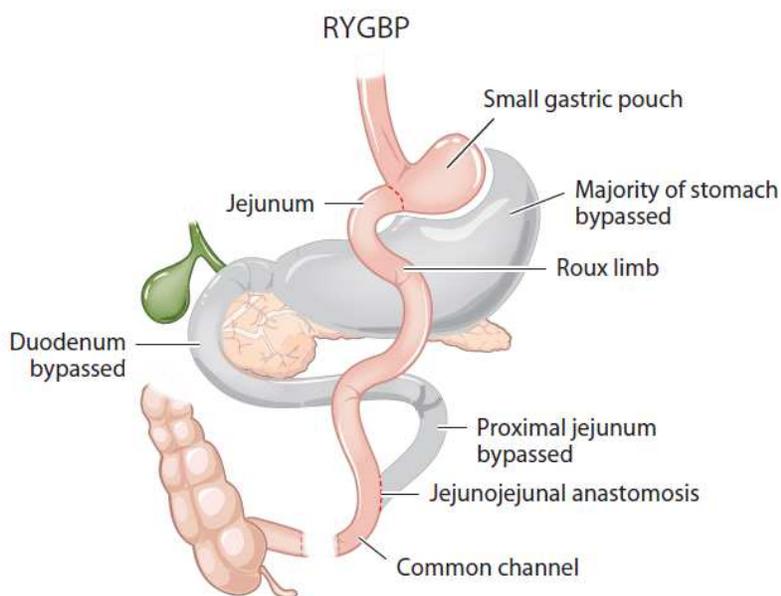


Figure VII. In the RYGB procedure, a small gastric pouch is created and anastomosed with the mid-jejunum. As a result, nutrients bypass the major part of the stomach, the duodenum and the upper part of the jejunum and enter the distal small intestine much faster. Figure adapted from (Manning and Batterham, 2014).

Effects of Roux-en Y gastric bypass on metabolism

Walter Pories announced in 1995 that bariatric surgery was a potential “cure” to diabetes (Pories et al., 1995). Since then, several studies have shown both short- and long-term beneficial metabolic effects of RYGB in obese diabetic and non-diabetic humans including:

- sustained weight loss for at least 15 years in most patients (20-30% of total body weight) (Sjostrom et al., 2012)
- an early (2-6 days) weight-independent improvement of glycemic control and insulin sensitivity (le Roux et al., 2007) (Wickremesekera et al., 2005).
- a reduction in obesity-related co-morbidities such as hypertension and cardiovascular diseases (Adams et al., 2007; Sjostrom et al., 2012) and remission of T2D (Adams et al., 2012)

The exact mechanisms for the immediate and long-term positive effect of RYGB on metabolic homeostasis remain unclear. The anatomical changes in the gastrointestinal system after RYGB allow nutrients to bypass the proximal part of the small intestine and reach the distal small intestine much faster, where high density of gut hormone-secreting enteroendocrine L-cells, source of GLP-1, oxyntomodulin, and peptide YY (PYY), are located. The altered transit of nutrients through the gastrointestinal tract causes secretory changes in these hormones. These observations have led to the hindgut (or lower gut) hypothesis (Pories et al., 1995) to explain the improvement of glucose tolerance and insulin resistance after RYGB. Fasting levels of GLP-1 remain unchanged following RYGB, but postprandial GLP-1 secretion increases several fold (Umeda et al., 2011; Falken et al., 2011; Jorgensen et al., 2012) as early as 2 days after surgery (le Roux et al., 2007). Thus, GLP-1 appears to be the key hormone in the ameliorating effect of RYGB on type 2 diabetes. The improvement in type 2 diabetes within the first days after surgery before any weight loss has led to the hypothesis that perioperative caloric restriction is responsible, primarily by increasing insulin sensitivity in the liver, maybe by a reduction in liver fat content (Madsbad et al., 2014).

There is considerable interest in the physiological mechanisms by which RYGB resolves the co-morbidities of obesity, including diabetes. Clarification of these mechanisms may lead to the development of new drugs for the treatment of T2D and other obesity related morbidities.

Effects of Roux-en Y gastric bypass on inflammatory mediators

A growing interest in a relationship between obesity-induced chronic inflammation and metabolic co-morbidities including insulin resistance, has prompted studies on immunological changes after bariatric

surgery. Indeed, changes in adipose-derived cytokines and immune cell populations could represent an alternative explanation to the post-operative improvement of glycemic control. Weight reduction resulting from bariatric surgery or nutritional intervention significantly improves the systemic and adipose tissue inflammatory states associated with obesity (Forsythe et al., 2008). The majority of obesity-related cytokines and their variations in relation to weight reduction are, however, far from established and additional studies are needed to determine whether reduced adipose-tissue inflammation following bariatric surgery is responsible for reduced systemic inflammation and improved insulin sensitivity.

Research to date has focused on common circulating pro-inflammatory cytokines, such as CRP, IL-6 and leptin, while changes in anti-inflammatory cytokines are more scarcely reported (Table I)

Adipose-derived cytokines	Pro (P) /anti (A)- Inflammatory	≤ 6 months	≥ 1 year
IL-6	P	↓→	↓
TNF- α	P	→↑	↓→
CRP	P	↓	↓
MCP-1	P	→	↓→
IL-8	P	No data	→
Leptin	P	↓	↓
Adiponectin	A	→↑	↑
IL-1ra	A	→↓	↓
IL-10	A	→	No data

Table I. Changes in circulating adipose-derived pro/anti-inflammatory mediators up to 6 months and 1 year or more after RYGB. More than one arrow means that data are conflicting; → = no change, ↓ = decreased, ↑ = increased. (Vendrell et al., 2004; Swarbrick et al., 2006; Lin et al., 2007; Swarbrick et al., 2008; Trakhtenbroit et al., 2009; Miller et al., 2011; Brethauer et al., 2011; Dalmás et al., 2011; Alvehus et al., 2012; Illan-Gomez et al., 2012; Viana et al., 2013).

The majority of inflammatory cytokines are decreased one year or more following RYGB (Table 1). In contrast, adiponectin is so far the only anti-inflammatory cytokine that has been reported to be increased after surgery, despite inconsistency in when the increase in adiponectin concentrations following RYGB is observed. Some studies have found increases in adiponectin appearing between one and three months

after surgery (Lin et al., 2007; Brethauer et al., 2011), while others report lack of increase until two years after RYGB (Trakhtenbroit et al., 2009), which may suggest that either a certain amount of weight loss, a period of weight stabilization or improvement of the metabolic instability is required before changes in plasma adiponectin are manifested. Nevertheless, increases in adiponectin often reflects surgery-induced weight loss (Faraj et al., 2003; Butner et al., 2010) and improvement of insulin resistance (Faraj et al., 2003; Swarbrick et al., 2006; Lin et al., 2007; Brethauer et al., 2011). Data on the other anti-inflammatory cytokines, such as IL-10 and IL-1ra following RYGB are few and inconclusive to date (Brethauer et al., 2011; Donath et al., 2013).

Leptin is a frequently measured adipokine in bariatric surgery follow-up studies, and the reduction in concentrations has been found as early as 1-2 weeks after RYGB (Rubino et al., 2004; Korner et al., 2009; Isbell et al., 2010; Falken et al., 2011; Jorgensen et al., 2012). Long-term RYGB studies show that changes in leptin correlate with the degree of weight loss after surgery implicating that leptin is a reliable marker of fat mass (Korner et al., 2009), however, the early changes in leptin before weight loss is obtained, suggest that other surgery-related factors in addition to loss of fat mass, as for example reduction in energy expenditure and activation of neuroendocrine responses, are involved in regulating leptin (Ahima et al., 1996; Landt et al., 2001).

The effects of bariatric surgery on IL-6 concentrations are conflicting, with some studies reporting decrease in IL-6 within six months after surgery (Vendrell et al., 2004), while others have not been able to detect any changes (Brethauer et al., 2011; Laimer et al., 2002; Vazquez et al., 2005; Miller et al., 2011). Reductions in IL-6 concentrations is more consistent one year after RYGB (Swarbrick et al., 2008; Viana et al., 2013) or other types of bariatric surgery procedures (Kopp et al., 2003; Illan-Gomez et al., 2012; Kopp et al., 2005) reporting one-year follow-ups. Changes in IL-6 have been found to correlate with improvements in BMI (Swarbrick et al., 2008), fasting plasma insulin, and HOMA (Illan-Gomez et al., 2012).

The incretin glucagon-like peptide-1 (GLP-1)

Incretins are gut-derived hormones with the primary function of promoting postprandial insulin secretion, of which GLP-1 is a prototypical example. This peptide is synthesized in response to incoming nutrients and secreted by enteroendocrine L-cells, present in the ileum and colon (Eissele et al., 1992). The expression and release causes i) insulin release from beta-cells in the pancreas, ii) reduction of gastric emptying and induction of satiety, and iii) inhibition of glucagon release from the α -cells in the pancreas (Asmar and Holst, 2010). GLP-1 and gastric inhibitory polypeptide (GIP), another incretin hormone, are responsible for the incretin effect, i.e. a greater degree of insulin secretion in response to oral than to an

isoglycaemic intravenous glucose infusion. In patients with T2D, the incretin effect is reduced or in some cases absent (Nauck et al., 1986). Administering pharmacological levels of GLP-1 or a GLP-1 receptor agonist in this patient group have shown to restore the insulin secretory function (Vilsboll et al., 2007), and this peptide hormone is an attractive and applied therapeutic approach in managing T2D. Furthermore, treatment with a GLP-1 receptor agonist induces a weight loss of about 2-4 kg.

Secretion and regulation of GLP-1

GLP-1 is produced via posttranslational processing of proglucagon in the L-cells. Recent studies have revealed that other gut endocrine cell types along the length of the intestinal tract, such as K-cells and I-cells, co-express GLP-1 and other peptide hormones, such as GIP, PYY, and cholecystokinin (CCK) (Habib et al., 2012). The proglucagon precursor is also present in α -cells, and is processed to glucagons, but these cells are also capable of producing and secreting bioactive GLP-1 (Nie et al., 2000; Thyssen et al., 2006). Ellingsgaard and co-workers recently reported increased GLP-1 production in rodent and human α -cells mediated by elevated IL-6 concentrations either via exercise or exogenous IL-6 administration (Ellingsgaard et al., 2011).

Ingestion of glucose, short-chain fatty acids, and amino acids stimulate vesicular GLP-1 release from the L-cells into circulation by direct receptor interactions (Diakogiannaki et al., 2012). The majority of secreted bioactive GLP-1 (7-36) is degraded by the DPP-4 enzyme cleaving alanine in position 2 in the N-terminal of bioactive GLP-1 to give inactive GLP-1(9-36amide) (Figure VIII) (Nauck et al., 2003; Drucker and Nauck, 2006). Only about 10-15 % of the secreted GLP-1 reaches the peripheral vessels in the active form.

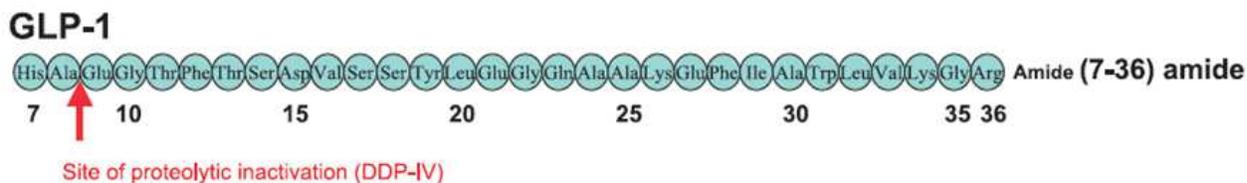


Figure VIII. Amino acid sequence and other structural characteristics of GLP-1. Red arrow show the site of proteolytic cleavage by DPP-4(IV). Figure adapted from (Nauck et al., 2003).

Due to DPP-4 cleavage, the plasma half-life of intact GLP-1 is only 1-2 minutes (Deacon et al., 1995) limiting the use of native GLP-1 in the clinic, and as earlier mentioned pharmacological inhibition of DPP-4, to increase levels of bioactive endogenous GLP-1 or administration of DPP-4 resistant GLP-1R agonists with prolonged half-life are successfully applied in the treatment of T2D.

Actions of GLP-1 receptor agonists

Pharmacological levels of GLP-1 have a diverse array of biological actions mediated either via direct target cell receptor interaction or via activation of central GLP-1Rs. The GLP-1R is a G-protein-coupled receptor (GPCR) with a relatively large extracellular N-terminal domain of >150 amino acids, containing three conserved disulfide bonds for structural stability and a cleavable signal peptide for plasma membrane translocation. The N-terminal receptor domain is responsible for the high affinity binding of endogenous peptide hormone ligands. The GLP-1 receptor signals primarily through G α s proteins to i) activate adenylate cyclases, ii) elevate cellular levels of cAMP, and iii) activation of PKA leading to downstream phosphorylation of cellular targets and release of calcium from intracellular stores via IP3 receptors on the endoplasmic reticulum (Holst, 2007). GLP-1Rs are expressed in various organs and tissues, including pancreatic islets, brain, kidney, heart, gastrointestinal tract (Holst, 2007; Park et al., 2007), and as recently found, on various murine and human immune cell populations (Hadjiyanni et al., 2010; Marx et al., 2010; Hogan et al., 2011).

The mechanisms of actions, including pancreatic and extrapancreatic effects of GLP-1R agonists are summarized in Figure IX.

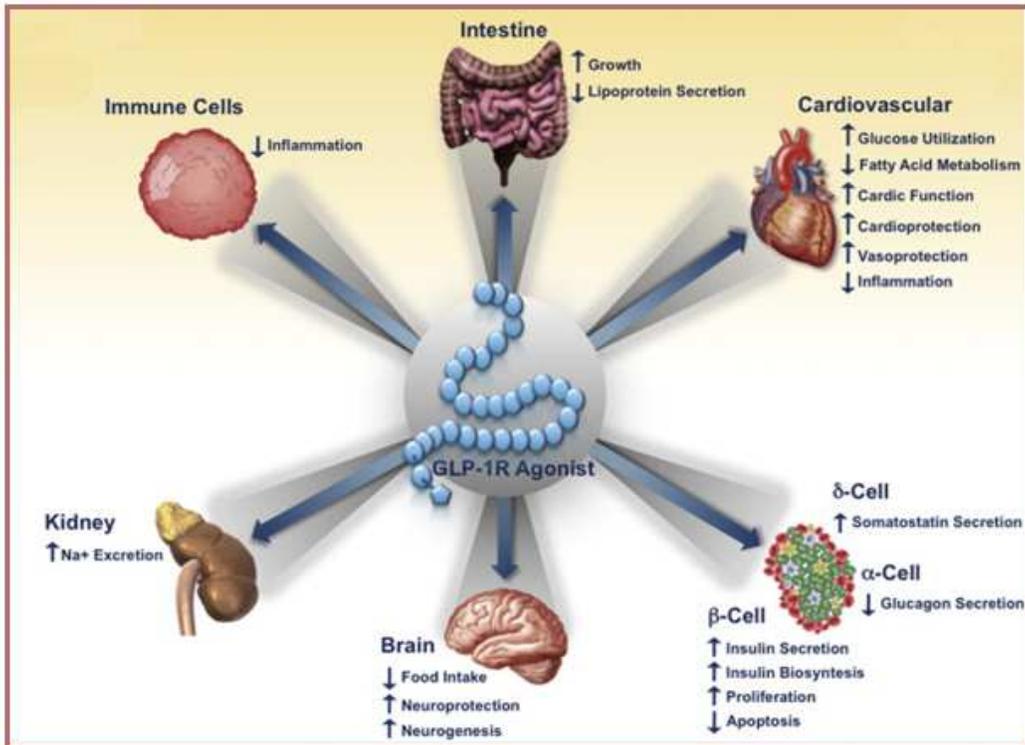


Figure IX. Direct pharmacological actions of GLP-1R agonists. The diverse biological actions of GLP-1R agonists covering pancreatic and extrapancreatic actions. Figure from (Campbell and Drucker, 2013).

The primary indication for GLP-1R agonist treatment is to improve glycemic control in diabetic patients via increased glucose-dependent insulin release and inhibition of glucagon secretion, but additionally, GLP-1R activation causes a range of other effects, some of which are beyond the glucose-lowering effects.

The pancreatic islets, where GLP-1R activation in animal studies increases β -cell proliferation and inhibits apoptosis leading to increased β -cell mass and functionality in terms of insulin biosynthesis and secretion. Moreover, glucagon release from the α -cells is inhibited (Buteau et al., 2004; Drucker, 2006; Asmar and Holst, 2010).

The brain, where GLP-1R-signaling increases satiety in brain (Kanoski et al., 2011) leading to reduced food intake. In animal studies have in addition a range of neuroprotective properties been reported as protection against Alzheimer disease and apoplexia cerebri (Hunter and Holscher, 2012).

The heart, where GLP-1R agonists have shown cardio-protective actions, such as improved cardiac function and decreased inflammation (Noyan-Ashraf et al., 2013). Both animal and human studies have indicated that treatment with GLP-1 or a GLP-1 receptor agonist reduces infarct size after an acute myocardial infarction (Drucker, 2006).

The intestines, where GLP-1R signaling affects intestinal lipoprotein metabolism (Anagnostis et al., 2011).

The kidneys, where GLP-1R agonists have shown to increase sodium excretion (Filippatos and Elisaf, 2013).

The immune system, where GLP-1R-signaling regulates various subpopulations of murine and human lymphocytes and GLP-1 agonists have shown to possess anti-inflammatory and immunoregulatory effects in various *in vitro* and *in vivo* settings, which will be explained in more details in the following section.

Anti-inflammatory and immunoregulatory effects of GLP-1

Administration of GLP-1R agonists has been associated with suppression of inflammation *in vitro* (Kim Chung et al., 2009; Hattori et al., 2010; Ishibashi et al., 2011) and *in vivo* (Hogan et al., 2014; Chaudhuri et al., 2012; Wu et al., 2011; Dozier et al., 2009).

In vitro studies have shown that suppression of the NF- κ B pathway, which can be induced by ROS, IL-1 β , TNF- α , and LPS, appears to be a general anti-inflammatory action of GLP-1 (Kodera et al., 2011; Hattori et al., 2010). NF- κ B-deficient mice are protected from high-fat diet induced insulin resistance (Wunderlich et al., 2008), possibly due to the inhibition of pro-inflammatory cytokine production (Schmidt et al., 2008; De Souza et al., 2005). Treatment of human umbilical endothelial cells with liraglutide reduced TNF- α -induced inflammatory responses, such as ROS and MCP-1 (Hattori et al., 2010; Shiraki et al., 2012). Similar, LPS-stimulated inflammatory responses were inhibited by exendin-4, a GLP-1 analog, in cardiomyoblasts (Chen et al., 2012), 3T3-L1 adipocytes (Lee et al., 2012), and in human peripheral mononuclear cells (PBMCs) (Hogan et al., 2014). In *ob/ob* mice, treatment with recombinant adenovirus producing GLP-1 inhibited macrophage infiltration and adipose tissue expression and production of IL-6, TNF- α , and MCP-1 (Lee et al., 2012).

Hadjiyanni and colleagues detected widespread expression of mRNA transcripts encoding the GLP-1R in immune cells from bone marrow, spleen, thymus and peripheral lymph nodes (Hadjiyanni et al., 2010). In human isolated CD4⁺ lymphocytes, exendin-4 reduced chemokine-induced migration in a concentration-dependent manner by inhibition of the PI3-K pathway (Marx et al., 2010), whereas thymocytes and

lymphocytes from GLP-1R knockout mice were hyper-proliferative in response to mitogenic stimulation (Hadjiyanni et al., 2010), supporting a role of GLP-1R signaling in regulating lymphocyte proliferation and/or migration in murine and human immune cells. Along the same line, GLP-1R activation by exendin-4 or liraglutide stimulated subpopulations of murine and human T-lymphocytes, by both increasing the frequency and improving the function of these cells in recently diagnosed diabetic NOD mice and obese humans (Xue et al., 2008; Hadjiyanni et al., 2008; Hogan et al., 2011).

The intriguing findings of GLP-1Rs on mouse keratinocytes (List et al., 2006) prompted studies on the effect of GLP-1R activation in the skin (Figure X).

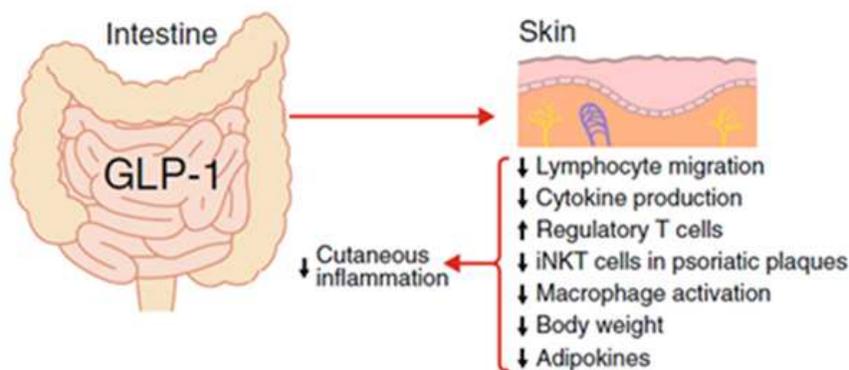


Figure X. Physiological effects of GLP-1 in the skin summarizing anti-inflammatory and immune-regulatory actions by GLP-1 that may result in down-regulating cutaneous inflammation. Figure adapted from (Drucker and Rosen, 2011).

Faurschou and co-workers did not find GLP-1Rs on human keratinocytes, but instead suggested that infiltrations with immune cells expressing the GLP-1Rs could explain previous observations (Faurschou et al., 2013). Experiments in psoriasis patients have shown that GLP-1 therapy with liraglutide or exendin-4 has resulted in an immediate and unexpected reduction in the emergence and size of psoriatic plaques (Hogan et al., 2011; Buysschaert et al., 2012; Faurschou et al., 2013; Ahern et al., 2013). In addition, changes in number and function of iNKTs, reduced macrophage activation, and decreased monocyte production of $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 occurred after commencing liraglutide therapy (6 and 10 weeks, respectively) and were independent of changes in glucoregulation and weight loss (Hogan et al., 2011; Ahern et al., 2013). Similar, exenatide, a synthetic version of Ex-4, administered for 12 weeks in T2D subjects reduced circulating markers of inflammation in mononuclear cells independent of body weight changes (Chaudhuri et al., 2012).

Although information on the immunological effects of GLP-1 and whether the GLP-1R exerts direct effects on immune cell populations are scarce, GLP-1R activation appears to affect multiple immune parameters that could improve adipose tissue and systemic inflammation.

OBJECTIVE AND HYPOTHESES

Rationale

The findings of a direct GLP-1-mediated effect on lymphocyte subpopulations *in vitro* and *in vivo* and the few but interesting case reports on clinical improvements in psoriasis patients treated with GLP-1 receptor agonists have supported immune-regulatory actions of GLP-1. In addition, the effects of gastric bypass surgery on inflammation and mediators of the immune system prompted us to investigate the link between gastric bypass, GLP-1, and immunological regulation of inflammatory cellular and soluble mediators. These assertions provided a basis for this PhD.

The RYGB procedure offers an interesting model for examining associations between immunological changes in the obese setting and subsequent improvement of glycemic control, potentially mediated by GLP-1. The elucidation of cellular and molecular mechanisms involved in a potential GLP-1-mediated resolution of inflammation may provide knowledge on the aspect of resolving inflammation as an essential part of improving insulin resistance and other obesity-related co-morbidities. A possible dual effect on both the immune system and the glucose metabolism of GLP-1-based therapeutics will optimize future treatment of diabetes and/or other obesity-related diseases.

Aim

The main aim of the PhD thesis is to investigate anti-inflammatory and immunomodulatory effects of increased endogenous GLP-1, and a GLP-1R agonist, in relation to inflammatory conditions, such as diabetes and obesity, and furthermore to investigate the impact of RYGB on markers and mediators related to the immune system.

Hypotheses

- GLP-1 has local and systemic anti-inflammatory effects
- The effect of GLP-1 in managing T2D is, in part, mediated through anti-inflammatory and immunoregulatory mechanisms
- The anti-inflammatory and immune-modulating actions of GLP-1 are mediated via Treg cells or other cells important in the immune system
- The metabolic improvements by RYGB in obesity and T2D involves resolution of inflammation and changes in the immune system

In accordance with the aim of the thesis, five studies are described. The studies are subdivided in *in vitro* and animal studies (study I and II) and in human studies (studies III-V).

In vitro and animal studies

Study I

Effect of GLP-1 on the functionality of human expanded Tregs

Study II

Investigation of anti-inflammatory effect of GLP-1 in the mouse PMA ear inflammation model

Human studies

Study III (**Manuscript 1**)

Effects of Roux-en-Y gastric bypass on fasting and postprandial levels of inflammatory markers in obese subjects with normal glucose tolerance or type 2 diabetes

Study IV (**Manuscript 2**)

Effect of Roux-en-Y gastric bypass on circulating invariant NKT cells, regulatory T-cells, and inflammatory markers in obese subjects

Study V (**Manuscript 3**)

Effect of 12-week treatment with glucagon-like peptide-1 receptor agonist liraglutide on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects with type 2 diabetes

In the succeeding section, an overview of the main results of study I – II is presented followed by a short discussion. For study III – V, only a brief summary of the aim, results and conclusion is provided as details are presented in the corresponding manuscripts 1, 2 and 3.

In the ‘Methods’ section, a thorough description of study I and II is provided followed by a shorter informative description of study III – V. Further details on these studies can be found in the corresponding Manuscripts 1, 2 and 3.

STUDY I¹

Effect of GLP-1 on the functionality of human expanded Tregs

The aim of the study was to investigate the effect of the GLP-1R agonist, liraglutide, on the suppressive effect of cell-culture expanded Tregs on co-cultured autologous PBMCs. Reduced expression of the T-cell activation surface markers CD154 and CD69 on responder T-cells (Tresp) from the PBMC population in the presence of Tregs, was used as a read out for Treg-mediated suppression.

T-cells immediately express CD154 and CD69 after TCR stimulation. CD69, a C-type lectin, is recognized as an early activation marker on leukocytes (Sancho et al., 2003), while CD154 (CD40 ligand), a co-stimulatory molecule expressed on activated T-cells that engages CD40 on APCs, induces APC activation and T-cell help to B-cells (Schonbeck et al., 2000).

Effect of GLP-1 on Treg-mediated suppression of CD69 and CD154 on responder PBMCs

To examine the effect of GLP-1 on T-cell suppression by Tregs, liraglutide was added daily during the last three days of Treg expansion and again during the 7 hour FastImmune suppression assay. Three different doses of liraglutide (100nM, 10nM, 1nM) were used. As a negative control, the oligonucleotide CpG-A that impairs Treg function by interacting with TLRs expressed on Tregs (Peng et al., 2005), was included.

The FastImmune suppression assay was performed with three independent donors of Tregs and autologous PBMCs. CD69 and CD154 suppression at different Treg to PBMC ratios for one donor (A) are shown in Figure 1. It is evident from these graphs that with lower Treg to PBMC ratio, the suppressive activity decreases as measured by an increased expression of CD69 and CD154 surface markers on CD4⁺ Tresp of the PBMC preparation.

¹Study was conducted by Kirsten Lindegaard Bovbjerg, Monika Gad and laboratory technicians at Bioneer A/S as part of Zealand Pharma activities in an innovation consortium (The TREG Consortium) at Bioneer A/S

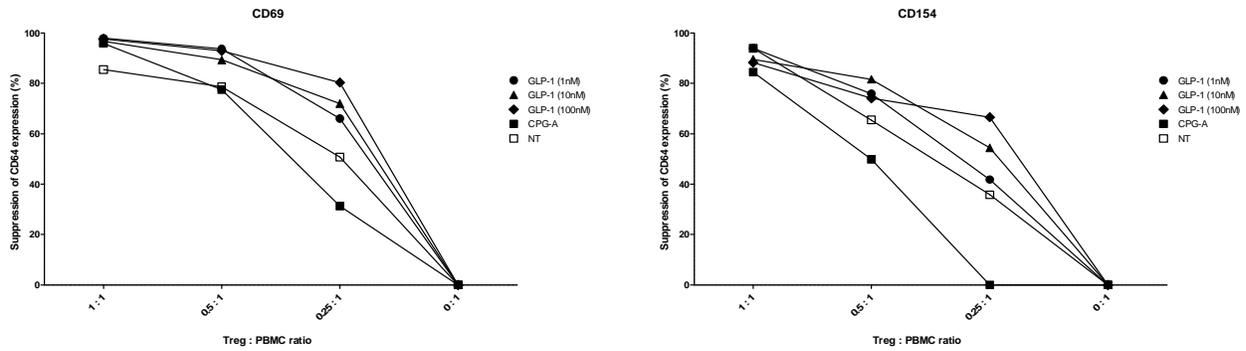


Figure 1. Percentage suppression of CD69 and CD154 expression on PBMCs by expanded Tregs represented in donor A. GLP-1: Glucagon-like peptide-1, CPG-A: negative control, NT: non-treated Tregs.

For donor A, a Treg to PMBC ratio of 0.25:1 showed enhanced dose-dependent suppressive action of Tregs when liraglutide was present (Figure 1). At this ratio, liraglutide concentrations of 1nM, 10nM, and 100nM led to suppression of CD69 by 66.0%, 72.0%, and 80.3%, respectively. Tregs alone (non-treated) suppressed CD69 expression by 50.7 %, while CpG-A suppressed CD69 expression by 31.3%. A similar suppression of CD154 by liraglutide was observed in donor A (Figure 1). The effect of liraglutide on Treg functionality was examined from two additional donors and Table 1 summarizes percent suppression of CD69 and CD154 in all three donors at a Treg to PBMC ratio of 0.25:1.

Because liraglutide treatment in donor B showed highest efficacy on the Treg suppressive function at a concentration of 10nM (table 1), this concentration was subsequently tested in duplicates in donor C. Tregs incubated with liraglutide did not experience enhanced suppression (CD69: 35.2%, 35.4%, CD154: 18.8% and 21.0%) compared to no treatment (CD69: 47.1% and CD154: 32.7%).

The peptide buffer used for dissolving and diluting liraglutide was included and indicated no effect on viability and number (data not shown). In addition, a scrambled peptide with no agonistic activity on the GLP-1R was included in donor C. The replicates of a scrambled peptide with no agonist activity on the scramble peptide were inconsistent.

Percent suppression at 0.25:1 Treg PBMC ratio	Donor A		Donor B		Donor C	
	CD154	CD69	CD154	CD69	CD154	CD69
No treatment	35.8	50.7	17.6	36.0	47.1	32.7
Liraglutide (1nM)	41.8	66.0	20.3	39.8		
Liraglutide (10nM)	54.4	72.0	29.9	46.7	35.2; 35.4	18.8; 21.0
Liraglutide (100nM)	66.6	80.3	17.2	30.3		
CpG-A	0	31.3	18.1	35.8	43.6	21.2
Vehicle			15.3	29	46.4	30.6
Scramble peptide					26.8; 58.3	14.5; 40.2

Table 1. Treg-mediated suppression in of CD69 and CD154 expression on Tresp from PMBCs from three independent donors at a Treg to PBMC ratio of 0.25:1.

Comments and Discussion

Accumulating evidence supports a pathogenic role of the adaptive immune response in diabetes, and therefore there is an ongoing surge in the investigation of down-regulatory mechanisms that could be therapeutically exploited. The role of Tregs has been the object of considerable attention. Tregs are pivotal for the maintenance of self-tolerance and their adoptive transfer protects from autoimmune diseases (Sakaguchi et al., 2009). Animal models suggest that Tregs play a critical role in controlling the development of both T1D and T2D (Kornete et al., 2013). Detailed functional characterization and potential clinical application of Tregs has been hampered by the paucity of circulating Tregs in peripheral blood. The ability to (rapidly) expand human Tregs is an attractive approach, and despite emerging techniques in the *ex vivo* expansion of Tregs, there is still lack of standardized and validated *in vitro* based screening platforms for Treg functionality. A broader understanding of Treg function will accelerate the potential clinical application in T-cell-mediated diseases.

GLP-1Rs have been found on murine Tregs (Hadjiyanni et al., 2010), and data obtained in mice suggest that GLP-1 has immune modulatory effects, affecting proliferation and function of Treg cells (Xue et al., 2008; Hadjiyanni et al., 2008) associated with a delay in diabetes onset (Hadjiyanni et al., 2008). Although human trials have never replicated data from animal models, data are encouraging.

In the present study it was demonstrated that human nTregs expanded for 14 days can suppress expression of the surface markers CD69 and CD154 on activated CD4⁺ Tresp from autologous PBMCs and that the percentage suppression of expanded Tregs was associated with the Treg:PBMC ratio. Thus, the data clearly shows that this surrogate measure of suppression was Treg dose dependent.

Data on donor A showed a concentration-dependent positive effect of liraglutide on the suppressive activity of Tregs on Tresp evident at a ratio of 0.25:1. This dose-dependency could not be confirmed in donor B; however, there was a potential effect at the intermediate liraglutide concentration (10nM) on Treg-mediated suppression which proved higher than non-treated Tregs. In donor C, there was no effect of liraglutide at the intermediate dose compared with non-treated Tregs. The reason for the discrepancy between the three donors might be explained by inevitably donor variability in human studies impacting the quantity and quality of the Tregs in their natural ability to suppress and how they respond to expansion and to compound treatment. Another explanation might be that the preliminary data in donor A were an artifact. Negative controls for specificity of liraglutide included determinations of the isolated effect of the vehicle on Treg function and on Tresp alone, and scrambled peptide effect on Treg function. These controls were, unfortunately, not included when collecting data in donor A. The presence of vehicle during Treg expansion and in the suppression assay displayed suppression equaling the non-treated Tregs in donor B and C, suggesting that any effect observed by liraglutide was not caused by the dissolving agent. The scramble peptide, however, which was tested in duplicates at 10mM in donor C gave contradicting data, suggesting that i) the presence of a peptide, regardless of specificity, induces an arbitrarily response, or ii) technical variations in the expansion process or in the preparation of the co-culture assay, i.e. different cell numbers per well (Ruitenberg et al., 2011), could have had an effect on suppression outcome.

As negative control for Treg suppression was CpG-A. Ruitenberg *et al.* has shown that CpG-A induces time-dependent decreases in the suppression of CD69 and CD154 on Tresp by Tregs (Ruitenberg et al., 2011). Peng *et al.* discovered that the poly-G tail portion of the CpG-A oligonucleotide was the ligand of TLR8 expressed on Tregs and that this interaction was responsible for inhibiting Treg-mediated suppression (Peng et al., 2005). Our data from donor A confirmed that the presence of CpG-A led to reduced suppression, but this effect was not present in the remaining two donors, which may further confirm the low quality of Tregs and collected data from these donors.

Based on this experiment it is unclear whether GLP-1 has an effect on Treg function (*in vitro*). The limitations of the study are the small sample size (n=3), large donor variability, and the explorative nature of this type of assay including lack of standardized positive controls i.e. compounds that increase Treg

suppressive function. Thus, this study should be interpreted with caution and additional donors would be required to interpret the effect of liraglutide, as high individual variations between donors must be taken into consideration when performing human experiments. A GLP-1 antagonist which would impede the effect of liraglutide on a potential Treg-mediated suppression would be an interesting compound to include in future experiments as well as cytokine measurements to explore if GLP-1 augments a Th2 versus Th1 response.

There is lack of studies examining *ex vivo* and *in vitro* effect of liraglutide on expansion and subsequent functionality of human Tregs. Xue and co-workers found that Tregs isolated from Ex-4-treated NOD mice showed significantly enhanced suppressive capacity compared to Tregs from untreated mice (Xue et al., 2008). When the same authors tested the *in vitro* effect of Ex-4 (1000ng/ml corresponding to 238nM) on Treg function on splenocytes isolated from untreated NOD mice, they found a trend toward increased suppressive function compared to untreated Tregs. The study included small sample size (n>5) and the assay was a long-term co-culture proliferation assay (5 days of co-culture with Tresp and Tregs) without previous expansion. Suppression was further more measured by ³H-thymidine incorporation. Thus, any comparisons between this study and the study by Xue and co-workers are difficult, but could imply that GLP-1 has minor effects on the frequency and function of Tregs.

STUDY II²

Investigation of anti-inflammatory effect of GLP-1 in the mouse PMA ear inflammation model

The objective of the study was to test whether GLP-1R activation by the GLP-1R agonist liraglutide has displayed anti-inflammatory effects in an *in vivo* inflammation model. Specifically, the ability of subcutaneously administered liraglutide to downregulate surrogate inflammation markers, including ear swelling, ear weight, and systemic biomarkers of inflammation (cytokines and the acute phase protein haptoglobin), in the murine PMA (phorbol 12-myristate 13-acetate) ear inflammation model was studied. Local ear inflammation was induced by PMA, a phorbol ester capable of activating a variety of immune cells, in particular mast cells and neutrophils. Topical application of PMA to the ear results in localized inflammation with signs including erythema, edema, infiltration of leukocytes and hyperplasia. Betamethasone (BM), a potent glucocorticoid, has been shown to reduce ear thickness and ear weight of PMA treated ears and was included as a positive control for anti-inflammatory effect. An anti-inflammatory effect of liraglutide would also be expected to reduce ear thickness, and/or ear weight. Likewise changes in inflammatory markers would indicate systemic anti-inflammatory effect. Body weight measurements were frequently done during the treatment period to confirm systemic exposure to liraglutide.

Experimental animals

All mice were monitored clinically and no abnormalities were observed. Mice were divided into four groups: group 1, control (acetone/water); group 2, 'PMA only' (0.040 mg/ml PMA + acetone/water); group 3, 'PMA and BM' (0.040 mg/ml PMA + 0.50 mg/ml BM); and group 4, 'PMA and liraglutide' (0.040 mg/ml PMA + 40 nmol/kg) (see "Methods", Table 2).

Body weight

No difference in mean body weight between the four groups was observed at day -2 before initiating liraglutide treatment ('Control', 19.58 ± 0.57 g; 'PMA only', 19.87 ± 0.19 g; 'PMA and BM', 20.08 ± 0.40 g; 'PMA and liraglutide', 19.27 ± 0.40 g) ($P=0.42$). Treatment with liraglutide for 9 days (day -2 to day 7) significantly reduced body weight by $-5.3 \pm 0.8\%$ compared to the remaining groups; 'control' group $-2.4 \pm 0.4\%$, 'PMA only' group $-1.3 \pm 0.7\%$, and the 'PMA and BM' group $-3.6 \pm 1.0\%$ ($P<0.01$).

²The PMA ear inflammation study was conducted at and in accordance with Pipeline Biotech A/S under Danish Animal Experiments Inspectorate License number 2011/561-1956 schedule C4.

Daily monitoring of body weight from day -2 to 7 showed that liraglutide induced a body weight reduction that reached a plateau after 3 days whereas the body weight of the remaining groups remained unchanged until day 5. On day 7 a decrease in the relative body weight was observed for all remaining groups, which could be due to stress mediated by the handling process (Figure 2). The body weight reduction confirmed systemic exposure of liraglutide.

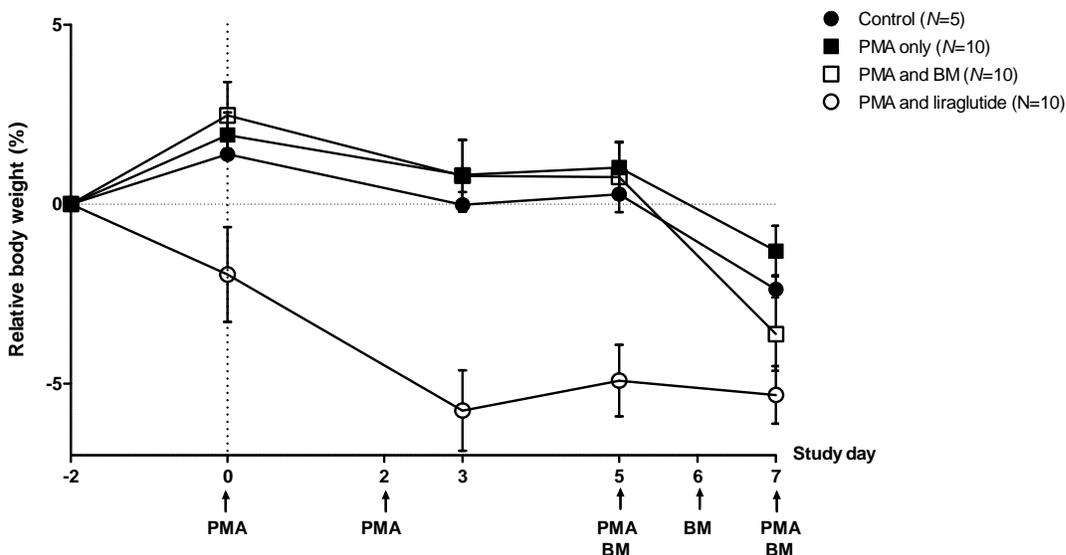


Figure 2. Relative body weight over time during the 9-day study period normalized to day -2 in the control group (solid circle, $N=5$), 'PMA only' (solid square, $N=10$), 'PMA and BM' (open square, $N=10$), and 'PMA and liraglutide' group (open circle, $N=10$). Each data point represents the average percent change in the body weight of mice relative to the weight at day -2 \pm SEM. Comparisons of change in body weight from day -2 to termination between all four groups using one-way ANOVA with Dunn's post test for multiple comparisons.

Ear thickness and ear weight

Local application of PMA to the ear of mice induces local inflammation with subsequent erythema (redness), edema (swelling) and hyperplasia (increase of immune cells). PMA was applied on the right ear at day 0, 2 and 5. BM treatment was initiated at day 5, and applied once daily at day 6 and 7. To evaluate ear inflammation, ear thickness of both ears of each mouse was measured once daily at day -2, 5, 6 and twice at day 7. The change in ear swelling (Δ ear thickness) was calculated by subtracting the thickness of the left ear (vehicle) from the thickness of the right ear (treatment). As a second terminal end point measurement of ear inflammation, ear biopsies were collected and weighed. Changes in ear biopsy

weights (Δ Ear weight) were calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment).

At study initiation at day -2 Δ Ear thickness were identical for both ears in all four groups (0.0 ± 0.0 mm (SEM)). As expected, no ear swelling was observed in the control group that received the vehicle during the entire study period whereas BM application at day 5 clearly inhibited ear swelling (figure 3). At the end of the study period, PMA challenged ears displayed significantly increased Δ ear thickness ('PMA only' group: 0.13 ± 0.00 mm) compared to the control group (0.00 ± 0.00 mm) ($P < 0.001$). Treatment with BM resulted in significant reduction of Δ ear thickness (0.05 ± 0.01 mm) compared to the 'PMA only' group ($P < 0.01$). Subcutaneous injections with liraglutide for 9 days had no effect on reducing Δ ear thickness (0.12 ± 0.01 mm) compared to the 'PMA only' group (figure 3).

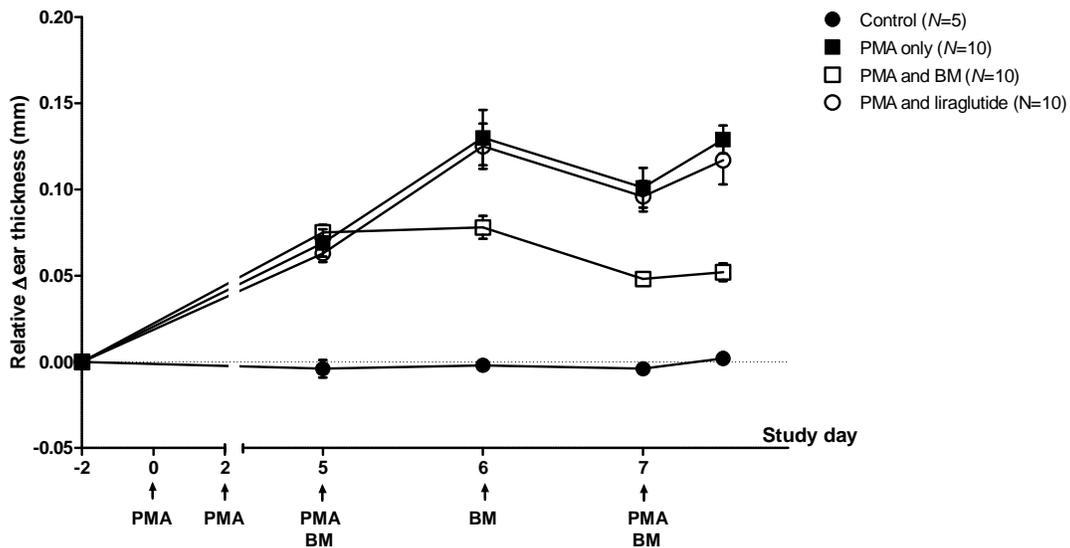


Figure 3. Development in Δ ear thickness from day -2 to termination baseline corrected to day -2 in the control (solid circle, $N=5$), 'PMA only' (solid square, $N=10$), 'PMA and BM' (open square, $N=10$), and 'PMA and liraglutide' group (open square, $N=10$).

Likewise, Δ Ear weight in the 'PMA only' group was significantly higher than in the control group ($P=0.003$) and treatment with BM efficiently reduced Δ ear weight compared to the 'PMA only' group ($P < 0.0001$). Treatment with liraglutide had no effect on ear weight (data not shown). The biopsy weight of the non-treated left ears was investigated to examine a potential systemic effect of PMA, BM or liraglutide. The BM treated group exhibited significantly lower left ear weight values ($P=0.0006$) compared to the 'PMA only' group indicating a systemic effect of the BM treatment. No effect of liraglutide was observed (data not shown).

Analysis of systemic inflammatory markers

A number of circulating pro- and anti-inflammatory markers were analysed from plasma obtained at termination to determine if PMA challenge, BM, and liraglutide treatment affected systemic inflammation parameters.

Generally PMA treatment induced high individual variations of the inflammation parameters within the groups. Treatment with PMA significantly increased concentrations of the acute-phase protein, haptoglobin (HP), compared to the control group ($P=0.013$) (Figure 4). There was no effect of BM or liraglutide in reducing the PMA-induced HP concentrations ($P=0.07$). The two high responders found in the ‘PMA and liraglutide’ group were not linked to other signs of infection or inflammation.

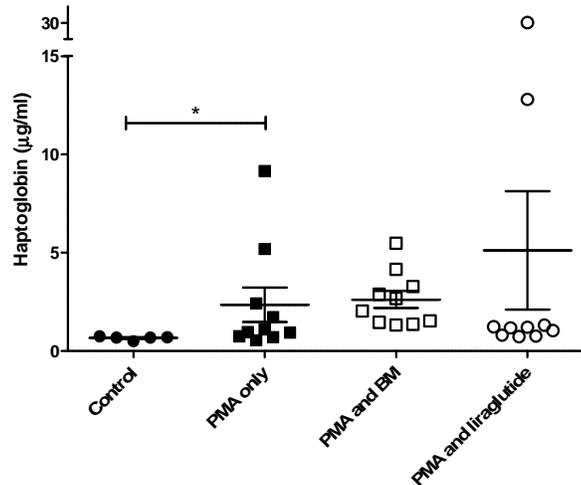


Figure 4. Haptoglobin concentrations in the control (solid circle, $N=5$), ‘PMA only’ (solid square, $N=10$), ‘PMA and BM’ (open square, $N=10$), and ‘PMA and liraglutide’ group (open circle, $N=10$). Data presented as mean \pm SEM. ‘PMA only’ group compared to control using t-test and PMA groups were compared to ‘PMA only’ group using one-way ANOVA using Dunn’s post test for multiple comparisons.

In addition to HP, plasma was analysed for the concentration of a selected panel of inflammation-related cytokines (IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p40, KC, and TNF- α). Differentially regulated cytokines are depicted in Figure 5A-D, expressed as an increase or decrease in concentrations relative to the mean of the normal control group³. Treatment with PMA enhanced circulating concentrations of IL-

³Samples were analyzed in two separate runs, each containing the control samples and 50% of the treatment groups. Data are expressed relative to the mean of the control group in each run due to differences in assay performance.

12p40 (B), KC (C) and TNF- α (D). Topical BM treatment significantly normalized concentrations of IL-12p40 ($P<0.001$) to a level below that of the control group and KC ($P<0.001$). BM treatment had no effect on the PMA-mediated increase in TNF- α and did not affect IL-1 β either (A). Similar, treatment with liraglutide appeared to normalize IL-12p40 and KC levels, and to reduce TNF- α concentration. Liraglutide significantly reduced IL-1 β compared to the ‘PMA only’ group reaching levels apparently lower than the control group.

IFN- γ levels for all groups were low and could not be detected for all groups. Levels of IL-2, IL-4, IL-5 and IL-10 did not change between the four groups (data not shown).

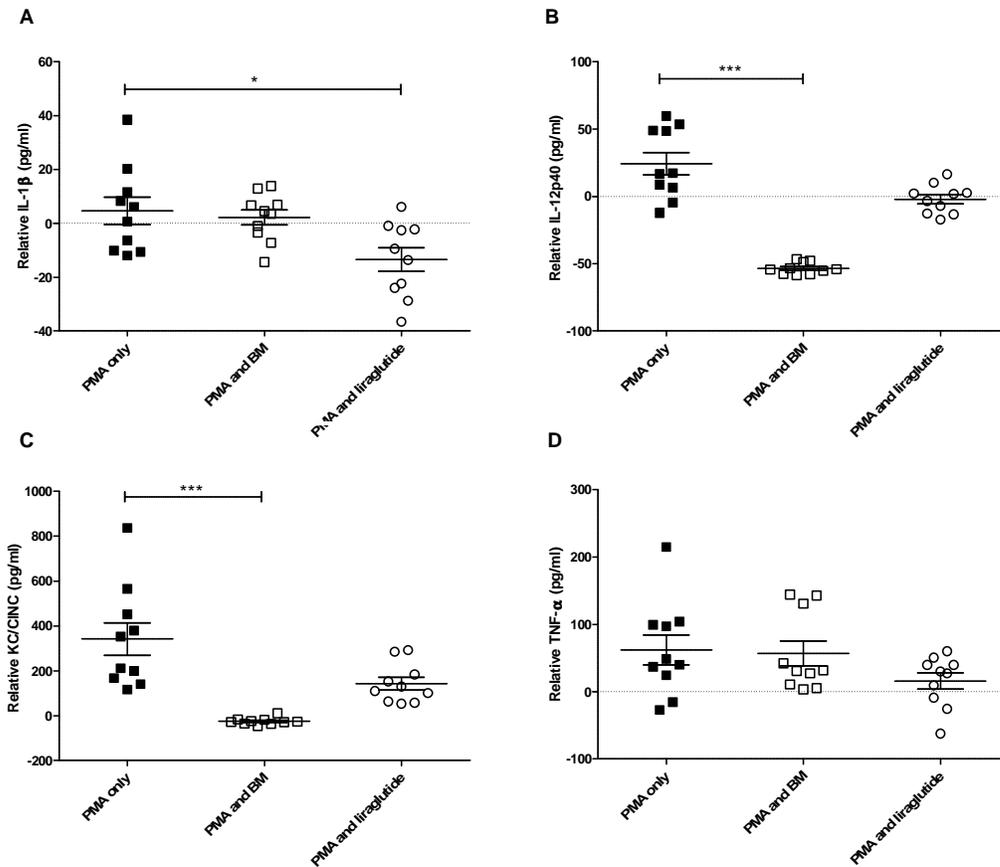


Figure 5. Relative concentrations of inflammatory markers in plasma normalized to the mean of the control group. ‘PMA only’ (solid square, $N=10$), ‘PMA and BM’ (open square, $N=10$), and ‘PMA and liraglutide’ group (open circle, $N=10$). Data presented as mean \pm SEM. Compared to the ‘PMA only’ group with one-way ANOVA using Dunn’s post test for multiple comparisons, $*P<0.05$, $***P<0.0001$.

Comments and Discussion

As membrane permeable specific activator of the protein kinase c (PKC) and hence of the NF- κ B pathway, PMA causes an extremely wide range of effects in a variety of cell types. Multiple applications of PMA to mouse ears cause erythema, edema, hyperplasia, and infiltration of leukocytes associated with a local up-regulation of pro-inflammatory cytokine production (Wershil et al., 1988; Holden et al., 2008).

PMA stimulates Ca^{2+} -dependent cytosolic phospholipase A_2 (cPLA₂) in neutrophils and macrophages (Qiu and Leslie, 1994), and cPLA₂ enzymes are critical regulators of the generation of pro-inflammatory leukotrienes and prostaglandins and shown to play a role in the pathogenesis of a number of inflammatory conditions, ranging from inflammatory bowel disease to psoriasis (Chiba et al., 2004; Funk, 2001). The PMA ear inflammation model is considered clinical relevant for selecting drug candidates for the treatment of inflammatory disorders and have thus been established as an *in vivo* model for the evaluation of various anti-inflammatory agents (Stanley et al., 1991; Burke et al., 2001; Malaviya et al., 2006). Emerging studies suggest that GLP-1 may play a role in the improvement of psoriasis (Drucker and Rosen, 2011), and investigation of potential anti-inflammatory effects of GLP-1 in this model has not been tested before.

The data presented here support that application of PMA resulted in skin inflammation indicated by a significant increase in ear thickness, which was reversed by topical treatment with BM. Systemic (s.c. in the skin of the back) injection of liraglutide for 2 days prior to PMA challenge and throughout the study period did not have an apparent effect on ear thickness or ear weight (see F

igure 3).

Topical application of PMA was also found to have systemic effects as it resulted in increased HP concentrations compared to untreated mice (Figure 4). No obvious effect on HP levels was seen after betamethasone treatment. In the liraglutide group, eight out of 10 mice exhibited lower HP concentration than mice in the other PMA-challenged groups, but two outliers contributed to controversy on a real effect of liraglutide in reducing HP concentrations. HP is a positive acute-phase protein produced mainly by liver cells but also from other tissues including the skin, although to a lesser extent (D'Armiento et al., 1997). HP concentrations rise progressively following acute tissue damage within 24-48 hours and fall quickly once the stimulus is removed (Morimatsu et al., 1991). As blood samples were collected 6 hours after the last PMA challenge and 56 hours after the preceding PMA challenge (Juge-Aubry et al., 2005) the HP response could have leveled off and not have been fully activated. Mean serum HP concentrations in healthy BALB/c mice are between 1 and 25 μ g/ml (Petersen et al., 2009), questioning the importance of

the minor group differences observed and, in part, the two high responders in the liraglutide treated group. In a THP-1 cell line (macrophage-like cells), HP secretion is stimulated by PKC (Oh et al., 2007), suggesting that the small but significant HP increase in the 'PMA only' group is dependent on PMA-induction. The reason for the rather low HP concentrations compared to the literature could be due to measuring HP in plasma versus serum. No studies can be found in the literature that has examined liraglutides effect on the HP response to localized inflammation, i.e. the effect liraglutide on systemic reaction to localized inflammation. There are, however, studies suggesting a link between a certain isoform of HP and diabetes-related CVD (Levy et al., 2010), and as GLP-1-based therapies are suggested to have cardioprotective effects (Burgmaier et al., 2013), a putative relationship between HP and GLP-1 deserves further attention.

Upon PMA challenge, liraglutide significantly suppressed IL-1 β while TNF- α , KC and IL-12 were moderately (non-significantly) decreased, while betamethasone treatment after PMA challenge significantly suppressed KC and IL-12 but not IL-1 β and TNF- α . Inflammatory macrophages are a major source of KC, the mouse ortholog of human IL-8 which plays a role in neutrophil infiltration, and TNF- α and IL-1 β , whose production is regulated by the NF- κ B pathway. These cytokines have also been suggested to be implicated in obesity-related insulin resistance (Neels et al., 2009; Wisse, 2004). *In vitro* studies in vascular endothelial cells have shown that liraglutide suppresses NF- κ B activation partly by increasing the protein level of I κ B proteins or by inhibiting degradation of I κ B α and/or by AMPK activation. (Hattori et al., 2010; Shiraki et al., 2012). Furthermore, Shiraki *et al.* found that treatment with liraglutide inhibited TNF- α -induced translocation of PKC- α primarily through inhibition of TNF- α -mediated signaling (Shiraki et al., 2012).

The PMA ear inflammation model is a widely used model for examining anti-inflammatory effect of compounds. Compounds can be tested in either acute or chronic inflammatory settings (Malaviya et al., 2006). In the present study, liraglutide was tested in the chronic inflammation model. The lack of effect observed, may be explained by the robust and "non-physiological" PMA-induced inflammation, which may not be suitable for studying minor to moderate effects as most likely is the case with liraglutide. The acute version of this model with duration of only 6 hours might have been a preferable choice instead. Topical application of PMA results in an acute inflammatory response, peaking around 6 hours and subsides by 24 hours (Stanley et al., 1991). It would therefore have been interesting to have obtained ear thickness measurements on day 1 or 2 before the second PMA induction. This could have indicated whether the more acute nature of the inflammatory response was responsive to liraglutide. Moreover, as mice were pre-treated with liraglutide in order to enhance efficacy by building up GLP-1 concentrations and possibly prime the immune system, the acute model could again have been preferable. Finally, in the

study by Malaviya *et al.* the authors found that cPLA₂ regulated PMA-induced chronic but not the acute inflammatory response in mice (Malaviya et al., 2006), which furthermore speak for the acute model as a more sensitive model for moderate anti-inflammatory agents.

Although betamethasone appeared to have systemic effects as measured by the weight of the left and untreated ear, another positive control, i.e. non-steroidal anti-inflammatory compounds, might have been a better compound for comparison. Betamethasone is a glucocorticoid, which is one of the most efficacious anti-inflammatory drugs by suppressing a wide spectrum of inflammatory processes, such as inhibition of PLA₂, leukocyte activity and pro-inflammatory cytokine production (Barnes, 1995).

There are various other interesting end points measurements besides ear thickness and cytokines, such as histopathological assessment of tissue inflammation in the ears to look for histological appearance of the PMA-induced inflammation and the effect of liraglutide/betamethasone and moreover could flow cytometry of the cellular tissue infiltrate give a phenotypic characterization of the immune cell infiltrations in the ear.

In conclusion, the preliminary data show that liraglutide did have a systemic effect on reducing the circulating concentration of the pro-inflammatory cytokine IL-1 β and to a certain extent on the acute phase protein haptoglobin in a model of chronic inflammation but did not have significant impact on reducing local inflammation (ear weight and thickness). Additional studies, preferably in an acute setting, would be interesting and required to determine if liraglutide has anti-inflammatory and immunoregulatory capabilities which may explain the improvement in psoriasis cases independent of regulation of glycemic control.

STUDY III – V

STUDY III (Manuscript 1)

Kirsten K. Lindegaard, Nils B. Jørgensen, Rasmus Just, Peter M.H. Heegaard, and Sten Madsbad.

Effects of Roux-en-Y gastric bypass on fasting and postprandial circulatory levels of inflammation-related biomarkers in obese subjects with normal glucose tolerance and in obese subjects with type 2 diabetes

Manuscript submitted to Journal of Clinical Immunology

The aim of the study was to examine if the Roux-en-Y gastric bypass (RYGB) procedure have effects on the immune system as measured by changes in circulating cytokines and adipokines in obese T2D and matched normal glucose tolerant (Sjostrom et al., 2012) subjects. The hypotheses were as follows: i) RYGB induces long-term beneficial immune changes causing a more anti-inflammatory profile, ii) T2D subjects have a higher inflammatory burden compared to NGT subjects, and iii) the postprandial response of cytokines and adipokines is changed after surgery. A reduction in IL-6, TGF- β , and leptin, and increase in adiponectin was found 1 year after surgery. No difference was observed between fasting levels of inflammatory markers in T2D and NGT subjects before or after surgery. Postprandial response of adiponectin was increased 1 year after surgery while that of leptin was decreased immediately after surgery in both T2D and NGT subjects but dependent on the fasting levels. The IL-6 response during meal intake was decreased in T2D subjects 1 year after surgery but unchanged in NGT subjects. Taken together, RYGB reduced pro-inflammatory biomarkers and increased anti-inflammatory mediators in diabetic and normal glucose tolerant obese subjects one year post-surgery.

STUDY IV (Manuscript 2)

Kirsten K. Lindegaard, Siv H. Jacobsen, and Sten Madsbad.

Effect of Roux-en Y gastric bypass on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects.

Manuscript in preparation

This study aimed at examining the Roux-en-Y gastric bypass (RYGB) effects on the immune system as measured by changes in circulating cytokines, adipokines and immune cells subsets in obese glucose tolerant subjects before and 3 months after surgery. The hypotheses were that RYGB induces i) a short-term change in inflammatory markers causing a more anti-inflammatory profile, ii) an increase in the

number of circulating iNKTs and Tregs and iii) a change in the postprandial response of immune cells and cytokines. A decrease in fasting leptin and an increase in IFN- γ and IL-10 were found after surgery. Flow cytometry analyses revealed no differences in the number of circulating Tregs or iNKTs after surgery compared to before. No postprandial changes occurred in circulating immune cells as measured 60 min and 120 min after food intake. Taken together, RYGB has a positive effect on insulin resistance and induces alterations in innate immune cell-driven inflammation despite no changes in the number of iNKT or Treg cells was detected 3 months post-surgery.

STUDY V (Manuscript 3)

Kirsten K. Lindegaard, Carsten Dirksen, Jens-Peter Stenvang, Sten Madsbad

Effect of 12-week treatment with glucagon-like peptide-1 receptor agonist liraglutide on circulating invariant NKT cells, regulatory T-cells, and inflammatory markers in obese subjects with type 2 diabetes.

Manuscript submitted to Scandinavian Journal of Immunology

The aim of the study was to investigate if GLP-1 (liraglutide) exerts immunomodulatory actions by examining the effect of exogenous administration of liraglutide for 6 and 12 weeks on circulating cytokines, adipokines and immune cells subsets in obese T2D human subjects. The hypotheses were that liraglutide therapy i) promotes a more anti-inflammatory cytokine profile and ii) increases the circulating number of iNKTs and Tregs. Liraglutide improved glucose metabolism by reducing HbA1c levels. There was, however, no observable change in cytokine concentrations between the three time-points, and no change in circulating iNKT or Tregs. Taken together this study can not support previous reported findings on anti-inflammatory and immunoregulatory effect of GLP-1R agonist treatment.

METHODS

Study I⁴

Effect of GLP-1 on functionality of human expanded Tregs

Isolation of peripheral blood mononuclear cells and enrichment of CD4⁺ cells

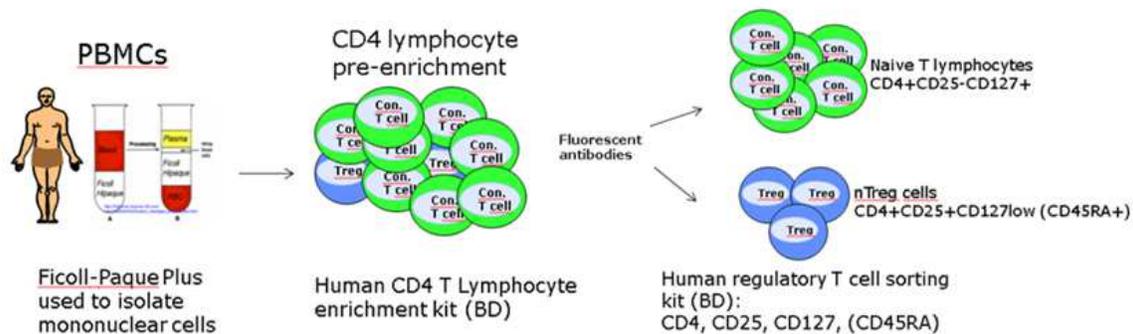
PBMCs were isolated from the buffy coat of healthy blood donors obtained from Rigshospitalet (Blodbanken, Rigshospitalet, Copenhagen, Denmark) by density gradient centrifugation with Ficoll-Paque Plus[®] (GE Healthcare Life Sciences, New Jersey, US). A fraction of the PBMCs (3×10^8 cells) were also cryopreserved in 90% FBS containing 10% DMSO (Gibco[®] Life Technologies, Grand Island, NY, USA) for later use as autologous responder cells in the BD FastImmune suppression assay. The remaining PBMCs ($2-4 \times 10^8$ cells) were passed through 70 μm cell strainer (BC Falcon, #352350) and resuspended in 1X BD IMag buffer (BD Bioscience, CA, US) to reach a concentration of 1×10^7 cells/ml. Pre-enrichment of CD4⁺ cells was performed using BD IMag[™] Human CD4 T Lymphocyte Enrichment Set-DM (BD Biosciences, CA, US, #557939) according to the manufacturer's instruction (Figure 6). Briefly, PBMCs was stained with Biotinylated Human CD4 T Lymphocyte Enrichment Cocktail. BD IMag[™] Streptavidin Particles Plus – DM was added to $2-8 \times 10^7$ cells/ml labeling the cells bearing the biotinylated antibodies. By use of BD IMagnet[™], negative selection was performed to enrich for the unlabeled T-cells. The negative selection was repeated four times to increase the yield of the enriched fraction. The combined enriched fraction was analyzed by flow cytometry and contained approximately 98% T-lymphocytes with no bound antibodies or magnetic particles.

Cell sorting of natural regulatory T-cells and conventional T-cells by FACS

The enriched fraction of CD4⁺ cells was washed with 1XPBS (w/o Ca²⁺ and Mg²⁺, Gibco[®] Life Technologies, #14190-094), resuspended in 1X PBS containing 0.1% human type AB serum (Gibco[®]Life-Technologies, #34005) to reach a density of $20-100 \times 10^6$ cells/ml and stained with PerCP–Cy5.5–anti–CD4, PE–anti–CD25, Alexa Fluor[®] 647–anti–CD127, and FITC–anti–CD45RA (Human Regulatory T Cell Sorting Kit, BD Biosciences, CA, US, #560753) for 30 min on ice according to the manufacturer's protocol.

⁴Study was conducted by Kirsten Lindegaard Bovbjerg, Monika Gad and laboratory technicians at Bioneer A/S as part of Zealand Pharma activities in an innovation consortium (The TREG Consortium) at Bioneer A/S

Flow cytometric analysis of samples was performed on a BD FACS Aria™ III flow cytometer (BD Biosciences, San Jose, CA) and natural (n)Tregs, identified as CD4⁺CD25⁺CD127^{low}CD45RA⁺ cells, and naive conventional T-cells, identified as CD4⁺CD25⁻CD127⁺ were sorted using a sequential sort gate strategy, as described in the Human Regulatory T Cell Sorting Kit instruction sheet. Briefly, a gate was placed around the CD4⁺ population followed by a gate on the CD25⁺CD127^{low} population and further gating of the CD45RA⁺ cells from this population. CD4⁺CD25⁺CD127^{low}CD45RA⁺ and CD4⁺CD25⁻CD127⁺ cells were acquired into polypropylene tubes (BD Falcon™, #14-959) with RPMI1640 media (Gibco®, Life Technologies) containing 10% heat-inactivated human AB serum. Post-sort analysis showed sorting purity greater than 98%. The CD4⁺CD25⁺CD127^{low}CD45RA⁺ nTregs and CD4⁺CD25⁻CD127⁺ conventional T-cells were cultured separately for 14 days to expand their numbers prior to use in the BD FastImmune suppression assay (see next section).



Figur 6. Isolation of PBMCs and subsequent CD4⁺ enrichment. Labeling and sorting of CD4⁺ enriched fraction with BD Human Regulatory T cell Sorting Kit antibodies into naïve conventional T-cells (CD4⁺CD25⁻CD127⁺) and natural Tregs (CD4⁺CD25⁺CD127^{low}CD45RA⁺) for later use in the BD FastImmune suppression assay.

Ex vivo expansion of regulatory T cells

Immediately after sorting cells into CD4⁺CD25⁺CD127^{low}CD45RA⁺ Tregs and CD4⁺CD25⁻CD127⁺conventional T-cells, cells were suspended in RPMI1640 culture media containing 2nM L-glutamine, 1% MEM vitamins, 1 mM natrium pyruvate, 1% MEM Non-Essential Amino Acids (NEAA), 10 mM HEPES, 100 U/ml penicilin, 100 ug/ml streptomycin, 50 uM 2-mercaptoethanol, and 10% heat-inactivated human AB serum, pH 7.4, at a density of 2-4×10⁵ cell/ml. The sorted T-cell subsets were expanded using anti-CD3/CD28-coated beads and IL-2 as previously described (Hoffmann et al.,

2004; Hoffmann et al., 2006). Expansion protocol for the Treg subset is outlined in Figure 7. The conventional T-cells underwent similar procedure for later use as control cells in the BD FastImmune assay.

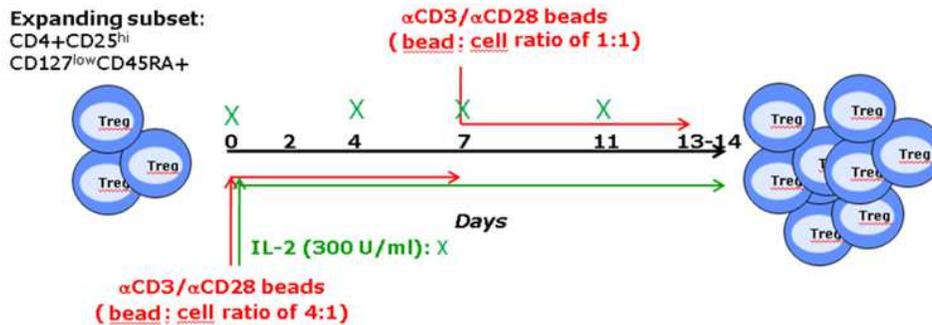


Figure 7. Expansion of $CD4^+CD25^+CD127^{low}CD45RA^+$ Tregs. Anti-CD3/CD28 beads were added to the Tregs at day 0 at a bead:cell ratio of 4:1, removed at day 7, and added at a bead:cell ratio of 1:1 at day 7 until termination of the expansion. Recombinant IL-2 was added to the expanding cells at day 0, 4, 7 and 11. Conventional T-cells followed similar procedure, but without addition of IL-2 and with other bead:cell ratios (see text).

At day 0, T-cell subsets were activated using Dynabeads® Human T-Activator CD3/CD28 (Invitrogen, Oslo, Norway) as follows: Tregs were mixed with anti-CD3/anti-CD28 beads at a bead:cell ratio of 4:1. For the conventional T-cell culture a bead:cell ratio of 1:1 was used. Approximately 1×10^4 cells/100 μ l were transferred to a pre-cooled (4°C, 1h) 96-well plate and placed in a 37 °C incubator with 5% CO₂. Recombinant IL-2 (rIL-2, Proleukin®, Chiron Corp, Emeryville, CA, US) was added to the expanding Treg subset on day 0, 4, 7, and 11 at a final concentration of 300 U/ml. At day 7, the anti-CD3/anti-CD28 beads were magnetically removed; the cells were counted and re-stimulated with anti-CD3/anti-CD28 beads at a bead:cell 1:1 ratio for Tregs and 1:10 for conventional T-cells.

Stimulation of expanding regulatory T-cells with GLP-1

The remaining three days of the expansion period (and again in the 7-hour co-culture, see next section), the GLP-1 analogue liraglutide (synthesized at Zealand Pharma A/S, Glostrup, Denmark) at three different concentrations (1nM, 10nM, and 100nM) and 10 μ g/ml CpG-A, a human TLR9 ligand with a poly-G oligonucleotide string (Type A CpG oligonucleotide, ODN 2336, InvivoGen, San Diego, CA, US)

was added (Figure 8). As controls for the specificity of liraglutide, the buffer used for dissolving and dilution of liraglutide was included as well as a scrambled peptide (Ac-DAla-DHyp-G-DTyr-DPro-G-NH₂) with no activity on the GLP-1R. The doses of liraglutide were selected based on published *in vitro* data on the functionality of a subset of T-cells (Hogan et al., 2011).

Over the course of the expansion, cell numbers were monitored and cultures were divided in order to maintain a cell concentration of approximately 5×10^5 cells/ml. At the last day of expansion, cells were harvested and anti-CD3/anti-CD28 beads were magnetically removed. Cells were washed with RPMI1640 culture media as described above and 1×10^6 cells/ml was transferred to a 6-well plate (5 ml/well) in media \pm 300 U/ml IL-2, counted and used in the FastImmune suppression assay described below.

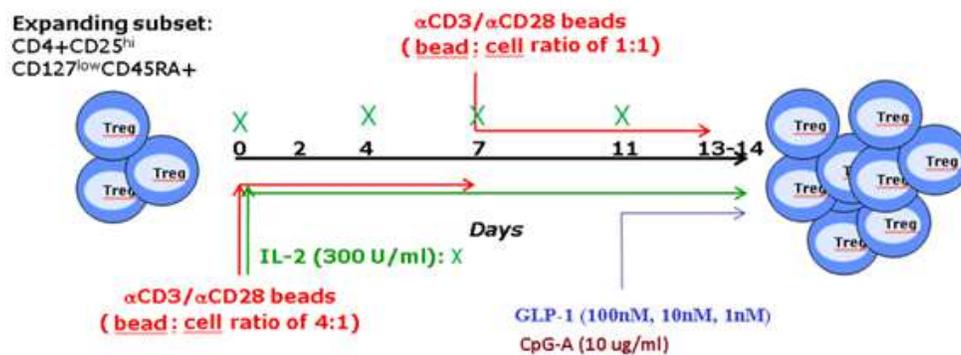


Figure 8. Addition of GLP-1 in three concentrations (100nM, 10nM, and 1nM) and CpG-A (10 ug/ml) to expanding Tregs the last three days of the expansion period.

Treg-mediated suppression of CD69 and CD154 on responder T-cells

Reduced expression of the T-cell surface activation markers CD154 and CD69 on PBMCs in the presence of Tregs can indicate Treg suppressive capacity (Figure 9).

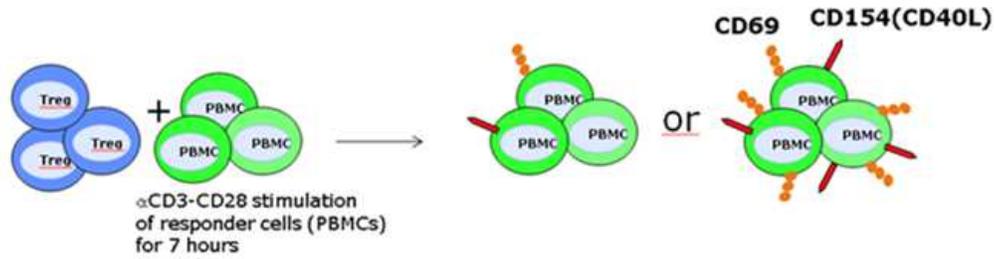


Figure 9. The BD FastImmune™ Regulatory T-cell Function Kit was used to determine the cell surface expression of CD69 and CD154 on PBMCs. In this kit, reduced expression of surface activation markers CD154 and CD69 on activated peripheral blood mononuclear cells (anti-CD3/anti-CD28 beads) in co-culture with Tregs may indicate Treg suppressive capacity, whereas increased expression of the surface markers may indicate lack of suppression.

The suppression assay is a short-term assay (7 hours) using activated PBMCs in the presence and absence of expanded Tregs. The assay was conducted using the Treg BD FastImmune™ Regulatory T-cell Function Kit (BD Biosciences, CA, US, #648956) and was performed using the reagents and instructions as described in the BD FastImmune assay (BD Biosciences, CA, US), briefly as follows.

On the day prior to the suppression assay, cryopreserved, autologous PBMC were thawed according to (Disis et al., 2006), resuspended in RPMI1640 culture media containing 2nM L-glutamine, 1% MEM vitamins, 1 mM sodium pyruvate, 1% MEM NEAA, 10 mM HEPES, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 uM 2-mercaptoethanol, and 10% heat-inactivated human AB serum, pH 7.4, and rested overnight in a 37 °C/5% CO₂ incubator. After the overnight rest, the PBMCs were counted and resuspended in RPMI 1640 culture media at a density of 2-5×10⁶ cells/ml.

The expanded Tregs were plated at 2-5×10⁵ cells/well in 100 µl volume in 96-well V-bottom polypropylene plates (BD Falcon™, #353263) and were then serially diluted so as to ultimately achieve a range of Treg to PBMC ratios of 1:1, 0.5:1, 0.25:1, and 0.125:1, while keeping the PBMC number constant. The plate was placed in a 37 °C/5% CO₂ incubator while preparing the activation agent consisting of anti-CD3/anti-CD28 beads and APC-anti-CD154. CD154 is transiently expressed on the cell surface, and the presence of anti-CD154 antibody will then remain on the cell surface even if CD154 is internalized (Chattopadhyay et al., 2005) .

After incubation, the activation agent was added at a concentration of 0.25 beads per PBMC (i.e. 1.25×10⁵ beads/5×10⁵ PBMC) and thoroughly mixed. Tregs, conventional T-cells and PBMCs with and

without the activation agent were included as controls. The plate was centrifuged (2-3 min, 250g) and CpG-A and GLP-1 was added in the same concentrations as during the expansion. The plate was incubated for 7 hours in the 37 °C/5% CO₂ incubator. After the 7-hour incubation period, the samples were either stained immediately or held overnight at 4 °C.

To prepare for the staining, the cells were pelleted in the 96-well plate (5 min, 500g) and washed with 1XPBS (w/o Ca²⁺ and Mg²⁺, Gibco® Life Technologies, #14190-094) containing 1% FBS and 0.15% sodium azide. An antibody cocktail of PerCp-CY5.5-anti-CD3/FITC-anti-CD4/PE-anti-CD25 reagent and PE-Cy7-anti-CD69 (all from BD FASTImmune™ Regulatory T-Cell Function Kit) was added to the residual volume and surface stained for 30-60 min at room temperature and in the dark. After washing the plate twice with 1XPBS/1% FBS/0.15% sodium azide, stained samples were analyzed on a FACS Aria™ III flow cytometer (BD Biosciences, CA, US) using BD FACSDiva™ software.

The gating strategy started with a lymphocyte gate, followed by gating on CD4⁺ cells. As Tregs also express CD69 and CD154 upon activation (anti-CD3/anti-CD28 beads were used both in the expansion assay and in the suppression assay), it was important to separate the Tresp from the Tregs. This was performed by a CD25 negative gating, allowing only the expression analysis to include only CD4⁺CD25⁻ events using an unstained, unstimulated PBMC sample. CD154 and CD69 activation marker staining was displayed as single color histograms of the CD4⁺ population.

Data analyses

Flow cytometry data were analyzed using FlowJo software (TreeStar). Suppression of activation was measured as a reduction in the frequency of CD154- or CD69-expressing cells and calculated using the following formula: $100 - [(\% \text{ Positive in presence of Treg} / \% \text{ Positive in absence of Treg}) \times 100]$.

STUDY II⁵

Efficacy study of subcutaneous administration of liraglutide on inflammation in the murine PMA ear inflammation model

PMA ear inflammation mouse model

The murine PMA (phorbol 12-myristate 13-acetate) ear inflammation model is a short term (7 days) chronic ear skin inflammation model commonly used for testing the effect of anti-inflammatory compounds on inflammatory diseases as previously described (Malaviya et al., 2006; Burke et al., 2001). Ear skin inflammation is induced by application of PMA, a phorbol ester capable of activating a variety of immune cells, mast cells in particular, resulting in localized inflammation with signs including erythema, edema, infiltration of leukocytes and hyperplasia (Wershil et al., 1988; Szallasi and Blumberg, 1989)(Figure 10).

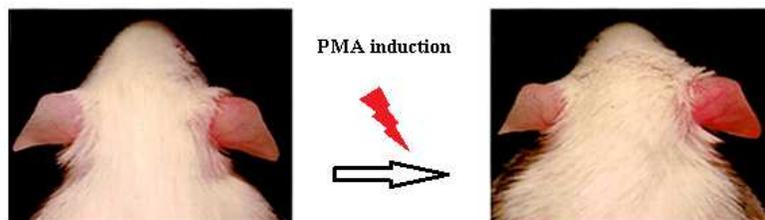


Figure 10. PMA induces local inflammation characterized by redness and swelling of the ear

Experimental animals

35 female SPF (Eller et al., 2011) BALB/c mice, between 7 and 8 weeks of age, were obtained from Taconic Europe A/S (Lille Skensved, Denmark). The mice were housed in a controlled environment (12-h light/12-h dark photoperiod and temperature $22\pm 2^{\circ}\text{C}$). Mice were provided free access to UV-sterilized water and standard mouse chow (Altromin®, Ringsted, Denmark). The animals were imported to the laboratory 6 days before the start up of the experimental procedures in order to assure proper acclimatization and daily records and decisions were made concerning animal welfare. The study was approved by the National Animal Experiments Inspectorate.

Preparation of liraglutide dose solution

⁵The PMA ear inflammation study was conducted at and in accordance with Pipeline Biotech A/S under Danish Animal Experiments Inspectorate License number 2011/561-1956 schedule C4.

Freeze-dried liraglutide (synthesized at Zealand Pharma A/S) was dissolved in sterile filtered 0.1% aqueous ammonia to a concentration of 450 nmol/ml (stock solution) by gentle swirling, yielding a clear solution by visual inspection. Stock solutions were diluted to 8 nmol/ml (dosing solution) in sterile PBS containing 1.06 mM KH₂PO₄, 155.17 mM NaCl, 2.97 mM Na₂HPO₄ •7H₂O, pH 7.4 (Gibco® Life Technologies, #10010). Dosing solutions were kept at ambient temperature and used on the day of preparation. Immediately after use the remaining dosing solution was stored below -18°C for possible later analysis.

PMA ear inflammation study protocol

The ear skin inflammation experiment was performed largely as previously described (Malaviya et al., 2006). PMA and BM doses were determined by Pipeline-Biotech (Pipeline-Biotech A/S, Rødning, Denmark) based on the literature and prior in-house efficacy optimization studies, and the liraglutide dose was chosen to mimic the human therapeutic dose.

Animals were divided into the following groups: group 1, control (acetone/water); group 2, PMA only (0.040 mg/ml PMA + acetone/water); group 3, PMA and BM (0.040 mg/ml PMA + 0.50 mg/ml BM); and group 4, PMA and liraglutide (0.040 mg/ml PMA + 40 nmol/kg) (Table 2).

Group No.	Treatment (N)	Ear challenge	Topical treatment (right ear)	S.c injection twice daily
1	Control (5)	Acetone/water	Acetone/water	Liraglutide vehicle
2	PMA only (10)	0.040 mg/ml PMA	Acetone/water	Liraglutide vehicle
3	PMA and BM (10)	0.040 mg/ml PMA	0.50 mg/ml BM	Liraglutide vehicle
4	PMA and liraglutide (10)	0.040 mg/ml PMA	Acetone/water	40 nmol/kg liraglutide

Table 2. Overview of treatment groups

Study design is summarized in Figure 11. To optimize efficacy of liraglutide, the dosing period began two days prior to PMA challenge, at day -2. Ear inflammation was induced on day 0, 2, 5 and 7 by twice daily topical application of 10 µl of a 40 µg/ml PMA solution in acetone/water (99:1) to the right ear of each mouse, 5 µl to each side of the ear, except for the control group, which received PMA vehicle (acetone/water) and served as control for the normal ear. The PMA and BM group was treated with

topical application of 20 μ l of a 0.50 mg/ml BM in acetone/water (99:1) to both sides of the right ear (10 μ l to each side of the right ear) within 20 min after PMA application performed twice daily at day 5 and 6 and once in the morning at day 7 and served as a positive control. Group 1, 2 and 4 were treated in the same way with the acetone/water (99:1) following the PMA treatment. Liraglutide, 40 nmol/kg (dose volume of 5ml/kg), was administered to group 4 by subcutaneous (s.c) injection in the back of the neck to each mouse twice daily at day -2 to 6 and once in the morning at day 7. The remaining groups were treated with 5 ml/kg sterile 0.1% ammonia and PBS (liraglutide vehicle) injected s.c. as with the liraglutide treatment (Table 2). After treatment of the ears the animals were placed in a cage different from their normal cage for a short time to draw the attention of the mice to the new environment and avoiding the mice licking test articles from each others ears.

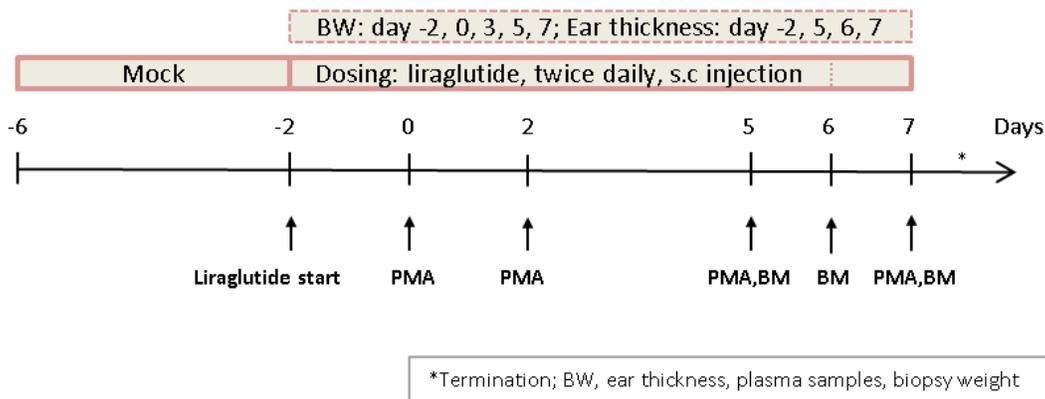


Figure 11. Study design

Body weight

Body weight was determined at day -2, 0, 3, 5, and 7 for each mouse.

Ear thickness

Ear thickness of both ears of each mouse was measured by a pressure sensitive digital slide gauge (Mitutoyo, Aurora, IL, ID-C1012CB, #543-274B) once at day -2, 5, 6 and 7 and at termination at day 7. Measurements were conducted before challenge with PMA and treatments. The digital micrometer is applying a pressure on the measurement point on the ear and the swelling will decrease with at high rate followed by a slow rate, and the thickness of the swelling will be recoded when the slow rate is started,

which will be within few seconds. Care was taken that there was no contamination due to use of the same digital micrometer for all ears. This was assured by alcohol cleaning of the micrometer and by measuring before any treatment of ears and in the following group order: group 1, 2, 4, and 3. Ear swelling was calculated by subtracting the thickness of the left ear (vehicle) from the right ear (treatment).

Ear weight

Six hours after the last PMA challenge on day 7 mice were killed by cervical dislocation. At termination, a 6 mm diameter ear punch biopsy from each ear was removed with a metal punch and weighed. Each biopsy was taken from the middle part of the each ear (both right and left). Ear edema was calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment), and was expressed as ear weight.

Analysis of systemic inflammatory markers

Blood was obtained as retroorbital blood samples collected into EDTA tubes under CO₂ anaesthesia and plasma was prepared immediately by centrifugation and stored at -80 °C until use. Plasma was analyzed by multiplex analysis using the mouse TH1/TH2 9-plex panel (MULTI-ARRAY®, MSD, MD, USA, #K15013C) on the Mesoscale Discovery platform. Choice of analytes, based on obtaining a broad range of general inflammation-related cytokines, included IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12, KC, and TNF- α . The acute-phase protein, HP was measured using a sandwich ELISA as described in (Petersen et al., 2009)⁶.

Statistical analysis

The net AUC, including the areas falling below baseline, was calculated by using the trapezoid method. Statistical evaluations of the data were carried out using one-way ANOVA (Kruskal Wallis) between PMA-groups unless stated otherwise followed by Dunn's post test to correct for multiple comparisons. Mann-Whitney U t-test was used when comparing control group to 'PMA only'. All statistical calculations were performed using prism 5.0 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant. Data are expressed as mean \pm SEM.

⁶Haptoglobin measurements was performed at National Veterinary Institute by employed lab technician

STUDY III – V

Study III (Manuscript 1)

Effects of Roux-en-Y gastric bypass on fasting and postprandial levels of inflammatory markers in obese subjects with normal glucose tolerance or type 2 diabetes

Study IV (Manuscript 2)

Effect of Roux-en Y gastric bypass on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects

Study V (Manuscript 3)

Effect of 12-week treatment with glucagon-like peptide 1 receptor agonist liraglutide on circulating invariant NKT cells, regulatory T-cells and inflammatory markers in obese subjects with type 2 diabetes

Study registration

Studies were approved by the Municipal Ethical Committee of Copenhagen⁷ and in accordance with the Helsinki-II declaration and the Danish Data Protection Agency. The studies were registered at www.clinicaltrials.gov⁸. Before initiating study III and IV, supplementary protocols were approved in addition to the main protocols. The initial protocol of study V comprised 15 patients, but due to difficulties in the recruitment process, we only managed to include 9 patients during the study period (January 2013 – April 2014).

Participants, patient material, and study design

Obese patients, type 2 diabetes or glucose-tolerant subjects were conducted from the Hvidovre Hospital bariatric surgery program or from the outpatient clinic at the Department of Endocrinology at Hvidovre Hospital. Study III (**Manuscript 1**) was based on stored sample material (plasma) obtained during the course of the original study (2008 – 2009). Samples in Study III (**Manuscript 1**) and Study IV (**Manuscript 2**) from gastric bypass patients were collected before and after surgery with sequential follow-ups depending on the respective study. Samples from a mixed meal test was included and selected based on the expected GLP-1 secretion pattern including baseline concentration (0 min), GLP-1 peak (45-60 min) and back to baseline (120-240 min). T2D patients commencing to liraglutide therapy (Victoza®, Novo Nordisk, Bagsværd, Denmark) were examined on two occasions: prior to and 12 weeks after

⁷ Study III: Reg. nr. H-A-2008-080-31742; Study IV: Reg. nr. H-2-2011-057; Study V: Reg. nr. H-2-2012-148

⁸ Study V: ID NCT01579981; Study V: ID NCT01559792; Study VI: ID NCT02201550

initiating the treatment. Doses of liraglutide were decided according to NICE (National Institute of Care Excellence) and as described in (Madsbad, 2009).

Immunological analyses

Analysis of circulating inflammatory cytokines by Mesoscale Discovery technology (Study III – V)

For immunoanalysis of circulating cytokines, the Mesoscale Discovery (MSD) technology using a high-throughput MULTI-ARRAY platform was chosen (MSD[®], Gaithersburg, MD, USA). The system uses plates with 10 carbon electrodes per well, each electrode being coated with a different capture-antibody enabling simultaneous measurement of up to 9 different analytes. The assay procedure follows that of a sandwich ELISA. The analytes of interest are captured on the electrode and are detected with an analyte-specific ruthenium-conjugated secondary antibody. Upon electrochemical stimulation, the ruthenium label emits light (luminescence) at the surface of the electrodes allowing the concentration of the analyte to be determined relative to the particular electrode.

An initial selection on which inflammation markers to include was based on a literature search on commonly reported cytokines and scarcely reported but interesting cytokines with respect to obesity and T2D. An introductory screen in Study III (**Manuscript 1**) of four patients (two with normal glucose tolerance and two with type 2 diabetes) contained the following inflammatory analytes: IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-15, IL-17, TNF- α , TGF- β , GM-CSF, IFN- γ , adiponectin and leptin and a panel was selected based upon the most responsive markers. Because of new assay development technique within multiplexing (The V-PLEX[®] technology, MSD), enabling higher sensitivity, Study IV (**Manuscript 2**) and Study V (**Manuscript 3**) included IFN- γ , IL-10, and MCP-1.

Flow cytometry analysis of blood samples (Study IV and V)

In Study IV (**Manuscript 2**), blood was left overnight at room temperature prior to PBMC isolation, whereas this procedure was undertaken within 2 hours from blood collection in Study V (**Manuscript 3**), due to optimization experiments indicating a larger cell yield when PBMC isolation was performed within 4 hours. Due to isotype and high background staining in some patient samples, it was decided in Study V (**Manuscript 3**) to replace the PE-anti-TCR V α 24-J α 18 antibody with a PE-anti-CD1d tetramer pre-loaded with α -GalCer (ProImmune Ltd, Oxford Science Park, UK) together with APC-anti-CD3 (clone: HIT3a) and Alexa Flour 488-anti-CD19.

DISCUSSION

Obesity-induced T2D is accompanied with a set of metabolic abnormalities comprising the metabolic syndrome, such as hypertension, dyslipidemia, and insulin resistance. Although the exact causes for the onset of clinical disease remain largely unknown, emerging evidence suggests that obesity-induced inflammation, especially in the adipose tissue, is involved in metabolic dysregulation and therefore plays an important role in the pathogenesis of this deteriorating disease. The exaggerated secretion of adipose-derived inflammatory cytokines in the obese state affects insulin resistance negatively by inactivating the insulin receptor and the IRS proteins through serine phosphorylation and activating inflammatory pathways through the pro-inflammatory transcription factor NF- κ B and/or JNK signaling (Wisse, 2004).

Bariatric surgery, including RYGB, has revolutionized the treatment of severe obesity and related comorbidities, in particular T2D. With surgery, large weight loss is accomplished with immediate improvement in glycemic control and insulin secretion. Studies have suggested that exaggerated postprandial GLP-1 secretion is a pivotal factor involved in both weight loss and improved glucose metabolism following RYGB (Dirksen et al., 2013). Thus, gastric bypass patients offer an interesting look into the possible effects of increased GLP-1 levels on the immune system and its links to the observed increase in insulin sensitivity.

The *in vitro* work, the animal study, and the three manuscripts constituting this thesis was undertaken to study GLP-1 effects on immune parameters. In **Study I**, the functionality of human expanded Tregs in the presence of GLP-1 was examined *in vitro*. **Study II** investigated anti-inflammatory effect of GLP-1 *in vivo* in the PMA-induced ear inflammation model. Three human studies were conducted that generated the three manuscripts in the thesis; two RYGB studies (**Study III, Manuscript I and Study IV, Manuscript II**) and one study in obese T2D subjects commencing liraglutide therapy (**Study V, Manuscript III**), to evaluate immunological changes either after surgery (1 week, 3 months, and 1 year) or after 12 weeks GLP-1 therapy.

Does GLP-1 treatment modulate Treg function *in vitro*?

Animal studies have shown that re-injecting expanded Tregs into *ob/ob* mice protected against weight gain and alleviated metabolic abnormalities (Illan 2010). Data from murine experiments support that GLP-1 has immunomodulatory effects by affecting proliferation and function of Treg cells (Xue 2008, Hadjiyanni 2008), and functional GLP-1Rs have been found on several immune subpopulations, including CD4+, CD4+CD25+ T-cells, and iNKT cells (Hadjiyanni et al., 2010; Marx et al., 2010; Hogan et al., 2011). This created a basis for testing a direct effect of GLP-1 on isolated populations of *ex vivo*

expanded human Tregs. We hypothesized that activation of the GLP-1R by a GLP-1R agonist would increase the ability of Tregs to inhibit activation of other T-cells (**Study I**).

We found that the GLP-1R agonist liraglutide could enhance suppressive function of Tregs in a dose-dependent manner ($N=1$). The data were, however, not reproducible in two succeeding donors. As discussed, the reason for the discrepancy between the three donors might be explained by the variations in patient material impacting the quality of the Tregs in their natural ability to suppress and how they respond to expansion and to compound treatment. Based on this experiment we were unable to make solid conclusions about whether GLP-1 has a direct role as modulator of Treg function (*in vitro*).

In vitro experiments that have examined GLP-1 in mixed or isolated immune cell populations and in adipocyte- or endothelium-derived cell lines have reported varying anti-inflammatory effects of GLP-1. When studying cell-specific responses and mechanisms, *in vitro* experiments are essential but do not necessarily reflect the *in vivo* situation. As the evidence for anti-inflammatory effect of GLP-1 *in vitro* builds upon unspecific details on mechanisms of action, the conclusions should be interpreted with caution due to limited translation into the *in vivo* situation, where multiple cell types and signaling molecules interact in the physiological environment.

Despite emerging techniques in the *ex vivo* expansion of Tregs and subsequent functional analysis, this assay platform is still in the explorative phase with regard to consistency and validation of functionality assessment. Based on the present experiment, the dose-dependent effect of liraglutide observed in one donor was not sufficient for determining whether GLP-1R activation *in vitro* causes enhanced suppressive function of Tregs.

Does GLP-1 treatment modulate circulating immune cells *in vivo*?

The observations of clinical improvement in psoriasis after initiating treatment with GLP-1R agonists associated with changes in number and function of iNKTs and the finding of functional GLP-1Rs on this immune subset have encouraged studies investigating functional or proliferative changes in this subset in relation to GLP-1R activation.

Accordingly, we performed a human study in obese T2D patients subjected to liraglutide therapy (**Manuscript 3**). We found that liraglutide treatment for 12 weeks reduced HbA1c and induced minor weight loss of 3 %, but no changes in the number of circulating Treg and iNKT cells was detected. There was a small statistically significant, but most likely biological and clinically insignificant, increase in IL-8. In this study, we were unable to reproduce the suggested effect on iNKT cells reported in work by the O'Shea group (Hogan et al., 2011; Ahern et al., 2013). As discussed in **Manuscript 3**, an activated

immune system may be a prerequisite for GLP-1 having an effect on the immune cell subset frequency and/or function. Psoriasis is an autoimmune-mediated inflammatory skin disease involving cells from both the adaptive and innate immune system, including Treg and iNKT cells, and an enhanced production of inflammatory cytokines detected locally and in circulation (Kagen et al., 2006; Davidovici et al., 2010; Sweeney et al., 2011). Hogan and co-workers reported higher circulating iNKT cell frequencies in obese psoriasis patients compared to our patient group, both before and especially after liraglutide therapy, which could suggest differences in the degree of immune activation and thus incompatibly between these two patient groups. T2D patients belong to a very heterogeneous group in relation to BMI, body fat distribution, including abdominal obesity, insulin resistance, degree of low grade inflammation, and amount of obesity-related co-morbidities, i.e. CVD. Indeed, immunoregulatory effect of GLP-1R agonist treatment may not be expected in all patients, emphasizing a limitation of the study presented in **Manuscript 3**, such as the small sample size and lack of obese control group receiving placebo. Assessment of circulating iNKTs in relation to clinical impact of a given disease is complicated by the paucity in human blood, by large individual variations, and lack of standardized antibodies for enumeration of these cells.

To conclude on the immune cell data obtained and described in **Manuscript 3**, no effect on circulating Treg or iNKT cells could be detected in obese T2D patients receiving liraglutide therapy.

Does GLP-1 have local and/or systemic anti-inflammatory effects?

In **Study II**, the GLP-1R agonist liraglutide was examined in the PMA ear inflammation model - a model of chronic inflammation. Investigation of anti-inflammatory effects of GLP-1 in this model has not been tested before. We found that treatment with liraglutide appeared to down-regulate specific parts of the systemic response to local inflammation, as detected by a significant suppression of PMA-induced IL-1 β secretion and a modest reduction in TNF- α , KC and IL-12 (non-significant). Liraglutide treatment did, however, not affect the primary study end-points relating to the local PMA-induced inflammation, such as ear thickness and ear weight.

The lack of effect on these parameters may be explained by the robust and “non-physiological” nature of PMA-induced inflammation, which limits the value of this model in studying minor to moderate immunoregulatory effects. The model is attractive when testing compounds with a proposed effect on the cPLA₂ enzymes that are stimulated by PMA but may not be sensitive enough for the detection of small anti-inflammatory changes as potentially mediated by GLP-1. Based on findings in the literature suggesting that liraglutide modifies cytokine production but do not suppress cytolytic activity of altered iNKT cells (Hogan 2011), and on our findings, both in the obese T2D patients on liraglutide therapy and in the PMA

experiment, the actions of GLP-1 may be of a more immunoregulatory character as opposed to immunosuppressive.

T-lymphocytes modulate immune responses through several suppressive mechanisms, and although no changes in the frequency of iNKT and Treg cells was detected, their function may have been affected by surgery (rationale behind **Study I**). Changes in the cytokine profile towards enhanced anti-inflammatory Th2 immunity and reduced Th1 responses may reflect functional changes of the cytokine-secreting immune cells. One may speculate that the acute version of the ear inflammation model with duration of only 6 hours might have been a better alternative to study GLP-1 immunoregulatory effects. As mice were pre-treated with liraglutide, the potential ability of liraglutide to prime and inhibit immune cells might have been revealed if we had obtained ear thickness measurements on day 1 or 2 after the first PMA challenge.

As described in **Manuscript 3**, we did not find any changes in IL-6, IL-10, TNF- α , TGF- β , adiponectin and leptin but a small increase in plasma IL-8 after 12 weeks of liraglutide therapy in humans. As shown in **Manuscript 1 and 2**, similar changes in IL-8 were registered after RYGB. The biological relevance and clinical significance of this increase can, however, be questioned. The circulating levels of IL-8 we have reported in **Manuscript 1, 2 and 3**, are all comparable to the concentrations found by Bruun and co-workers in obese subjects. After 20 weeks and 24 weeks of weight loss (~15%), the same authors measured a 30% increase in IL-8, equal to our findings. Thus, regardless of the amount of weight loss (GLP-1 induced a weight loss of 3% see **Manuscript 3** and RYGB induced weight loss of 13% see **Manuscript 1 and 2**), IL-8 secretion appears to be slightly affected. Compared to the aforementioned GLP-1-mediated decrease in KC (the mouse ortholog of human IL-8), a GLP-1-specific effect on IL-8 appears questionable.

One explanation for the GLP-1-induced effect on cellular and molecular immune mediators observed in some studies and not in others might be that the anti-inflammatory effect of liraglutide therapy is indirect, either through weight loss *or* by improvement in metabolic parameters, such as glucose and insulin that may have an impact on cytokine secretion as well. In **Manuscript 3**, we found that liraglutide improved glucose metabolism but without significant effects on cytokine secretion.

Is RYGB associated with changes in the immune system – and if so, are these changes mediated by GLP-1?

Bariatric surgery restores glycemic control and improves insulin secretion and obesity-related comorbidities. Reduction in the risk of developing obesity-related co-morbidities by gastric bypass may

involve resolution of inflammation characterized by decreases in pro-inflammatory cytokines (e.g IL-6 and leptin) and increases in anti-inflammatory adipokines such as adiponectin. The approximately 10-fold increases in postprandial GLP-1 secretion following gastric bypass procedures may be involved in the beneficial metabolic effects both directly via the classical glucoregulatory pathways and indirectly via anti-inflammatory and immune-regulatory mechanisms.

The hypothesis behind the work described in **manuscript 1 and 2** was that the metabolic improvements observed after RYGB was linked to an anti-inflammatory profile, mediated, in part, by immunological activity of GLP-1. **Manuscript 2** included evaluation of Tregs and iNKTs and cytokines, which was not measured in **Manuscript 1**.

In **Manuscript 1**, we reported a decrease in fasting and postprandial concentrations of the pro-inflammatory cytokines IL-6, leptin, and the regulatory cytokine TGF- β , while the anti-inflammatory adipokine adiponectin was increased one year post-surgery. Two patient groups were examined; obese patients with type 2 diabetes and subjects with normal glucose tolerance, with no significant differences in the levels of inflammatory mediators between the two groups before, 1 week, 3 months, and 1 year after surgery. The improvement of glucose metabolism after one year was correlated with the decrease in IL-6 and TGF- β concentrations, but only in the T2D group. As discussed in the manuscript, this could be due to either a major improvement of HbA1c values in this group as compared to the normal glucose tolerant group, but could also imply a role for IL-6 in glycemic control when insulin resistance is present. Swarbrick and coworkers found a similar decrease in circulating IL-6 concentrations one year after RYGB to be associated with the relative improvement of insulin sensitivity (swarbrick 2008).

Previous bariatric surgery studies have rarely examined both pro- and anti-inflammatory cytokines and the majority of these studies often include only a single follow-up measure. A strength of the study described in **Manuscript 1** is the inclusion of several time-points, which provides a more comprehensive examination of RYGB effects on the immune system as both short- and long-term changes are measured. Our main results on the short-term effects were a decrease of leptin to a level sustained at all post-operative follow-ups and a marked but non-significant increase in IL-6 due to huge patient variation. The immediate decrease in leptin occurs independently of loss of fat mass, and may be explained by a neuroendocrine response to fasting as suggested in (landt, M + Ahima). The short-term effects of RYGB could be a result of caloric restriction rather than the surgical procedure itself.

A further strength of the data presented in **Manuscript 1** was that dynamic meal-induced gastrointestinal hormone and cytokine responses were investigated. The enhanced postprandial GLP-1 secretion occurring

immediately after RYGB may influence functional capabilities of immune cell subsets expressing the GLP-1R whereby cytokine secretion is altered. Considering the hypothesis of a direct effect of GLP-1 on the circulating immune cells, the inclusion of a meal test was an interesting approach in studying acute effects on immune cell cytokine secretion. The results did, however, not reveal any correlations between postprandial GLP-1 and cytokine responses.

Manuscript 2 sought to examine the short-term effects (3 months) of RYGB on changes in iNKTs and Tregs and whether changes in cytokine secretion, as observed and reported in **Manuscript 1** could be attributed to these changes. Lynch and co-workers found enhanced levels of circulating iNKTs following weight loss induced by RYGB, suggesting one potential mechanism by which the massive weight loss after bariatric surgery may re-establish insulin sensitivity (Lynch 2012). No changes in Tregs or iNKTs could be detected three months after surgery although insulin resistance was significantly improved as described in **Manuscript 2**. We did, however, find increases in the anti-inflammatory cytokine IL-10 (113%), an unexpected increase in IFN- γ (24%), and an expected significant decrease in leptin. IFN- γ and IL-10 are prototypical Th1- and Th2-derived cytokines secreted by T-lymphocytes in order to regulate immune responses. The presence of IL-10 is important for the immunosuppressive effect of Tregs, while iNKTs are potent inducers of IFN- γ . IFN- γ has shown cytotoxic effect on beta-cells in autoimmune diabetes (Chong et al., 2001), but studies on beta-cell function after RYGB suggest improved function (Jorgensen et al., 2013). The finding on IFN- γ presented in **Manuscript 2** may not be clinically relevant but could nevertheless illustrate functional changes in the cytokine-secreting cells. Although we did not observe changes in the frequency of iNKT and Treg cells, their function may have been affected by surgery as reflected by changes in Th1 and Th2 immune responses. Functional analyses of immune cell subsets following RYGB could be an attractive supplement to improve understanding of the effects of RYGB and weight loss on inflammation.

CONCLUSION

This PhD work was undertaken to investigate anti-inflammatory and immune-modulating effects of GLP-1 in different experimental model systems. Overall, no strong immunosuppressive effects of GLP-1 were detected in the *in vitro* and *in vivo* test systems applied. While direct immunoregulatory effects of GLP-1 on Treg and iNKT cells is an attractive hypothesis we were not able to detect changes in these cells in the non-clinical or clinical studies conducted. The lack of changes may be due to limitations in the experimental setup and/or large heterogeneity in patient material. Indeed, GLP-1 may only affect Tregs

and iNKTs in exacerbated inflammatory situations such as during a flare in psoriasis. While initial positive data was obtained in one single donor in the *in vitro* Treg studies, the findings could not be reproduced in subsequent samples. In the PMA animal model, we did observe a systemic immunoregulatory effect of GLP-1 on various pro-inflammatory cytokines, but no suppressive effect on the local ear inflammation. The model might have been too severe to allow GLP-1 to exert a significant local effect, despite slight changes in systemic inflammatory markers. The effect of liraglutide treatment on circulating inflammatory markers was minimal in patients with T2D. A slight increase in plasma IL-8 was detected following 12 weeks of treatment while the other markers were unchanged.

RYGB was associated with changes in some immune mediators, such as IFN- γ , IL-10 and leptin. It is not possible to determine from our study design the influence of weight loss or RYGB *per se* on the observed changes and whether alterations in cytokine secretion is related to functional changes in the immune cells or due to other influencing factors such as post-surgical stress responses as recently suggested (Lin and Gletsu-Miller, 2013).

FUTURE PERSPECTIVES

Despite observations of only limited effects of GLP-1 on different immune parameters in the different test systems used in this PhD thesis it remains relevant to further study potential immunoregulatory effects of GLP-1 treatment. Moreover, controversy exists in the literature regarding the absolute effects and specific mediators of potential GLP-1-mediated immune suppression. Moreover, it will be important to understand if GLP-1 treatment can alter the iNKT or Treg cells in subtypes of patients with T2D such as reported in patients with psoriasis. Moreover, RYGB studies in animals lacking a functional GLP-1R can be used to further elucidate a potential anti-inflammatory role of GLP-1 following this procedure.

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MANUSCRIPT 1

Effects of Roux-en-Y gastric bypass on fasting and postprandial circulatory levels of inflammation-related biomarkers in obese subjects with normal glucose tolerance and in obese subjects with type 2 diabetes

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ABSTRACT WORD COUNT: 250

ABSTRACT

Purpose: To explore the hypothesis that Roux-en-Y gastric bypass (RYGB) reduces circulating levels of pro-inflammatory cytokines, while increasing anti-inflammatory cytokines in obese subjects with type 2 diabetes and in glucose tolerant obese subjects.

Methods: Thirteen obese subjects with type 2 diabetes and twelve matched obese normal glucose tolerant subjects were examined before, one week, three months, and one year post-surgery. IL-6, leptin, adiponectin, IL-8, TGF- β and the incretin hormone GLP-1 were measured in the fasting state and during a liquid meal. Insulin resistance was evaluated by HOMA-IR.

Results: Weight loss did not differ between the two groups. Pre-surgery, HbA1c was higher and HOMA-IR lower in type 2 diabetic patients, however, converged to the values of glucose tolerant subjects one year post-surgery. Circulating biomarker concentrations did not differ between the two groups at any time point. One week after surgery, circulating IL-6 and IL-8 were increased, while adiponectin and leptin were reduced compared with pre-surgical concentrations. TGF- β levels did not differ before and one week after surgery. Three months post-surgery, IL-8 was increased, leptin was reduced, and no change was observed for IL6, TGF- β , and adiponectin. One year post-surgery, concentrations of IL-6, TGF- β and leptin were significantly reduced compared to before surgery, while adiponectin was increased. No correlation between the postprandial GLP-1 responses and cytokines levels was observed before or after RYGB surgery.

Conclusion: RYGB reduced pro-inflammatory biomarkers and increased anti-inflammatory mediators in diabetic and normal glucose tolerant obese subjects 1 year post-surgery. IL-8 did not differ before and after surgery.

Key words; RYGB, weight loss, type 2 diabetes, glucose tolerant subjects, cytokines

ABBREVIATIONS

CVD: Cardiovascular disease

BMI: Body mass index

RYGB: Roux-en-Y gastric bypass

GLP-1: Glucagon-like peptide-1

HOMA-IR: Model assessment of insulin resistance

HbA1c: Glycated hemoglobin

HPLC: High pressure liquid chromatography

SEM: Standard area of the mean

SD: Standard deviation

AUC: Area under the curve

IL-: Interleukin-

TGF- β : Transforming growth factor beta- β

MSD: Meso Scale Discovery

NGT: Normal glucose tolerant

T2D: Type 2 diabetes

INTRODUCTION

Obesity is associated with chronic low-grade inflammation, which is thought to be involved in the development of obesity-related co-morbidities, such as insulin resistance, type 2 diabetes and cardiovascular diseases (CVD) [1].

The adipose tissue is considered the prime organ for mediating obesity-induced inflammation by secreting high levels of pro-inflammatory molecules, including IL-6 secreted by immune cells residing in the stromal vascular fraction. This is thought to contribute to the development of insulin resistance and deterioration of glucose homeostasis [2-4]. Leptin is primarily produced by adipocytes and plasma levels are elevated in obese subjects [5]. Leptin is a central mediator in regulating food intake and energy expenditure [6] and has been shown to inhibit insulin secretion from pancreatic beta cells [7]. In addition, studies have indicated an immunological role of leptin by inducing the production of IL-6 and other acute-phase reactants [8;9]. Adiponectin is also produced predominantly by adipocytes, and plasma levels of adiponectin are inversely correlated with body mass index (BMI) [10;11]. Decreased levels of adiponectin have been linked to impaired insulin sensitivity and increased risk of CVD [12;13]. The chemokine IL-8 has been found to be produced and released from human adipocytes [14], in addition to playing a central role in inflammatory processes, including atherosclerotic processes as a neutrophil chemoattractant [15;16]. Circulating levels of IL-8 are increased in obesity [17;18], in type 1 diabetes and type 2 diabetes patients compared to lean healthy subjects [19] and to obese subjects with normal glucose tolerance [20;21]. Transforming growth factor beta (TGF- β), an immunoregulatory cytokine, has also been found to be associated with obesity and type 2 diabetes [22-25].

Several studies in obese subjects have reported dietary-induced weight loss to improve insulin sensitivity, increase adiponectin levels and reduce circulating levels of leptin, IL-8, and IL-6 [26-29]. Bariatric surgery, including Roux-en-Y gastric bypass (RYGB), restores hepatic and peripheral insulin sensitivity and often causes remission of type 2 diabetes in most cases before any substantial weight loss is achieved [30;31]. There is a marked increase in hepatic insulin sensitivity within one week post-surgery, while peripheral insulin sensitivity is only improved about three months post-surgery after a major weight loss [32]. Several studies have shown that bariatric surgery decreases fasting plasma leptin [33-38] and IL-6 [39-42], while increasing levels of adiponectin independently of weight reduction [35;43].

Whether a more favorable inflammatory profile is linked to the immediate improvement of insulin resistance and resolution of type 2 diabetes after RYGB is not known. In the present study, we explore the hypothesis that the RYGB induced improvement in insulin resistance is associated with decreasing pro-inflammatory and increasing anti-inflammatory circulating cytokine levels. We examined short- and long-term changes of IL-6, leptin, adiponectin, IL-8, and TGF- β from before RYGB surgery, one week, three months and one year after RYGB in obese patients with type 2 diabetes and in obese subjects having normal glucose tolerance. We hypothesized that the fasting levels of pro-inflammatory cytokines would be higher in the type 2 diabetes group before surgery compared with the normal glucose tolerance group, and that the levels in both groups would decrease and equalize after surgery leading to a more anti-inflammatory profile in both groups.

In a recent study in mice, Ellingsgaard and co-workers demonstrated that enhancing circulating IL-6, either through high-fat diet or exercise, increased the secretion and production of glucagon-like peptide-1 (GLP-1) [44]. As the exaggerated postprandial secretion of GLP-1 takes place immediately after surgery [37;45]; we also studied levels of inflammatory markers in the postprandial period and related it to postprandial GLP-1 responses.

MATERIALS AND METHODS

Study population

Study population and study design have been described before [45]. Briefly, thirteen obese subjects with type 2 diabetes and twelve matched obese subjects with normal glucose tolerance were recruited from the Hvidovre Hospital bariatric surgery program (Hvidovre, Denmark). They all met the Danish criteria for bariatric surgery (age > 20 years and BMI > 40 kg/m² or > 35 kg/m² with comorbid conditions such as type 2 diabetes or hypertension), and had accomplished a mandatory preoperative 8 % diet-induced total body weight loss before inclusion. Patients were excluded if they had been receiving incretin-based therapies or insulin, anti-thyroid medication or anorectic agents within 3 months prior to the study.

Study design and experimental protocol

Participants were examined before and at one week, three months, and one year postoperatively. Antidiabetic medication was paused in diabetic subjects 72 h prior to the preoperative meal test. After surgery, none of the type 2 diabetes subjects received any antidiabetic medication.

Patients were studied after a 10-12-h overnight fasting period and were weighed and then ingested in a 30 min period a liquid meal consisting of 200 ml of Fresubin Energy Drink [300 kcal, carbohydrate (E% 50), protein (E% 15), fat (E% 35), Fresenius KabiDeutschland, Bad Homburg, Germany]. Blood was sampled from a catheter in an antecubital vein at -10, -5, 0, +15, +30, +45, +60, +90, +120, +180, and +240 min compared to the start of the meal. In the present study, only samples collected at time 0, 45, 60, 120 and 240 min were analyzed, and sample at time 0 min was used as fasting baseline level.

Surgical procedure

Surgery was performed at the Department of Gastroenterology at Hvidovre Hospital (Hvidovre, Denmark) as previously described [38].

Sample collection and laboratory analysis

Blood samples were collected in clot activator tubes or EDTA tubes. Determination of plasma insulin, GLP-1 and glucose was carried out as described in [45]. HbA1c was determined using HPLC (Tosoh Bioscience, Tokyo, Japan). For protein analyses, venous blood was sampled into EDTA tubes and kept on ice until centrifuged at 4°C, 10 min, 2000g. Plasma aliquots were stored at -80°C until analyzed. Total plasma concentrations of inflammatory markers were determined by electrochemiluminescent sandwich immunoassays (Meso Scale Discovery (MSD), Gaithersburg, MD, USA) enabling simultaneous quantification of multiple analytes. The assays were performed according to the manufacturer's protocol using an MSD instrument (SECTOR Imager 2400, MSD). All samples for each individual subject were run on the same assay plate and the same batch of kits was applied for all samples. Samples with values greater than the upper limit of quantification were diluted and reanalyzed. In the fasting state IL-6, leptin, adiponectin, IL-8, and TGF- β were measured, and in the postprandial samples IL-6, leptin, adiponectin were measured.

Statistical analyses

Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) as $\text{Insulin}_{\text{fasting}} \times \text{Glucose}_{\text{fasting}} / (22.5 \times 6.945)$. To assess the possible effect of RYGB on the postprandial cytokine response, total AUC for IL-6, adiponectin, and leptin were calculated by using the trapezoidal method (GraphPad

Prism v. 4). Statistical analyses were carried out using Mann-Whitney *U*-test for comparisons between groups. For comparisons of pre- and post-surgical time-points, Friedman's nonparametric repeated measures ANOVA with Dunn's multiple comparison post test was applied and included only patients with complete data set (blood sample drawn at all four study time-points). Relationship between variables was assessed by non-parametric Spearman rank correlation. Statistical analysis was performed using GraphPad Prism v. 4 (San Diego, CA, USA). The level of significance was set at $P < 0.05$.

Ethics statement

Written informed consent was obtained from all participants. The study was approved by the Municipal Ethical Committee of Copenhagen (Reg. nr. H-A-2008-080-31742) and was in accordance with the Helsinki-II declaration and the Danish Data Protection Agency. The study was registered at www.clinicaltrials.gov (ClinicalTrials.com ID NCT01579981).

RESULTS

Study population

One subject with type 2 diabetes could not be studied 1 week after RYGB because of anemia. One subject with normal glucose tolerance was excluded from the 3 months follow-up data set, due to excessively high fasting insulin and C-peptide concentrations, indicating a non-fasting state. One subject with normal glucose tolerance was not examined at 1 year follow-up due to pregnancy (Figure 1).

Twelve subjects with type 2 diabetes were treated with ≥ 1 oral antidiabetic medication, and one subject was diet-treated only before surgery. After RYGB none of the type 2 diabetes patients received any anti-diabetic medication.

Changes in weight and glucose metabolism

Data are presented in Table 1. RYGB-induced weight loss in type 2 diabetes subjects: one week: $2.1\% \pm 1.6\%$, three months: $13.2\% \pm 3.7\%$, one year: $22.2\% \pm 8.8\%$ and in normal glucose tolerance: 1 week: $2.5\% \pm 1.3\%$, three months: $15\% \pm 4.5\%$, and one year: $25.2\% \pm 8.0\%$. Body weight and BMI did not differ between the two groups before or after surgery. Fasting glucose concentrations decreased continuously in type 2 diabetes subjects after surgery

($P=0.0001$), whereas in the normal glucose tolerant subjects, it was reduced one week after surgery and thereafter remained unchanged ($P=0.01$). Fasting insulin was significantly lower in both groups after surgery at one week in the type 2 diabetes group and at three months in the normal glucose tolerance group.

HbA1c values were significantly reduced in type 2 diabetes subjects at the one-year follow-up ($P=0.0007$), while no changes occurred in normal glucose tolerant subjects. Before surgery, HbA1c levels were lower in normal glucose tolerance subjects compared to type 2 diabetes subjects ($P=0.0002$), but three months and one year after surgery, HbA1c levels did not differ significantly between the groups (three months: $P=0.06$, one year: $P=0.24$). HOMA-IR was lower in the normal glucose tolerance group before surgery ($P=0.002$), and 1 week ($P=0.001$), and 3 months ($P=0.05$) after surgery compared to the type 2 diabetes group, but the levels did not differ between the two groups at the 1 year follow-up.

Fasting concentrations of inflammatory cytokines

Data are presented in Figure 2. We did not find any significant differences in cytokine concentrations between the two groups at any of the four study time-points (data not shown), and therefore the two groups will be analyzed as one group. One week after surgery, circulating IL-6 and IL-8 levels were increased, of which only IL-8 reached statistical significance, while adiponectin and leptin levels were reduced compared with pre-surgical concentrations. TGF- β levels did not differ before and one week after surgery. At the three-month follow-up, IL-8 was increased, leptin was reduced, and no changes occurred in IL-6, TGF- β , and adiponectin compared to before surgery. One year after surgery, however, concentrations of IL-6, TGF- β and leptin were significantly reduced compared to before surgery, while adiponectin was increased. IL-8 did not differ from before and one year after surgery.

Correlation analysis was performed on data collected before and one year after RYGB in the whole study population and in the two groups separately (Table 2). In the whole study group, before surgery, IL-6 was the only cytokine, that showed a correlation with BMI ($r=0.54$, $P=0.01$) and HOMA-IR ($r=0.52$, $P=0.02$), but after RYGB, the correlation failed to reach statistical significance (BMI: $r=0.40$, $P=0.08$ and HOMA-IR: $r=0.17$, $P=0.48$). Moreover, post-surgical IL-6 values correlated with TGF- β concentrations ($r=0.46$, $P=0.04$). Leptin concentrations correlated with insulin concentrations ($r=0.47$, $P=0.03$), and with body weight ($r=0.49$, $P=0.03$) and BMI ($r=0.58$, $P=0.01$) after surgery. Adiponectin was inversely correlated with body weight before ($r=-0.51$, $P=0.01$) and after surgery ($r=-$

0.59, $P=0.004$), and with BMI ($r=-0.53$, $P=0.01$), HOMA-IR ($r=-0.43$, $P=0.04$) and insulin ($r=-0.47$, $P=0.03$) after surgery. There was a positive correlation between levels of fasting GLP-1 and IL-8 after surgery ($r=0.62$, $P=0.004$).

Postprandial circulating concentrations of cytokines

The postprandial responses of cytokines and GLP-1 are presented in Figure 3a-f and as total AUC in Table 3. Total AUC for the different cytokines in type 2 diabetes patients and normal glucose tolerant subjects did not differ significantly at any study time-point and the two groups will be analyzed as one group. An increase in total AUC for IL-6 was observed one week after surgery followed by a decrease to preoperative response levels after three months and after one year (Fig. 3a). Total AUC for adiponectin was significantly increased one year after surgery compared with both before, one week and three months after surgery ($P<0.0001$) (Fig. 3b). At all post-operative follow-ups, a significant reduction in leptin was observed with no significant differences between the responses after surgery (Fig. 3c) ($P<0.0001$). Postprandial GLP-1 responses were markedly increased at all three post-surgical follow-ups (Fig. 3d) ($P<0.0001$). No correlations between the postprandial GLP-1 response and postprandial IL-6 secretion or other cytokine responses were observed before or after RYGB surgery (data not shown).

DISCUSSION

Low-grade chronic inflammation associated with obesity is considered a major risk factor for the development of obesity-related comorbidities such as insulin resistance and CVD and resolution of the unfavorable inflammatory state could offer a potential explanation for the health improvement associated with major weight loss after bariatric surgery. Previous RYGB studies have focused primarily on well-characterized pro-inflammatory markers and have rarely prospectively examined acute and long term changes of both pro- or anti-inflammatory markers and to our knowledge, only one other study has investigated the postprandial response of cytokines in RYGB-operated subjects.

In the present study, we have assessed fasting and postprandial responses of IL-6, IL-8, TGF- β , leptin, and adiponectin before as well as one week, three months, and one year after RYGB in 13 obese patients with type 2 diabetes and in 12 matched obese subjects with normal glucose tolerance.

We found that RYGB induces changes in circulating concentrations of cytokines in both obese patients with type 2 diabetes and in obese subjects with normal glucose tolerance by decreasing fasting plasma concentrations of the pro-

inflammatory cytokine IL-6, leptin, and the regulatory cytokine TGF- β , and by increasing the anti-inflammatory adiponectin. IL-8 did not differ before and 1 year after surgery. For all investigated cytokines, the changes in fasting levels were similar to the postprandial concentrations of the same cytokine in both type 2 diabetes subjects and normal glucose tolerant subjects and the dynamics in the postprandial responses did not change between pre- and post-surgery. No significant changes in adiponectin and leptin levels were observed postprandially, while a small but significant increase in IL-6 was observed during the meal both before and after surgery. Fasting and postprandial concentrations were similar in the two groups at all time-points. As expected, GLP-1 showed a marked increased postprandial response immediately after surgery.

Fasting cytokine response

Fasting concentrations of cytokines did not differ between type 2 diabetic patients and glucose tolerant subjects at any study time-point, which may in part be explained by the mandatory preoperative 8% diet-induced total weight loss before surgery and that both groups displayed morbid obesity with a mean BMI of about 42 kg/m². Nevertheless, insulin resistance evaluated by HOMA-IR was more pronounced in type 2 diabetic patients before and the first three months after surgery. One year after RYGB, insulin resistance did not differ between the two experimental groups.

Our main findings were a reduction of fasting circulating levels of leptin and adiponectin one week after RYGB, accompanied by an increase in IL-6 and IL-8, while TGF- β levels did not change one week after surgery. One year post-operatively, IL-6, TGF- β , and leptin circulatory levels were significantly reduced, while adiponectin was increased compared to before surgery. IL-8 did not differ before and one year after surgery.

Significant correlations present before surgery and one year after RYGB were an inverse correlation of adiponectin with body weight, while IL-6 and leptin were found to correlate positively with body weight after RYGB but not before. In addition, before surgery, plasma concentrations of IL-6 were found to be correlated with leptin and with HOMA-IR, and after RYGB to be correlated with circulating TGF- β . The levels of adiponectin after RYGB were found to be associated with measures of insulin sensitivity [fasting insulin and HOMA-IR]. The decrease in IL-6 and in TGF- β observed after RYGB was associated with improvement of glucose metabolism as demonstrated by a decrement in HbA1c values (see Table 2).

The reductions in IL-6 concentrations one year after RYGB is in agreement with some other RYGB studies [40;41] and consistent with other weight loss intervention studies, either through dietary restriction [29] or other types of bariatric surgery procedures [39;42;46] reporting one-year follow-ups. Other studies have reported decreased IL-6 within six months after surgery [47], while other investigators have not been able to detect any changes [33;35;48;49]. The maximal weight loss after RYGB is obtained between 1 to 1.5 year after surgery [50], and evidence seems to suggest that at least two years is required post-surgery for stabilization of the inflammatory profile [51]. Lack of effect on inflammation related circulating biomarkers could therefore be explained by the short duration of some studies. In addition, the surgical procedure and subsequent healing process might elicit an inflammatory response delaying the weight induced reduction in biomarkers until several months after surgery. Another explanation for a delay in reduction of pro-inflammatory mediators may be that the restrictive diet following surgery resembles a state of starvation, which has been shown to initiate certain pro-inflammatory responses [52].

We hypothesized that type 2 diabetic subjects would have a higher inflammatory burden as reflected in a more inflammatory circulatory biomarker profile. Significantly higher concentrations of IL-6 have been detected in type 2 diabetic subjects compared to obese healthy subjects [53]. The role of IL-6 in type 2 diabetes and in insulin resistance is, however, debatable [54]. We observed that pre-surgical IL-6 concentrations in the type 2 diabetes group were higher compared to normal glucose tolerant subjects, although not significantly so, which also has been shown in other studies [39;46]. It has been suggested that IL-6 has an effect on the secretion of GLP-1 by intestinal L-cells [44]. In the present study no association between postprandial GLP-1 and IL-6 or other cytokine responses was demonstrated. We did, however, find a significant correlation of IL-6 with body weight after RYGB together with an association between the one-year post-surgical decrease in IL-6 and improved HbA1c values only in type 2 diabetes subjects (Table 2). This observation may imply a role of IL-6 in obesity and in glycemic control when insulin resistance is present, although a clear conclusion about causality cannot be obtained from the present study.

The increase in fasting concentrations of the anti-inflammatory adiponectin is consistent with the findings of a number of RYGB studies [33;35;43]. Brethauer and co-workers found an increase in adiponectin appearing three months after surgery [33], while Trakhtenbrot and co-workers report lack of increase until two years after RYGB

with no changes observed in a cohort of type 2 diabetes subjects undergoing gastric banding. Subjects in the RYGB group lost significantly more fat mass than the gastric banding group [55]. The inverse correlation of adiponectin with body weight before and one year after surgery, but not at the one-week and three-month follow-ups (data not shown), may suggest that either a certain amount of weight loss, a period of weight stabilization or improvement of the metabolic instability is required before changes in plasma adiponectin are manifested.

The decrease in adiponectin immediately after surgery, has, to our knowledge, not been reported before. In a study with obese subjects undergoing liposuction, decreases in adiponectin in addition to increases in IL-6 was reported and was suggested to indicate that IL-6 suppresses adiponectin secretion [56]. *In vivo* studies in obese subjects have shown that low levels of plasma adiponectin were inversely correlated with circulating IL-6 [27;57]. While *in vitro* studies have demonstrated downregulation of adiponectin mRNA in adipose tissue fragments incubated with IL-6 [27;58], adiponectin have also been shown to increase insulin sensitivity through an IL-6-dependent pathway [59]. We did, however, not observe any correlation between adiponectin and other inflammatory cytokines after surgery.

We found a reduction in circulating leptin as early as 1 week after surgery and before significant changes in body weight; a finding which is supported by Isbell and co-workers and others with 2- and 3-week post-surgical follow-ups [34;36;38;60;61]. Reduction of circulating leptin during caloric restriction has been suggested to be an essential component of the neuroendocrine response to fasting [62;63], and the immediate decrease in leptin could therefore potentially be ascribed to post-surgical caloric restriction rather than the RYGB procedure itself.

To our knowledge, the response of IL-8 and TGF- β has not previously been studied in relation to bariatric surgery. We found an increase in IL-8 concentrations 1 week and 3 month after RYGB, but after 1 year the concentrations had returned to preoperative levels. The observed increase in IL-8 after RYGB is of interest as this chemokine potentially is involved in the pathogenesis of atherosclerosis and CVD [16], and thus, we would have expected that a health promoting intervention like RYGB should result in a lowering of IL-8 levels. Similar to our finding, Bruun and coworkers reported an increase in IL-8 after 20 weeks and 24 weeks, respectively, of dietary-induced weight loss of approximately 15% [17;26]. Other cell types besides adipocytes involved in acute and chronic inflammatory responses could add to the secretion of IL-8 [64] and this could evidently also be the case after acute weight loss. In addition, other substances released from the adipose tissue during weight loss, such as organochlorine compounds,

may also stimulate the release of IL-8 [65] and of other adipose tissue-derived cytokines [66]. The immediate increase after RYGB could also be a surgery-related stress response, as recently suggested [67].

Studies measuring circulating TGF- β are somewhat limited due to TGF- β being present in an inactive form requiring proteolytic activation. Furthermore, TGF- β is released during the coagulation process complicating comparisons between measurements obtained in serum versus plasma [68]. These analytic difficulties might explain to some degree the diverging conclusions on TGF- β in diabetes research. Our data on TGF- β suggest elevated levels in the obese state as the RYGB-induced weight loss induced a decrease in circulatory TGF- β concentrations. Herder and co-workers found that enhanced levels of TGF- β were linked to increased risk of type 2 diabetes [24]. The relevance of a reduction in TGF- β is, however, difficult to evaluate as the influence of weight loss on TGF- β concentrations and the metabolic impact of either increasing or decreasing these concentrations in humans are unknown.

Postprandial cytokine response

The prandial levels of cytokines followed the changes in fasting plasma levels. During the meal no significant postprandial changes in concentrations was observed for adiponectin and leptin. Previous studies on postprandial adiponectin response in diabetic and/or obese subjects have shown a decrease [69], no change [28;70;71] or increase [72]. Similarly, postprandial leptin concentrations have shown a decrease [70;73], no change [74], or an increase [75] in obese and/or diabetic subjects.

A small transient decrease in IL-6 concentrations was observed immediately after food intake followed by an increase lasting for the remainder of the meal test reaching final concentrations above baseline. The initial decrease and the following increase in postprandial IL-6 concentrations in obese and/or type 2 diabetics have been demonstrated before [53;76-80].

The present study has limitations; firstly participants were included after a preoperative weight loss of about 8%, likely to improve several metabolic parameters, especially hepatic insulin sensitivity. Nevertheless, postoperative metabolic improvements were still observed, although the magnitude probably had been greater if participants had not been subjected to the preoperative diet. Secondly, the study was based on the assumption that a postprandial response would be a more sensitive measure of endocrine changes and therefore the study was powered to take advantage of this, meaning that small postoperative changes in fasting circulatory levels may not reach statistical significance. Thirdly, it is not possible to determine from our study design the importance of the changes in

cytokines for the improvement in hepatic and peripheral skeletal muscle insulin resistance and glucose metabolism. Finally, we did not include a control group subjected to the same postoperative diet, which would be of major interest provided that diet-adherence can be controlled.

In conclusion, in the present study, short and long-term changes in cytokines and metabolic variables were reported in obese patients with type 2 diabetes and in subjects with normal glucose tolerance after RYGB surgery. Pre-operative levels of inflammatory mediators did not differ between the two groups. RYGB reduced fasting and postprandial concentrations of the pro-inflammatory cytokines IL-6, leptin, and the regulatory cytokine TGF- β to a similar extent in both groups. The anti-inflammatory adiponectin was increased, while IL-8 did not differ before and after surgery.

FIGURES

TITLES AND LEGENDS

Figure 1 title: Trial Profile

Figure 1 text: Numbers of subjects initially enrolled are depicted. Subject disposition (number of diabetic vs normal glucose tolerant subjects, subjects who were excluded or withdrawn, and subjects who were lost to follow up) are also shown.

Figure 2a-f title: Fasting plasma concentrations of inflammatory cytokines and GLP-1 in the whole study population

Figure 2a-f text: Fasting plasma concentrations of (a) IL-6 ($N=18$), (b) IL-8 ($N=18$), (c) TGF- β ($N=22$), (d) adiponectin ($N=20$), (e) leptin ($N=18$), and (f) GLP-1 ($N=21$) before (Pre), one week, three months, and one year after Roux-en-Y gastric bypass. Data presented as mean \pm SEM. Comparisons between time-points was made using ANOVA (non-parametric Friedman test for repeated measures) followed by Dunn's multiple test for statistical differences, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Figure 3 title: Postprandial cytokine and GLP-1 response in the whole study population

Figure 3 text: Postprandial response of (a) IL-6, adiponectin (b), leptin (c), and GLP-1 (d) in the whole study population before (Pre), one week, three months, and one year after Roux-en-Y gastric bypass. Data presented as mean \pm SEM

TABLES

TITLES AND LEGENDS

Table 1 Title: Characteristics of type 2 diabetic subjects and normal glucose tolerant subjects before (Pre), 1 week, 3 months, and 1 year after Roux-en-Y gastric bypass

Table 1 text: Data are presented as mean \pm SD. BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; NGT, normal glucose tolerance; T2D, type 2 diabetes. **P*-value for overall comparison of time-points within groups, **P*<0.05, ***P*<0.01, ****P*<0.0001; †*P*-value for comparison between groups, †*P*<0.05, ††*P*<0.01, †††*P*<0.0001

Table 2 title: Significant correlation coefficients of 1-year changes between anthropometric, clinical and inflammatory variables in the whole study population and in type 2 diabetes subjects and normal glucose tolerant subjects

Table 2 text: Data are presented as *r*: correlation coefficient of correlation, and the sample size in parentheses. NGT, normal glucose tolerant subjects; T2D, type 2 diabetes subjects; BMI, body mass index; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance. Data were analyzed by Spearman correlation test. **P*<0.05, ***P*<0.01

Table 3 title: Total AUCs for IL-6, adiponectin, leptin and GLP-1 in response to meal intake measured in the whole study population before (Pre-RYGB), 1 week, 3 months, and 1 year after (post-RYGB) Roux-en-Y gastric bypass

Table 3 text: Data are presented as mean with 95% confidence interval (CI). Comparisons between time-points was made using ANOVA (non-parametric Friedman test for repeated measures) followed by Dunn's multiple test for statistical differences, *a*: different from pre-surgical levels, *b*: different form one-week level, and *c*: different from one year level

FIGURES

Figure 1.

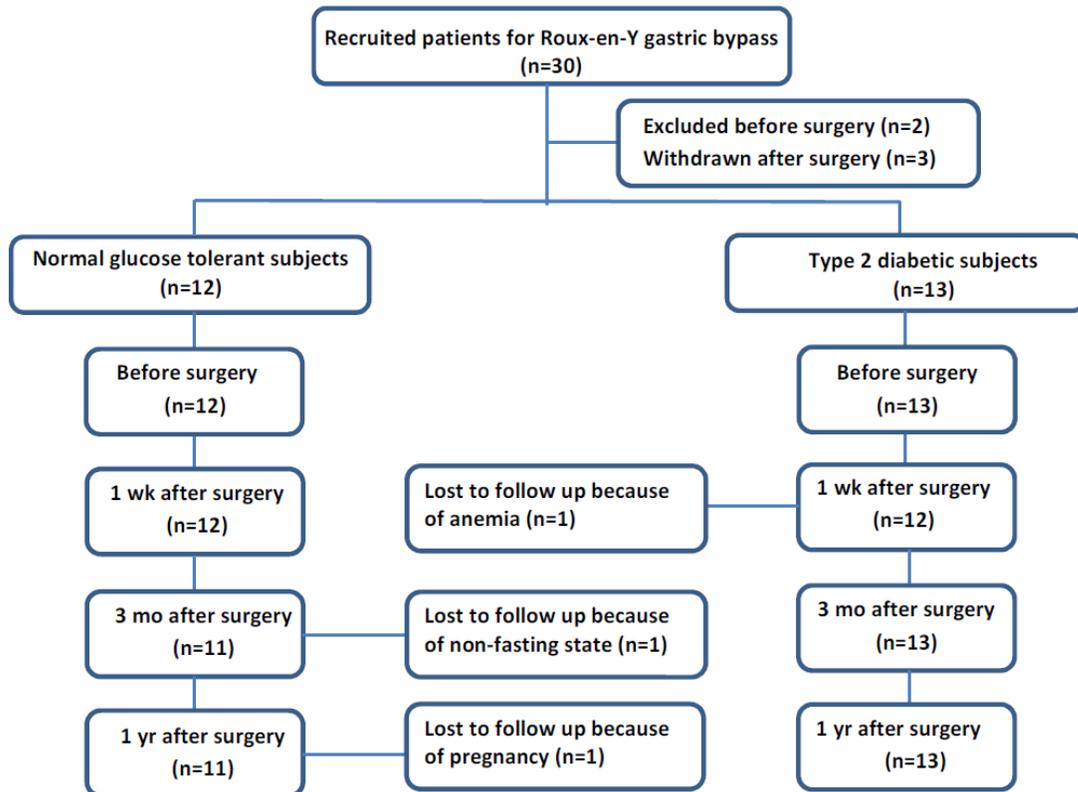


Figure 2a-f

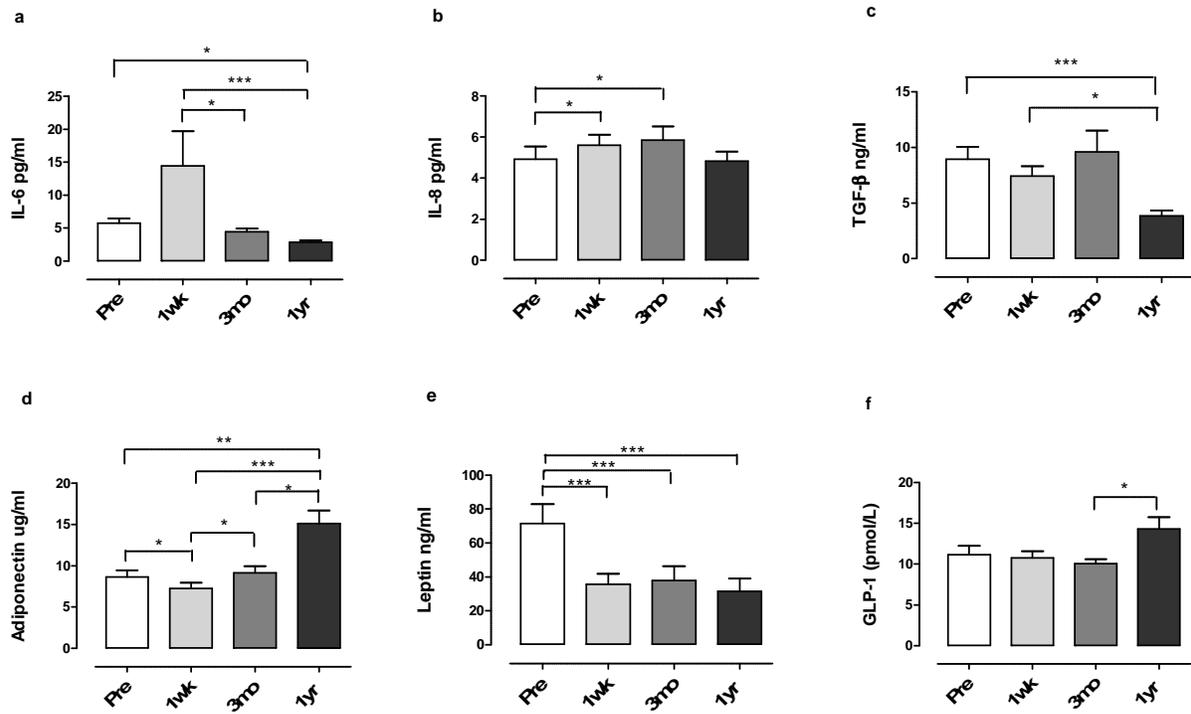


Figure 3a-d.

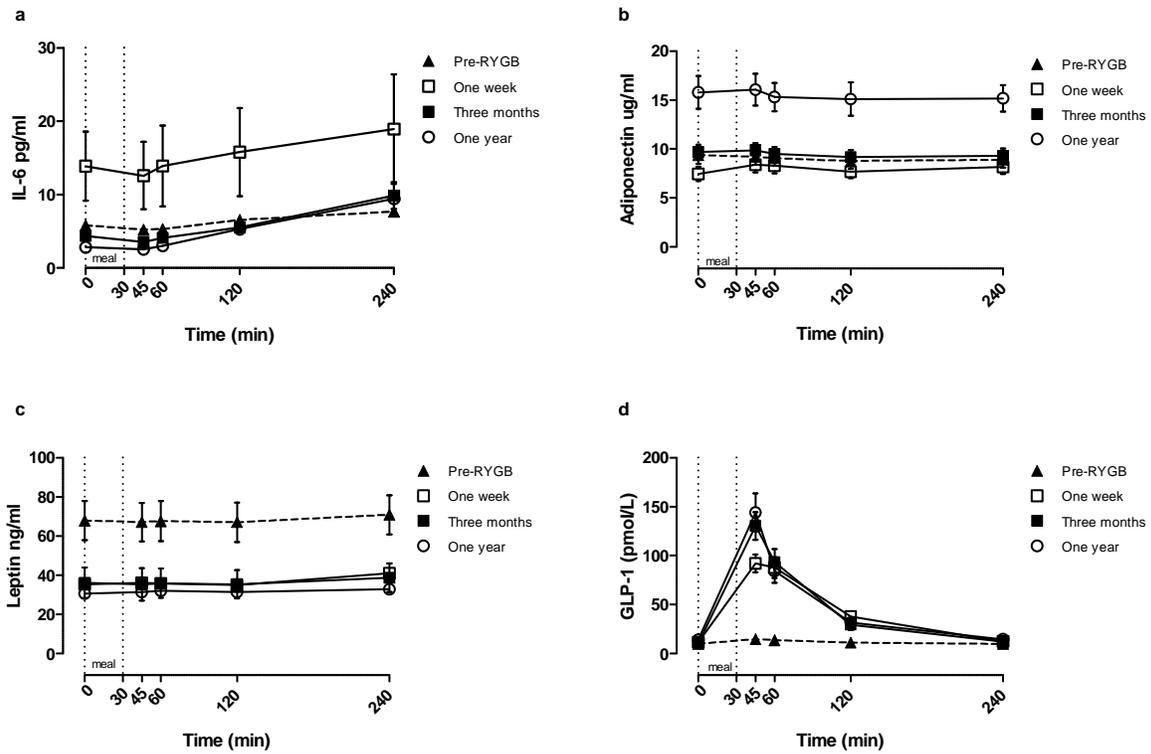


Table 1.

		Pre	1 week	3 months	1 year	*P value
Weight (kg)	<i>N</i> (T2D ; NGT)	13; 12	12; 12	13; 11	13; 11	
	T2D	129 ± 14	127 ± 13	112 ± 15***	101 ± 20***	<0.0001
	NGT	127 ± 15	124 ± 15	106 ± 13**	95 ± 16***	<0.0001
	†Pvalue	0.57	0.29	0.25	0.49	
BMI (kg/m ²)	<i>N</i> (T2D ; NGT)	13; 12	12; 12	13; 11	13; 11	
	T2D	43.2 ± 5.3	42.5 ± 5.5	37.2 ± 5.8***	34.8 ± 7.9***	<0.0001
	NGT	41.5 ± 4.8	40.5 ± 4.5	34.4 ± 3.4**	31.3 ± 4.8***	<0.0001
	†Pvalue	0.34	0.4	0.12	0.42	
Glucose (mmol/L)	<i>N</i> (T2D ; NGT)	13; 12	12; 12	13; 11	13; 11	
	T2D	8.9 ± 2.3	7.0 ± 1.1*	6.9 ± 1.6**	6.3 ± 1.6***	0.0001
	NGT	5.5 ± 0.7†††	5.0 ± 0.6†††	4.9 ± 0.4**†††	5.0 ± 0.3††	0.01
	†Pvalue	<0.0001	0.0002	0.001	0.007	
HbA1c (%)	<i>N</i> (T2D ; NGT)	13; 12		13; 11	13; 10	
	T2D	7.0 ± 0.9		5.9 ± 0.8	5.6 ± 0.6***	0.0007
	NGT	5.5 ± 0.4†††		5.4 ± 0.3	5.4 ± 0.3	0.22
	†Pvalue	0.0002		0.06	0.24	
Insulin (mmol/L)	<i>N</i> (T2D ; NGT)	13; 12	12; 12	13; 11	13; 11	
	T2D	125.0 ± 77.0	72.9 ± 32.1*	58.1 ± 35.4***	46.7 ± 27.1***	<0.0001
	NGT	82.2 ± 28.3	49.4 ± 13.5	42.8 ± 13.8**	35.8 ± 16.5***	0.0001
	†Pvalue	0.29	0.053	0.6	0.45	
HOMA-IR	<i>N</i> (T2D ; NGT)	13; 12	12; 12	13; 11	13; 11	
	T2D	6.6 ± 3.6	3.2 ± 1.5*	2.5 ± 1.7**	1.8 ± 1.1***	<0.0001
	NGT	2.9 ± 1.1††	1.6 ± 0.5††	1.3 ± 0.5*†	1.1 ± 0.5***	0.0002
	†Pvalue	0.002	0.001	0.047	0.11	

Table 2

Variable 1	Variable 2	Whole group	NGT	T2D
IL-6	HbA1c	0.47*(19)	-	0.82**(11)
	TGFβ	0.65**(20)	0.70*(8)	0.71*(11)
IL-8	GLP-1	0.59**(20)	0.80*(9)	-
	Adiponectin	-	0.67*(9)	-
TGF-β	HbA1c	-	-	0.64*(13)
Adiponectin	Leptin	-0.58**(21)	-0.68*(9)	-
	GLP-1	0.47*(21)	0.87**(9)	-
Leptin	BMI	0.65**(21)	0.75*(9)	-
	HOMA-IR	-	0.72*(9)	-
	Insulin	0.51*(21)	-	-

Table 3

Total AUC	Mean (95% CI)	<i>P</i>
IL-6 (pg/ml × 240min) <i>N</i>=18		
Pre-RYGB	1525 (1151; 1899)	0.0006
1 week post-RYGB	3897 (530; 7264)	
3 months post-RYGB	1471 (1099; 1843)	
1 year post-RYGB	1312 (803; 1821) ^b	
Adiponectin (µg/ml × 240min) <i>N</i>=18		
Pre-RYGB	2006 (1615; 2398)	<0.0001
1 week post-RYGB	1891 (1505; 2278)	
3 months post-RYGB	2188 (1801; 2575) ^b	
1 year post-RYGB	3541 (2821; 4261) ^{a,b,c}	
Leptin (ng/ml × 240min) <i>N</i>=18		
Pre-RYGB	16997 (11138; 22856)	<0.0001
1 week post-RYGB	8883 (5600; 12166) ^a	
3 months post-RYGB	9113 (4981; 13245) ^a	
1 year post-RYGB	7883 (3904; 11862) ^a	
GLP-1 (pmol/l × 240min) <i>N</i>=21		
Pre-RYGB	2742 (2433; 3051)	<0.0001
1 week post-RYGB	10257 (7962; 12551) ^a	
3 months post-RYGB	11606 (8833; 14379) ^a	
1 year post-RYGB	11642 (8398; 14886) ^a	

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MANUSCRIPT 2

Effect of Roux-en-Y gastric bypass on circulating invariant NKT cells, regulatory T-cells, and inflammatory markers in obese subjects

Kirsten K. Lindegaard, Siv H. Jacobsen, and Sten Madsbad. *In preparation for submission*

TITLE: Effect of Roux-en-Y gastric bypass on circulating invariant NKT cells, regulatory T-cells, and inflammatory markers in obese subjects

RUNNING TITLE: Immune parameters in obese following Roux-en-Y gastric bypass

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Abstract

Aim: To study the effect of Roux-en Y gastric bypass (RYGB) on circulating regulatory T (Treg) and invariant natural killer T (iNKT) cells, and anti-inflammatory and pro-inflammatory cytokines.

Methods: Changes in circulating Treg and iNKT cell ratios and plasma inflammatory markers were investigated in ten obese glucose-tolerant patients undergoing laparoscopic RYGB before and 3 months after RYGB in the fasting state and during a meal test.. Insulin resistance was evaluated by the HOMA-IR index.

Results: RYGB induced a significant weight loss of 18.5 ± 0.6 kg and improved insulin resistance. Fasting circulating Treg and iNKT cell percentage did not differ before and after surgery. Fasting plasma concentrations of IL-10 were increased by 113% ($P=0.004$), IFN- γ by 24% ($P=0.04$), while leptin was decreased by 72% ($P=0.004$). IL-8 increased by 14% (not significant). Postprandial total AUC for Treg cells and iNKTs did not differ before and after RYGB. A significant increase in total AUC for IL-10 and IL-8 was observed after surgery ($P=0.008$ and $P=0.03$), while the postprandial IFN- γ response was unchanged ($P=0.1$). Total AUC for leptin was significantly decreased after RYGB ($P=0.004$). RYGB did not result in any changes in the fasting or postprandial responses of IL-6, TNF- α , MCP-1, and adiponectin.

Conclusion: RYGB and weight loss improve insulin resistance and is associated with alterations in circulating concentrations of innate immune cell-driven inflammation related factors, however no changes in the number of iNKT or Treg cells were observed 3 months post-surgery.

Key words: RYGB, inflammation, regulatory T (Treg) cells, invariant natural killer T (iNKT)

Abbreviations

RYGB: Roux-en-Y gastric bypass

Treg: Regulatory T-cells

iNKT: invariant Natural Killer T cells

HOMA-IR: Homeostasis model assesment

IL-: Interleukin-

IFN- γ : Interferon-gamma

AUC: Area under curve

TNF- α : Tumor necrosis factor- α

MCP-1: Monocyte chemoattractant protein-1

GLP-1: Glugon-like Peptide-1

GLP-1R: Glucagon-like Peptide-1 Receptor

FFM: Fat-free Mass

BMI: Body Mass Index

IQR: Interquartile Range

Th1: T-Helper Cell Type 1

Th2: T-Helper Cell Type 2

M1: Macrophage Type 1

M2: Macrophage Type 2

Introduction

Obesity-induced inflammation, especially in the adipose tissue, liver and skeletal muscles, is involved in metabolic dysregulation and in the pathogenesis of insulin resistance and subsequent type 2 diabetes (Hotamisligil, 2006). Macrophages are important mediators of obesity-induced insulin resistance (Dalmas et al., 2011). T-cells are capable of regulating macrophage activation by secreting Th1 cytokines, such as IFN- γ that enhances the differentiation of macrophages into a pro-inflammatory M1 phenotype, or Th2 cytokines such as IL-10 and IL-4 to promote an anti-inflammatory M2 macrophage profile (Lee and Lee, 2014).

Invariant natural killer T (iNKT) cells, a subset of innate-like T-lymphocytes, and regulatory T (Treg) cells, an adaptive T-lymphocyte subset, are reduced in adipose tissue and circulation of obese mice and humans and this reduction is associated with tissue inflammation and glucose intolerance (Feuerer et al., 2009; Yun et al., 2010; Deiluiis et al., 2011; Lynch et al., 2012; Wagner et al., 2013).

Roux-en-Y gastric bypass (RYGB) surgery induces sustained weight loss, improves insulin sensitivity, and reduces concentrations of circulating pro-inflammatory cytokines such as IL-6, MCP-1, and leptin, while increasing adiponectin concentrations (Brethauer et al., 2011; Isbell et al., 2010; Miller et al., 2011; Beckman et al., 2011; Falken et al., 2011; Jacobsen et al., 2012; Kopp et al., 2005; Swarbrick et al., 2008; Viana et al., 2013; Illan-Gomez et al., 2012), (Miller et al., 2011; Swarbrick et al., 2006). RYGB also enhances circulating levels of certain gastrointestinal hormones, particularly the postprandial secretion of glucagon-like peptide-1 (GLP-1) (Madsbad et al., 2014). In addition, changes in circulating iNKT cells have been found following RYGB (Lynch et al., 2012). GLP-1 receptors (GLP-1R) have been found on several T-lymphocytes, including iNKT and Treg cells, and treatment with a GLP-1R agonist in obese subjects with T2D enhanced iNKT cell numbers in circulation (Hogan et al., 2011; Ahern et al., 2013), suggesting one potential mechanism by which bariatric surgery re-establishes insulin sensitivity.

Thus, the evidence of a direct contribution of obese adipose tissue in systemic inflammation and of a RYGB-mediated effect on molecular and cellular inflammatory markers suggest that alterations in immune cell subsets and inflammation mediators could be important for some of the health benefits observed following RYGB. We therefore hypothesized that RYGB increases circulating iNKT and Treg cells, increases anti-inflammatory and reduces pro-inflammatory cytokines, altogether reflective of improved metabolic function and reduced adipose tissue inflammation.

Methods

Study participants

Study population and study design have been described before (Jacobsen et al., 2013). The study included ten obese glucose-tolerant patients undergoing laparoscopic RYGB at Hvidovre Hospital (Copenhagen, Denmark). They all met the Danish criteria for bariatric surgery (age > 20 years and BMI > 40 kg/m² or > 35 kg/m² with comorbid conditions such as type 2 diabetes or hypertension), and had accomplished a mandatory preoperative 8% diet-induced total body weight loss before inclusion. One patient experienced postoperative complications and was excluded from the study. Written, informed consent was obtained from all patients, and the study was approved by the Municipal Ethics Committee of Copenhagen in accordance with the Helsinki-II Declaration and was registered at www.clinicaltrials.gov (ID NCT01559792).

Study design

Patients were studied within 2 weeks before and 3 months after RYGB. Dual energy x-ray absorptiometry was used to assess fat-free mass (FFM). On each occasion, the patients arrived after an overnight fast (10-12 h) and a mixed meal (200 ml, 1,650 kJ (394 kcal): carbohydrate 50%, protein 15%, fat 35%, consisting of glucose (50 g glucose), rapeseed oil [14.1 g] and casein protein [15.2 g]) was ingested. The meal was consumed slowly over 30 min and blood was sampled prior to the meal (0 min), and 60 min and 120 min after the meal start.

Sample collection and analysis

Plasma insulin, glucose, and HOMA-IR were analyzed and calculated as previously described (SHJ 2011). Total plasma concentrations of inflammatory markers were determined by Mesoscale Discovery (MSD, Gaithersburg, MD, USA) with the SECTOR Imager 2400 (MSD)(electrochemiluminescent assay). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Circulating lymphocytes were counted and enumerated by labeling PBMCs with anti-TCR V α 24-J α 18-PE antibody (clone 6B11) (Biolegend, San Diego, CA, USA), anti-CD3-APC (Biolegend), and anti-CD19-Alexa Flour 488 (BD Biosciences, San Diego, CA, USA) for iNKT identification and anti-CD4-PerCP-Cy5.5, anti-CD25-PE, anti-CD127-Alexa Flour®647, and anti-CD45RA-FITC (Human Regulatory T cell Sorting Kit, BD Biosciences, San Diego, CA, USA) prior to flow cytometry using a Moflo MLS (DakoCytomation, Fort Collins, CO, US) or a MofloAstrios (Beckman Coulter Inc., Florida, US) cell sorter. Exclusion of dead cells was performed using the LIVE/DEAD® Fixable Violet Dead Cell Stain

Kit (Invitrogen, Carlsbad, CA, USA). Samples were analyzed using Summit v4.3 or v.6.2 software, respectively.

Statistical analyses

To measure meal responses, the total area under the curve (AUC) was calculated employing the three data points and using the trapezoidal method (GraphPad Prism v. 4). Data collected before and after RYGB were compared using the Wilcoxon signed rank test. The level of significance was set at $P < 0.05$. Data are expressed as mean \pm SEM or as medians [interquartile range (IQR)].

Results

Changes in weight and glucose metabolism

Patients (three men, nine women) had a pre-operative BMI of 39.2 ± 1.8 kg/m². RYGB induced a significant weight loss of 18.5 ± 0.6 kg and a reduction in body fat percentage ($-3.5 \pm 1.1\%$). Fasting glucose and insulin concentrations were significantly lower after surgery (5.2 ± 0.2 vs 4.9 ± 0.1 mmol/l, $P=0.008$; 125 ± 16 vs 66 ± 7 pmol/l). HOMA-IR was significantly reduced 3 months after RYGB (4.3 ± 0.6 vs 2.1 ± 0.2 , $P=0.008$).

Changes in fasting and postprandial immunological parameters

The median (IQR) of circulating Treg cell percentage did not differ before and 3 months after RYGB (3.8% (2.5 – 4.4) vs 3.8% (3.3 – 4.6)). RYGB did not increase iNKT percentage significantly, although median (IQR) changed from 0.020% (0.013 – 0.058) to 0.045% (0.008 – 0.060) ($P=0.57$). The median (IQR) of plasma concentrations of IL-10 was significantly increased by 113% (from 0.52 pg/ml (0.25 – 1.69) to 1.11 pg/ml (0.39 – 2.96), $P=0.004$) and IFN- γ by 24% (from 3.4 pg/ml (2.7 – 4.7) to 4.2 pg/ml (3.4 – 9.7), $P=0.04$), while that of leptin was decreased by 72% (from 75.3 ng/ml (36.9 – 112.1) to 21.2 ng/ml (9.2 – 32.2), $P=0.004$). IL-8 increased by 14% (from 4.3 pg/ml (3.0 – 5.2) to 4.9 pg/ml (4.4 – 5.9), $P=0.1$), but failed to reach statistical significance.

Total AUC for Treg cells and iNKTs did not differ before and after RYGB (Table 1). A significant increase in total AUC for IL-10 and IL-8 was observed after surgery ($P=0.008$ and $P=0.03$), while the postprandial IFN- γ response was unchanged ($P=0.1$). Total AUC for leptin was significantly decreased after RYGB ($P=0.004$).

RYGB did not result in any changes in the fasting concentrations of IL-6 (1.8 pg/ml (1.2 – 3.2) vs 2.0 pg/ml (1.1 – 3.6)), TNF- α (1.4 pg/ml (0.9 – 1.6) vs 1.5 pg/ml (1.0 – 2.1)), MCP-1 (158 pg/ml (137 – 195) vs 173 pg/ml (152 – 207)), and adiponectin (8.5 μ g/ml (5.3 – 16.4) vs. 7.5 μ g/ml (6.2 – 13.6)) or in the postprandial responses of these cytokines (data not shown).

Changes in fasting and postprandial responses are summarized in Table 2.

Discussion

The present study suggests that RYGB induces changes in systemic concentrations of cytokines in obese subjects with normal glucose tolerance by increasing fasting plasma concentrations of the Th1 and Th2 cytokines, IFN- γ and IL-10, while decreasing the pro-inflammatory adipokine leptin.

Plasma IFN- γ has, to our knowledge, not been reported in human RYGB studies previously. Obese IFN- γ -deficient animals have reduced expression of pro-inflammatory cytokines and immune cell infiltration in the adipose tissue and better glucose tolerance than control animals consuming the same diet (Rocha, VZ). IFN- γ expression in adipose tissue has been shown to be reduced in obese mice undergoing bariatric surgery (Zhang et al., 2011), and we would therefore have expected to find decreased IFN- γ concentrations in our patient group. Brethauer and co-workers reported no changes in IL-10 6 months after RYGB, but significant increases in adiponectin were found (Brethauer et al., 2011).

IL-10 and IFN- γ are major prototypical Th2- and Th1-derived cytokines, respectively, and secreted by T-cells in order to regulate immune responses. Treg cells are capable of suppressing Th1 responses in the presence of IL-10 (von Boehmer H., 2005; Sakaguchi et al., 2009), while iNKT cells induce both Th1 and Th2 immunity depending on their state of activation and the surrounding cytokine milieu (Bendelac et al., 2007; Berzins et al., 2011).

Adoptive transfer of iNKT cells into diet-induced obese mice has resulted in increased adipose tissue expression of IL-10 and adiponectin, while reducing leptin, coherent with improvements in glucose metabolism (Lynch et al., 2012). Macrophage differentiation is affected by IL-10 (and IL-4) toward an anti-inflammatory M2 phenotype (Lee and Lee, 2014), while IFN- γ induces a shift from an M2 to a pro-inflammatory M1 phenotype characteristic of the obese and dysfunctional adipose tissue. In human cultured adipocytes, IL-10 is suggested to act as a protective against TNF- α -induced insulin resistance (Lumeng et al., 2007), while IFN- γ suppresses the secretion of insulin-sensitizing adiponectin (Simons et al., 2007). The lack of increase in adiponectin after RYGB in our patient group might be explained by the increase in circulating IFN- γ .

Although we did not observe changes in the frequency of iNKT and Treg cells, their function may have been affected by surgery. Hogan and coworkers demonstrated that addition of GLP-1 to iNKT cell cultures resulted in alterations in the release of cytokines depending on the activation state of the iNKT cells. The effect of GLP-1 could be blocked by a GLP-1R antagonist suggesting a direct GLP-1R-signaling mechanism (Hogan et al., 2011). In addition, GLP-1 therapy in obese T2D patients reduced concentrations of CD163, indicative of macrophage activation, and increased IL-10 secretion by macrophages (Hogan et al., 2014). The enhanced postprandial GLP-1 secretion occurring immediately after RYGB may therefore influence functional capabilities of immune cell subsets expressing the GLP-1R. Functional analyses of these cells following RYGB could be an attractive supplement to improve understanding of the effects of RYGB and weight loss on inflammation.

Our study is limited by the small sample size and by only one follow-up. Inclusion of more patients would have improved the statistical analysis and may have resulted in significant changes in cytokines and immune cells. Furthermore, it is not possible to determine from our study design the influence of weight loss or RYGB *per se* on the observed changes and whether alterations in cytokine secretion is related to functional changes in the immune cells or due to other influencing factors such as post-surgical stress responses as recently suggested (Lin and Gletsu-Miller, 2013). Lastly, weight loss and intermediate metabolism are only in steady state 1-2 years after RYGB, and follow-up studies more than 1 year after surgery will therefore be of interest in order to understand the full effect of RYGB on inflammation.

In conclusion, RYGB has a positive effect on glucose metabolism and insulin sensitivity and induces alterations in innate immune cell-driven inflammation despite the fact that no changes in the number of iNKT or Treg cells were detected 3 months post-surgery.

Table 1. Fasting and postprandial response in circulating iNKT and Treg cells (N=9)

	Pre-op	Post-op
% iNKT of lymphocytes		
Fasting	0.020 (0.013 – 0.058)	0.045 (0.008 – 0.060)
60 min after meal	0.020 (0.013 – 0.058)	0.045 (0.008 – 0.063)
120 min after meal	0.025 (0.015 – 0.053)	0.030(0.010 – 0.060)
% Treg of lymphocytes		
Fasting	3.8 (2.5 – 4.4)	3.7 (3.3 – 4.6)
60 min after meal	3.5 (2.6 – 4.5)	4.2 (3.4 – 4.3)
120 min after meal	3.5 (2.9 – 4.3)	4.0 (3.3 – 4.2)

Data presented as median(IQR).

Table 2. Indication of changes in immune parameters following Roux-en-Y gastric bypass and during a meal test (N=9)

Immune parameter	Fasting Pre- vs Post-op	Postprandial Pre- vs post-op (tAUC)
Treg cells	→	→
iNKT cells	→	→
IL6	→	→
TNF- α	→	→
IL-8	→	↑ ($P=0.03$)
IL-10	↑ ($P=0.004$)	↑ ($P=0.008$)
IFN- γ	↑ ($P=0.04$)	→
MCP-1	→	→
Leptin	↓ ($P=0.004$)	↓ ($P=0.004$)
Adiponectin	→	→

Arrows indicate → = no change, ↑ = increase, or ↓ = decrease.

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MANUSCRIPT 3

Effect of 12-week treatment with glucagon-like peptide-1 receptor agonist liraglutide on circulating invariant NKT cells, regulatory T-cells, and inflammatory markers in obese subjects with type 2 diabetes.

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Abstract

Rationale: Emerging evidence suggests direct modulatory effect of the glucagon-like peptide-1 (GLP-1) on innate and adaptive T-lymphocyte subsets and on the inflammatory cytokine profile. Therefore, we investigated the effects of GLP-1 receptor (GLP-1R) agonist treatment on iNKT and Tregs cells and on pro-inflammatory and anti-inflammatory cytokines in obese type 2 diabetes (T2D) patients.

Methods: Nine obese T2D patients were examined prior to and after 12 weeks treatment with the GLP-1R agonist liraglutide. Circulating Treg and iNKT cells were determined by flow cytometry and plasma IL-6, IL-8, IL-10, TNF- α , TGF- β , adiponectin and leptin were measured using electrochemoluminescence (Meso Scale Discovery). Body weight and HbA1c were also recorded.

Results: Liraglutide reduced HbA1c and induced a minor weight loss. Percentage of circulating Treg and iNKT cells did not differ before and after treatment with liraglutide. Plasma IL-8 was increased, while no change in IL-6, IL-10, TNF- α , TGF- β , adiponectin and leptin could be detected after 12 weeks treatment.

Conclusions: Circulating iNKT and Treg cells are unchanged following 12-weeks treatment with liraglutide. A minor increase in the pro-inflammatory chemokine IL-8 after liraglutide treatment was measured, while no changes in other pro- or anti-inflammatory cytokines could be detected. In the present study, we could not support previous findings on anti-inflammatory and immunoregulatory effect of GLP-1R agonist treatment.

Key words: Type 2 diabetes, liraglutide, Treg cells, iNKT cells, cytokines

Abbreviations

T2D: Type 2 Diabetes

iNKT: invariant natural killer T cells

Treg: regulatory T-cells

GLP-1: Glucagon-like peptide-1

GLP-1R: GLP-1 receptor

FBS: Fetal bovine serum

IQR: Interquartile range

TNF- α : Tumor necrosis factor alpha

TGF- β : Transforming growth factor beta

MCP-1: Monocyte chemoattractant protein-1

PAI-1: Plasminogen activator inhibitor-1

hs-CRP: high sensitivity C-reactive protein

Th1: T-helper type 1

Th2: T-helper type 2

INTRODUCTION

Obesity-associated type 2 diabetes (T2D) is characterized by a state of chronic, low-grade inflammation with an excessive secretion of pro-inflammatory mediators, such as IL-6, TNF- α , and leptin from the adipose tissue and decrease in the anti-inflammatory adipokine adiponectin [1;2]. Inflammatory responses in the adipose tissue are regulated by an altered composition of immune cells with a lower ratio of cells with anti-inflammatory and immunoregulatory phenotypes (e.g. alternatively activated macrophages and T-helper type 2 (Th2) cells) and accumulation of pro-inflammatory immune cells [e.g. classically activated macrophages and T-helper type 1 (Th1) cells] [3]. Accumulating evidence suggests a direct role of a range of these cellular and molecular immune mediators in the development of insulin resistance [4]. Reduced ratios of invariant natural killer T (iNKT) cells and regulatory T (Treg) cells have been reported in adipose tissue and circulation in obese mice and humans compared to lean control groups [5-9]. Increasing these cell populations in murine models of obesity and/or diabetes has been shown to protect against diet-induced obesity, to attenuate inflammation, and to restore glucose homeostasis and improve insulin sensitivity [5;8;10;11]. Resolution of inflammation, potentially by harnessing the anti-inflammatory properties of iNKT and Treg cells, may therefore offer a novel treatment paradigm for T2D.

Glucagon-like peptide-1 (GLP-1) is an intestinally produced peptide hormone essential for glucose homeostasis by stimulating insulin secretion, inhibiting glucagon secretion and reducing food intake [12]. Furthermore, GLP-1 action is impaired in T2D patients, which is the background for treating T2D patients with GLP-1 receptor (GLP-1R) agonists, including liraglutide (Victoza®) [13;14]. The finding of GLP-1Rs on several immune cell populations, including iNKT and Treg cells [15-17] has suggested a possible immunomodulatory role of GLP-1 and GLP-1R agonists. A growing body of literature describes anti-inflammatory activities of GLP-1 affecting both molecular and cellular parameters independently of weight loss [18-22]. Studies in transgenic GLP-1 receptor knockout mice and in NOD mice treated with exendin-4, a GLP-1R agonist, have reported altered frequency of Treg cells in various tissues [15;23;24] and recently, the O'Shea group reported data from two human clinical case studies in obese, diabetic

psoriasis patients on liraglutide therapy showing an enhanced number of circulating iNKT cells after 6 or 10 weeks of treatment [17;25]. The same group reported, in a study of obese T2D subjects treated with liraglutide for 8 weeks, decreased monocyte secretion of the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 and increased secretion of adiponectin [26].

We hypothesized that the positive effect of GLP-1R agonist treatment on immune- and/or obesity-related diseases, such as psoriasis and T2D may, in part, be due to a GLP-1-mediated effect on specific cellular and soluble mediators of the innate and adaptive immune system. Thus, we investigated whether treatment for 12 weeks with the GLP-1R agonist liraglutide would increase circulating populations of iNKT and Treg cells in obese T2D patients concomitantly with a decrease in pro-inflammatory cytokines and increase in anti-inflammatory cytokines.

METHODS

Study participants

Obese patients with T2D planned to initiate treatment with liraglutide (Victoza®) were recruited from the outpatient clinic at the Department of Endocrinology at Hvidovre Hospital (Denmark). Patients were excluded if they had any known acute or chronic inflammatory disorder including infections, received anti-inflammatory drugs, were smokers or consumed > 14 units of alcohol/week, or had previously undergone bariatric surgery. The study was approved by the Municipal Ethical Committee of Copenhagen (Reg. nr. H-2-2012-148) in accordance with the Helsinki-II declaration, and was registered with ClinicalTrials.gov (NCT02201550) and with the Danish Data Protection Agency. A written informed consent was obtained from all patients before entering into the study.

Study design

Patients were examined on two occasions: prior to liraglutide treatment (Pre-GLP1) and 12 weeks after initiating the treatment (Post-GLP1). On each visit, which took place in the morning, body weight was

measured and fasting blood samples for HbA1c and immunological analyses were drawn. Patients were instructed to self-administer liraglutide by subcutaneous injection once daily. The dose was titrated to a level of 1.8 mg daily if tolerated by increasing the dose stepwise by 0.6 mg once weekly. Liraglutide 1.8 mg daily is the highest approved dose to use in subjects with type 2 diabetes mellitus.

Sample collection and laboratory analyses

HbA1c was determined using HPLC with a cation exchange column (Tosoh Bioscience, Tokyo, Japan). For protein analyses, venous blood was sampled into EDTA tubes and centrifuged at 4°C, 10 min, 2000g. Plasma aliquots were stored at -80°C until analyzed. Peripheral blood mononuclear cells (PBMCs) were isolated within 2 hours from blood collection by density gradient centrifugation with Ficoll-Paque Plus[®] (GE Healthcare Life Sciences, New Jersey, USA) followed by washing with HBBS supplemented with 5% fetal bovine serum (FBS) and 25mM HEPES buffer solution (Gibco[®] Life Technologies, Grand Island, NY, USA), pH 7.2. Cells were resuspended in HBBS (Gibco[®] Life Technologies), counted and stained with the appropriate antibodies.

Flow cytometry

iNKT cells were enumerated by staining with a combination of CD1d-PE α -GalCer-loaded tetramer (ProImmune Ltd., Oxford, UK) and anti-CD3-APC (Biolegend, San Diego, CA, USA). B-cells were depleted using anti-CD19-Alexa Flour[®] 488 (BD Biosciences, San Diego, CA, USA). Treg cell analysis was performed by labeling PBMCs with anti-CD4-PerCP-Cy5.5, anti-CD25-PE, and anti-CD127-Alexa Flour[®] 647, (Human Regulatory T cell Sorting Kit, BD Biosciences, San Diego, CA, US). Cells were acquired using a Moflo MLS (DakoCytomation, Fort Collins, CO, US) or a Moflo Astrios (Beckman Coulter Inc., Florida, US) cell sorter. Samples were analyzed using Summit v4.3 or v.6.2 software, respectively. Compensation was performed on single stained samples using the automated compensation

feature in the software. For each patient, a live/dead stain was included using the LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA).

Cytokine measurements

Total plasma concentrations of inflammatory markers were determined by electrochemiluminescent sandwich immunoassays (Meso Scale Discovery (MSD), Gaithersburg, MD, USA). The assays were performed according to the manufacturer's protocol using a dedicated MSD reader (SECTOR Imager 2400, MSD).

Data analysis and statistics

Treg and iNKT cell numbers are expressed as a percentage of lymphocytes. One patient at the 12 weeks follow-up failed due to technical difficulties during the iNKT flow analysis, and the patient was excluded from the this data set. All statistical analyses were performed using GraphPad Prism v5. The non-parametric Wilcoxon signed rank t-test was applied. All data are expressed as median and interquartile range (IQR). A P value <0.05 was considered statistical significant.

RESULTS

Patient characteristics

Ten patients with type 2 diabetes were recruited for the study of which one did not complete the 12 weeks study period due to severe nausea and vomiting leading to early discontinuation of liraglutide treatment. Patient characteristics of the 9 patients completing the whole study period are shown in table 1. All, except one patient with newly diagnosed T2D, received metformin either as monotherapy (n=3) or in combination with glimepiride (n=2), sitagliptin (n=1), insulin glargine (n=1), or NPH insulin (n=1). Liraglutide therapy was added to the existing treatments except for sitagliptin that was discontinued 14 days prior to initiation of liraglutide therapy.

When initiating the study, three of the nine participants were severely obese (BMI>40 kg/m²). After 12 weeks treatment with liraglutide, BMI and body weight were reduced, although not significantly so ($P=0.1$). Liraglutide therapy resulted in a significant decrease in HbA1c from a median (IQR) of 64 mmol/mol (59.5 – 73.0) to 51 mmol/mol (47.0 – 60.5) ($P<0.008$) (Table 1).

Immune cell and cytokine analysis

Data on immune cells and cytokines are outlined in table 2.

After 12 weeks of liraglutide therapy, the median (IQR) of circulating Treg cell percentage changed from 5.54% (3.54 – 6.34) to 4.61% (3.88 – 6.15), (not significant). The median (IQR) iNKT cell percentage showed minor increase from 0.011 (0.0025 – 0.083) to 0.014 (0.0037 – 0.070), (not significant). Median plasma concentrations of IL-8 was significantly increased by 32% ($P=0.02$). IL-10 increased by 14%, TNF- α and TGF- β by 10%, adiponectin by 17% and leptin by 15%, while a decrease in median plasma concentrations of IL-6 by 20% was measured; all the changes failed to reach statistical significance.

The iNKT data set revealed large individual differences. After liraglutide treatment, five out of eight patients experienced an 8 – 335% increase compared to before treatment and the remaining three patients experienced a reduction in iNKT cell numbers of 20%, 22%, and 37%. For the circulating number of Treg cells, four out of nine patients had reduced Treg cell frequencies ranging from 15 – 31%, one patient showed no change, and four patients showed an increase ranging from 9 – 22% following liraglutide treatment.

No correlations between anthropometric and clinical data could be established at any time point.

DISCUSSION

This study demonstrated that treatment for 12 weeks with the GLP-1R agonist liraglutide improved HbA1c significantly but had no apparent effect on a range of circulating molecular markers of

inflammation, except for a small significant increase in IL-8, most likely without any biological and clinical significance. No alterations in circulating numbers of Treg and iNKT cells measured in nine obese T2D patients were detected following liraglutide treatment. Weight loss was minor (3%) and within the range of what has been reported in other short-term liraglutide studies [14;21;25;26].

The few but convincing case reports on improvements in psoriasis with GLP-1R agonist therapy suggest anti-inflammatory effect of this anti-diabetic agent [17;25;27]. The clinical improvement appears to occur independently of changes in glucose control and/or weight loss. Work by the O'Shea group suggested that GLP-1 acted directly on iNKT cells, increasing their frequency in circulation in obese T2D patients with psoriasis correlating with an immediate improvement of psoriatic lesions [17;25]. Degree of weight loss, body weight, and HbA1c, in addition to dose of liraglutide in these patients were similar to our study population, while duration of the mentioned studies were somewhat shorter, and thus, an explanation for the lack of effect by liraglutide on iNKT cell frequency in our study may be due to our patients not having psoriasis. Psoriasis is an autoimmune-mediated inflammatory skin disease involving cells from the adaptive and innate immune system, including iNKT cells, and an enhanced production of inflammatory cytokines detected locally and in circulation [28-30]. An activated immune system may be a prerequisite for GLP-1 having an effect on iNKT cell frequency and/or function. Circulating iNKT cell frequencies reported in the study by Hogan and co-workers were higher than we have observed, both before and especially after liraglutide therapy, which could suggest differences in the degree of immune activation and thus incompatibly between patient groups. In general, however, iNKT cell numbers suffer from high individual variations most likely due to genetic influence, complicating comparisons of groups with small sample sizes [31].

The chemokine IL-8 has been found to be produced and released from human adipocytes [32], in addition to playing a central role in inflammatory processes, including atherosclerotic processes as a neutrophil chemoattractant [33;34]. Circulating levels of IL-8 are increased in obesity [35;36], compared to lean healthy subjects and associated with insulin resistance [35;37]. Bruun and co-workers reported a 30% increase in IL-8, similar to our finding, after 20 weeks and 24 weeks, respectively of a dietary-induced

weight loss of 13%. The circulating levels stated in these studies are comparable to the concentrations of IL-8 found in the present study. Other cell types besides adipocytes involved in acute and chronic inflammatory responses could add to the secretion of IL-8 [38] Biological and clinical relevance of this finding is, however, questionable.

Chaudhuri and co-workers showed that treating obese T2D subjects with exenatide, another GLP-1R agonist, for 12 weeks was associated with weight-independent reductions in plasma levels of pro-inflammatory cytokines, including MCP-1 and IL-6 [21]. In a clinical study in 165 T2D patients, liraglutide for 14 weeks decreased plasminogen activator inhibitor-1 (PAI-1), a pro-inflammatory marker, and, to some degree, high sensitivity C-reactive protein (hs-CRP), a marker of systemic inflammation, while no changes in adiponectin, leptin, IL-6 and TNF- α were reported. In addition, body weight was reduced and glycemic control reestablished [39].

Reductions in circulating Treg cells have been reported in obese non-diabetic and diabetic subjects compared to lean controls and was found to inversely correlate with measures of adiposity, inflammation, and glucose intolerance [9]. Experiments with GLP-1R knockout mice or NOD mice treated with exendin-4 resulted in alterations in the number of Treg cells in various tissues, although data have not been consistent between studies [23;24].

One explanation for the GLP-1-induced effect on cellular and molecular immune mediators observed in some studies and not in others might be that the anti-inflammatory effect of liraglutide therapy is indirect, either through weight loss *or* by improvement in metabolic parameters, such as glucose and insulin that may have an impact on cytokine secretion as well. There is also the possibility that liraglutide for longer duration or at a higher dose may be required to cause changes in cytokine concentrations. Another point is that the same immunomodulatory effects of GLP-1 agonist treatment may not be expected in all type 2 diabetes patients, since it is a very heterogeneous group in relation to BMI, body fat distribution including abdominal obesity, insulin resistance, degrees of low grade inflammation and amount of obesity related co-morbidities, i.e. cardiovascular diseases.

Despite previous studies having reported robust immunomodulatory effects of GLP-1R agonist treatment in small groups of well-characterized patients with psoriasis, a limitation of the present study is the small sample size and lack of a control group.

In conclusion, the present study indicates that the number of circulating iNKT and Treg cells are unchanged following 12-weeks treatment with liraglutide. The pro-inflammatory chemokine IL-8 was increased after liraglutide treatment, while no changes in other pro- or anti-inflammatory cytokines could be detected.

TABLES

Table 1. Characteristics of the nine participants with type 2 diabetes before (Pre-GLP-1) and 12 weeks after liraglutide treatment (Post-GLP-1)

	Pre-GLP-1	Post-GLP-1	<i>P</i> -value
Gender	5 men, 4 women	-	-
Age (years)	58 (54 – 66)	-	-
Weight (kg)	104.7 (88.1 – 131.6)	101.5 (84.3 – 133.5)	0.10
BMI (kg/m ²)	32.3 (30.2 – 45.2)	31.8 (28.9 – 44.6)	0.10
HbA1c			
mmol/mol	64.0 (59.5 – 73.0)	51.0(47.0 – 60.5)**	0.01
%	8.0 (7.6 – 8.9)	7.7 (6.5 – 7.7)**	0.01

Data are expressed as median and interquartile range, *N*=9. BMI, body mass index; HbA1c, glycated hemoglobin. Comparisons between time-points using Wilcoxon signed-rank test, ***P*<0.01.

Table 2. Changes in immune cells and cytokines in type 2 diabetes subjects before (Pre-GLP1) and 12 weeks after (Post-GLP1) liraglutide treatment

	Pre-GLP-1	Post-GLP-1	<i>P</i> value
Treg (% lymphocytes)	5.54 (3.54 – 6.34)	4.61 (3.88 – 6.15)	0.57
iNKT (% lymphocytes) [#]	0.011 (0.0025 – 0.083)	0.014 (0.0037 – 0.070)	0.38
IL-6 (pg/ml)	2.4 (1.8 – 3.0)	1.9 (1.8 – 3.1)	0.57
IL-8 (pg/ml)	4.0 (2.8 – 5.9)	5.3 (4.1 – 8.0)*	0.02
IL-10 (pg/ml)	0.57 (0.33 – 1.29)	0.70 (0.29 – 1.05)	0.13
MCP-1 (pg/ml)	180.8 (159.5 – 204.6)	180.3 (152.0 – 214.3)	0.82
TNF- α (pg/ml)	2.0 (1.5 – 2.4)	2.2 (1.7 – 2.4)	0.36
IFN- γ (pg/ml)	4.9 (3.9 – 5.6)	4.7 (4.2 – 6.1)	0.65
TGF- β (ng/ml)	3.8 (3.3 – 5.6)	4.2 (3.1 – 6.8)	0.73
Adiponectin (ug/ml)	5.2 (4.2 – 7.8)	6.1 (4.4 – 7.8)	0.25
Leptin (ng/ml)	40.7 (12.6 – 90.6)	47.1 (11.4 – 81.1)	0.95

Data are expressed as median and interquartile range, $N=9$; [#] $N=8$. Treg, regulatory T-cells; iNKT, invariant natural killer T-cells. Comparisons between time-points using Wilcoxon signed-rank test, * $P<0.05$.

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