Adipokines and Myeloid Cell Immune responses in Periodontal disease and Diabetes

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List of Abbreviations

ACE	Angiotensin I-converting enzyme
ACTH	adrenocorticotropic hormone
AdipoR	adiponectin receptor
AGEs	advanced glycation end-products
AIDS	acquired immunodeficiency syndrome
AP-1	activator protein-1
APPL1	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
Akt	Ak transforming
AMP	5' adenosine monophosphate
ANOVA	analysis of variance
ASP	Acylation stimulating protein
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDP	common dendritic cell progenitor
CETP	cholesteryl ester transfer protein
CK2	casein kinase 2
CLP	common lymphoid progenitor

CMP	common myeloid progenitor
CRP	C-reactive protein
CT	cycle threshold
CX_3CR1	CX_3 chemokine receptor 1
CXCL5	chemokine (C-X-C motif) ligand 5
DAPI	4'-6-Diamidino-2-phenylindole
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSAs	electrophoretic mobility shift assays
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transportation
Ets	E twenty-six
EU	endotoxin unit
FACS	fluorescence activated cell sorting
FcγRIII	fragment crystallizable γ receptor III
FCS	fetal calf serum

FDA	food and drug administration
FITC	fluorescein isothiocyanate
FL	fluorescence
FSC	forward scatter
GCF	gingival crevicular fluid
GK	Goto-Kakizaki
GM-CSF	granulocyte macrophage-colony stimulating factor
GMP	granulocyte/macrophage progenitor
GP	general practitioner
HbA1c	glycated haemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEK	human embryonic kidney
HLA	human leukocyte antigen
HMW	high molecular weight
HRP	horseradish peroxidase
HSC	haematopoietic stem cells
hsCRP	high-sensitivity CRP
ICAM	intercellular adhesion molecule
IDF	International Diabetes Federation
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	Interleukin

IL-1Ra	interleukin-1 receptor antagonist
IP	immunoprecipitation
IQR	interquartile range
IRAK-M	interleukin-1 receptor associated kinase M
IRS	insulin receptor substrate
JAK	janus kinase
kDa	kiloDalton
LAL	limulus amebocyte lysate
LBP	LPS-binding protein
LMW	low molecular weight
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAL	MyD88-adapter-like
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
M-CSF	macrophage-colony stimulating factor
MDP	macrophage/dendritic cell progenitor
MEP	megakaryocyte/erythrocyte progenitor
MFI	median fluorescence intensity
MMPs	matrix metalloproteinases
MMW	middle molecular weight

mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response gene (88)
n	number
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NEFA	nonesterified fatty acid
NF-κB	nuclear factor of kappa light chain gene enhancer in B cells
NK cell	natural killer cell
n.s.	not significant
ObR	*ob* receptor
OD	optical density
PAI-1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
PBEF	pre-B-cell colony-enhancing factor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PESA	periodontal epithelial surface area
P. gingivalis	*Porphyromonas gingivalis*
PI3K	phosphoinositide 3-kinase
PMA	phorbol myristate acetate

PMN	polymorphonuclear leukocytes
PRAT	protein associated with TLR
PVDF	polyvinylidene fluoride
R1/2	region 1/2
RACK1	receptor of activated C kinase 1
RAGEs	receptor for advanced glycation endproducts
RANTES	regulated upon activation normal T cell expressed and secreted
RD	reagent diluent
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
SEAP	secreted form of embryonic alkaline phosphatase
Ser	serine
siRNA	small interfering RNA
Spi-1	spleen focus forming virus (SFFV) proviral integration oncogene spi1
SSC	side scatter
STAT	signal transducer and activator of transcription
T1DM	type I diabetes mellitus
T2DM	type II diabetes mellitus

TBS-T	Tris-buffered saline Tween-20
TEMED	tetramethylethylenediamine
T_h	T helper
TIR	Toll/IL-1 receptor
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNF-α	Tumor necrosis factor –α
TRAM	TRIF-related adaptor molecule
T_{reg}	T regulatory
TRAF	TNF Receptor Associated Factor family of protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tris	tris (hydroxymethyl) aminomethane
VitD3	Vitamin D_3
v/v	volume in volume
w/v	weight in volume

Abstract

Type 2 diabetes mellitus (T2DM) is a risk factor for periodontal disease, however the pathogenic links between the two diseases are not completely understood. Both diseases are considered to be inflammatory conditions and, therefore, immune mediators likely play a role in the shared susceptibility between the two diseases. Adipokines have numerous immunological properties and their concentrations are altered in diabetes. Myeloid cells are key leukocytes in responses to periodontal pathogens such as *Porphyromonas gingivalis*. Therefore, the present study aimed to investigate the role of leptin and adiponectin in myeloid cell immune responses and in T2DM patients as potential mediators in an immunological link between diabetes and periodontal disease.

Leptin increased both *E. coli* and *P. gingivalis* LPS-induced TNF-α expression in monocytes. Although leptin had no effect on TLR4 expression, leptin did upregulate TLR2 and enhanced monocyte differentiation. Together, these results indicate the potential of leptin to alter monocyte immune responses to periodontal pathogens.

Male but not female T2DM patients with gingivitis or chronic periodontitis had increased serum leptin concentrations in comparison to matched, non-diabetic controls. Serum adiponectin concentrations were not affected by periodontal status. In contrast, gingival crevicular fluid (GCF) adiponectin concentrations were higher in periodontal disease in both T2DM patients and non-diabetic controls. GCF adiponectin concentrations were a predictor of periodontal status independent of BMI or gender. Serum adipokine concentrations were not affected by periodontal treatment. A decrease in GCF adiponectin concentrations was observed in T2DM patients and non-diabetic controls after periodontal treatment.

In conclusion, findings of the present study demonstrate a role for leptin in monocyte differentiation and immune responses towards periodontal pathogens. In addition, analyses of clinical samples revealed that leptin and adiponectin are a potential link between diabetes and periodontal disease. In particular, GCF adiponectin concentrations may represent a diagnostic marker for diabetes and periodontal disease.

Chapter 1 Introduction

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The increase of obesity in modern western societies and developing countries, which is mainly due to a combination of greater food consumption and a more sedentary lifestyle (Kopelman, 2000), is widely accepted as a major public health problem (Ogden *et al.*, 2006; Hyde, 2008). Thus, obesity is closely associated with the development of systemic diseases such as coronary heart disease, osteoarthritis, hypertension or diabetes mellitus (Kopelman, 2000), which in turn often are risk factors themselves for the development of further long-term complications. Amongst these systemic diseases, diabetes especially is linked to a number of serious complications, including diabetic neuro- and nephropathy, retinopathy or other micro-and macrovascular diseases (Southerland *et al.*, 2005). Furthermore, periodontal disease is another complication of diabetes (Southerland *et al.*, 2005). However, although a number of studies support diabetes as a risk factor for increased incidence or severity of periodontal disease (Nelson *et al.*, 1990; Ryan *et al.*, 2003; Heitz-Mayfield, 2005; Moles, 2006), a mechanistic link between the two diseases is still not completely understood. For example, no differences in the oral microflora between diabetics and non-diabetics with periodontal disease can be detected (Yuan *et al.*, 2001; Lalla *et al.*, 2006). One may assume that the impaired vascular repair and a modified collagen metabolism due to the hyperglycaemic state cause vascular changes not only systemically but also in gingival tissues (Brennan, 1989; Tepper *et al.*, 2002; Bhatwadekar *et al.*, 2008). These altered physiological mechanisms interfere with the transportation of nutrients and leukocyte migration, resulting in impaired tissue turnover and delayed wound healing and are therefore likely to contribute to an increased risk or exacerbation of periodontal disease in diabetes (Goova *et al.*, 2001; Ryan *et al.*, 2003).

Apart from physiological changes, altered immune functions presumably represent one of the most important biological links between diabetes and periodontal disease, as both diseases are inflammatory conditions (Kornman *et al.*, 1997; King, 2008). Indeed, the diabetic hyperglycaemic state not only contributes to vascular modifications, through the formation of advanced glycation end-products (AGEs) it also results in excessive activation of pro-inflammatory mediators, thus contributing to the overall inflammation in the host response to periodontal pathogens (King, 2008). However, hyperglycaemia is not the sole contributor to altered diabetic immune functions. The increased adipose tissue mass that often is part of diabetes pathology is a source of numerous inflammatory mediators as well. Previously, adipose tissue was only regarded as a fat and energy storage unit for the body. Actually, more recent research has demonstrated that adipose tissue is an active endocrine organ, producing a variety of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6 or monocyte chemotactic protein-1 (MCP-1) and hormones with immune-modulatory properties such as leptin, adiponectin or visfatin (Kershaw and Flier, 2004).

Although the concentrations of adipokines are often drastically changed during diabetes since they are directly correlated to the amount of adipose tissue or regulated by insulin (Maffei *et al.*, 1995b; Considine *et al.*, 1996; Hotta *et al.*, 2000; Kieffer and Habener, 2000; Pickup *et al.*, 2000), their potential role in an immunological link between diabetes and periodontal disease is incompletely understood. The pro-inflammatory cytokines and chemokines produced by the adipose tissue are thought to act combined with AGEs to enhance the inflammatory environment and thus increase the susceptibility to periodontal disease (Nishimura *et al.*, 2007). Yet, although other adipokines such as leptin and adiponectin reach circulating levels in the nanogram or

even microgram range (Hotta *et al.*, 2000) and thus could potentially be an important link between diabetes and periodontal disease, these adipokines have received surprisingly little attention in the field. This might be due to the rather recent discovery of either the adipokines themselves or their immune-regulatory properties.

Leptin and adiponectin have been shown to directly modulate the secretion of pro-inflammatory cytokines and to alter immune responses of myeloid cells to lipopolysaccharide (LPS) (Loffreda *et al.*, 1998; Neumeier *et al.*, 2006). Thus, altered concentrations of these adipokines during diabetes potentially induce a hyperresponsive phenotype in monocytes and other myeloid cells, pre-conditioning them to an exacerbated immune response towards periodontal pathogens. Together with the pro-inflammatory systemic state induced by AGEs and with the pro-inflammatory cytokines and chemokines produced by the adipose tissue, the adipokines leptin and adiponectin could hence be closely associated with the increased incidence and severity of periodontal disease during diabetes, their immune-modulatory properties making them potential candidates for an important role in an inflammatory link between the two diseases. Therefore, the main focus of the present work will be on the immunological actions of these adipokines on leukocytes, in particular their action in the modulation of myeloid cell immune responses to LPS, and an assessment of their association with clinical periodontal disease in diabetic patients.

1.1 Adipokines

1.1.1 Structure, components and physiology of adipose tissue

Adipose tissue is a complex structure composed of adipocytes and a variety of other cell types such as connective tissue, vascular cells and immune cells (Kershaw and Flier,

2004), which are also known as the stromal-vascular fraction (Riordan *et al.*, 2009). Furthermore, both sympathetic and parasympathetic innervations were demonstrated for adipose tissue, allowing for a brain–adipose tissue crosstalk (Bartness and Song, 2007; Kreier and Buijs, 2007). Although one of its main function is the storage of lipids (Frayn *et al.*, 2003), adipose tissue also acts as an endocrine organ, producing a very heterogeneous group of molecules which is known as adipokines. These include chemokines such as the monocyte chemotactic protein-1 (MCP-1) or IL-8, cytokines such as TNF-α or IL-6, enzymes for the production of steroid hormones and glucocorticoids and metabolic hormones such as leptin, adiponectin, visfatin or resistin (Table 1.1). The term "adipokine" sometimes is misleading, since throughout the literature it is not only used to describe products of the adipose tissue, but also in exclusive reference to molecules secreted predominantly by the adipocytes themselves. However, Fain *et al.* (2004) demonstrate that, with the exception of leptin and adiponectin, over 90 % of all products from the human adipose tissue originate from the stromal-vascular fraction. Thus, the majority of cytokines and chemokines produced by the adipose tissue presumably do not derive from adipocytes, but rather from resident macrophages within the adipose tissue (Zeyda *et al.*, 2007). Furthermore, even though leptin and adiponectin could be considered as "true" adipokines in a sense that they are primarily secreted by the adipocytes (Maffei *et al.*, 1995a; Scherer *et al.*, 1995), they are produced in minor quantities by some other tissues as well (see 1.1.2, 1.1.3). To avoid misconception, the present study refers to adipokines as any molecule secreted by the adipose tissue, be it is predominantly produced by adipocytes or by other cell types within the adipose tissue. However, the main focus will be on the "true" adipokines leptin and adiponectin.

Group	Adipokines
Cytokines and chemokines	TNF-α, IL-6, MCP-1, IL-8
Fibrinolytic proteins	PAI-1, Tissue factor
Complement-related proteins	Adipsin (complement factor D), Complement factor B, ASP
Lipids and proteins involved with lipid metabolism	LPL, CETP, Apolipoprotein E, NEFAs
Enzymes involved in steroid metabolism	Cytochrome P450-dependent aromatase, 17βHSD, 11βHSD1
Proteins of the renin-angiotensin system	Renin, Angiotensinogen, ACE
Metabolic hormones with immune functions	Leptin, Adiponectin, Resistin, Visfatin

Table 1.1 Overview of a range of different groups of adipokines

Adipokines are a heterogeneous group of proteins with a variety of functions in the metabolism and immune system. The table shows a classification of adipokines according to the function they are mainly associated with (after Kershaw and Flier, 2004). MCP-1: monocyte chemotactic protein, PAI-1: plasminogen activator inhibitor-1, ASP: acylation stimulating protein, LPL: lipoprotein lipase, CETP: cholesteryl ester transfer protein, NEFAs: nonesterified fatty acids, ACE: angiotensin I-converting enzyme.

Through the production of several inflammatory mediators and the resident adipose tissue macrophages which account for more than 10 % of the stromal-vascular fraction (Curat *et al.*, 2004), adipose tissue is strongly associated with the immune system. This is further emphasized by its close anatomical relationship to lymph nodes. The outer capsule of lymph nodes consists of only a very loose collagen layer which is interspersed with numerous fine lymph vessels. These vessels enter the lymph node through almost the total surface area and are permeable for large molecules and even small cells (Pond, 2005). Thus, products from the surrounding adipose tissue can penetrate the lymph node and potentially not only exert their effects on immune cells in circulation but directly on immune cells in the lymph node as well. Furthermore, during a localised inflammatory response, an upregulation of TNF-α receptor expression on adipocytes and an increase in vascularisation was only observed in adipose tissue in close contact with lymph nodes. No change was detected in other adipose tissue deposits (MacQueen and Pond, 1998; Macqueen *et al.*, 1999), revealing a specialization of adipose tissue surrounding the lymph nodes. In addition, adipose tissue itself can actively participate in an immune response. Adipocytes express a range of functional toll-like receptors (TLRs) and can respond to pro-inflammatory stimuli such as LPS (Batra *et al.*, 2007; Poulain-Godefroy and Froguel, 2007; Kopp *et al.*, 2009). Also, through expression of MCP-1, monocytes are recruited to adipose tissue where they differentiate into macrophages. Interestingly, a positive relationship between macrophage number and adipose tissue mass was established (Weisberg *et al.*, 2003; Curat *et al.*, 2004). Thus, in the course of an increasing body mass index (BMI) during obesity, the number of adipose tissue macrophages increases as well. Furthermore, it was demonstrated that these newly recruited adipose tissue macrophages display increased inflammatory properties such as an overexpression of pro-inflammatory

cytokine and chemokine genes compared to resident adipose tissue macrophages (Weisberg *et al.*, 2003; Lumeng *et al.*, 2007). Together, these and other findings led to the recognition of obesity as a systemic inflammatory condition (Gil *et al.*, 2007).

1.1.2 Functions of leptin in the metabolism and immune system

The adipokine leptin is a 16 kDa protein (Halaas *et al.*, 1995) and was first described as the product of the *ob* gene (Zhang *et al.*, 1994b). Mice homozygous for a mutation in the *ob* gene (*ob*/*ob* mice) (Ingalls *et al.*, 1950) show increased food intake, reduced activity and develop severe obesity and diabetes (Ingalls *et al.*, 1950; Pelleymounter *et al.*, 1995). However, after injections with recombinant leptin, in *ob*/*ob* mice but also in wild type mice with diet-induced obesity, reductions in food intake and improved weight loss is observed (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). Later, it was revealed that leptin acts as a satiety hormone in the hypothalamus where it regulates food consumption and energy expenditure (Flier and Maratos-Flier, 1998; Friedman and Halaas, 1998) through activation of the sympathetic nervous system (Satoh *et al.*, 1999).

The discovery of leptin as a satiety hormone and the promising experiments in mice with leptin as a weight reducing agent led to the belief that leptin could be applied as an anti-obesity drug in humans as well. Indeed, recombinant leptin is used very effectively to treat patients who suffer from leptin deficiency, a rare genetic disorder caused by mutations in the human leptin gene (Mazen *et al.*, 2009). These patients develop similar symptoms as the *ob*/*ob* mice, including severe obesity and dysfunctions in glycaemic control (Montague *et al.*, 1997; Ozata *et al.*, 1999; Mazen *et al.*, 2009). However, although recombinant leptin was successful in treating human leptin-deficiency induced obesity, in contrast to mice it had only marginal effects on humans with diet-induced

obesity (Heymsfield *et al.*, 1999). It appears that humans with diet-induced obesity are unresponsive to increased leptin concentrations due to hypothalamic leptin resistance and thus new obesity research focuses on improving leptin sensitivity rather than using the adipokine itself for obesity treatment (Ozcan *et al.*, 2009). Apart from its role as a satiety hormone, leptin is also associated with a number of metabolic functions. For example, leptin is involved in the regulation of adipose tissue lipogenesis (Buettner *et al.*, 2008) and cholesterol breakdown (O'Rourke *et al.*, 2001). In addition, several studies demonstrate a role for the adipokine in bone metabolism, however results are conflicting. Some authors report a positive association of leptin with bone mineral density and describe inhibitory effects on osteoclast generation (Holloway *et al.*, 2002; Tamura *et al.*, 2007), while others observe inhibitory effects on osteoblast activity (Pogoda *et al.*, 2006) and negative associations of leptin with bone mineral density (Kassem *et al.*, 2008). Possibly, a dose-dependent effect of leptin provides an explanation for these controversial results (Martin *et al.*, 2007) yet the exact role of leptin in bone regulation remains obscure.

Leptin circulates the serum of healthy individuals at an average concentration of 3-12 ng/ml (Hotta *et al.*, 2000), with a strong gender difference resulting in women having higher leptin concentrations than men (Nystrom *et al.*, 1997; Perkins and Fonte, 2002). Furthermore, leptin is positively correlated to the BMI (Maffei *et al.*, 1995b; Considine *et al.*, 1996). Although adipocytes are the main source of leptin (Maffei *et al.*, 1995a), a variety of other cells and tissues contribute to the overall leptin production. Thus, leptin can also be produced by the stomach (Bado *et al.*, 1998), salivary glands (Bohlender *et al.*, 2003), the placenta and trophoblast, (Masuzaki *et al.*, 1997; Meissner *et al.*, 2005), brain and pituitary gland (Morash *et al.*, 1999) and T cells (Fantuzzi *et al.*, 2005; De

Rosa *et al.*, 2007). The significance of these other leptin sources is largely unknown. However, an autocrine effect or an increase in leptin concentration potentially alters leptin functions locally and for T cell-derived leptin a specific anti-proliferative effect on T regulatory cells (T_{reg}) was demonstrated (De Rosa *et al.*, 2007). Several different mechanisms contribute to the regulation of leptin secretion and concentration. For example, leptin levels are inversely correlated to the pituitary-adrenal secretion of adrenocorticotropic hormone (ACTH) and cortisol and humans have higher leptin concentrations during the night then throughout the day (Sinha *et al.*, 1996a; Licinio *et al.*, 1997). In addition, serum leptin levels also increase after food consumption (Elimam and Marcus, 2002). For example, by suppressing food cravings during the night and further food consumption after a meal these regulatory mechanisms correspond well with the role of leptin as a satiety hormone. As mentioned above, leptin concentrations exhibit a strong gender difference. However, the sexual dimorphism cannot be attributed to gender specific differences in adipose tissue distribution (Rosenbaum *et al.*, 2001). Rather, the sex hormones appear to be key regulators of leptin concentrations. Thus, estrogen was found to increase leptin production (Shimizu *et al.*, 1997), whereas a strong negative correlation with testosterone is observed in men (Nystrom *et al.*, 1997). Furthermore, leptin expression is induced during inflammation. Injections of mice or hamsters with LPS, TNF-α or IL-1β resulted in increased serum leptin concentrations and increased adipose tissue leptin mRNA expression (Grunfeld *et al.*, 1996; Sarraf *et al.*, 1997; Faggioni *et al.*, 1998). However, in the same setting, injections with the anti-inflammatory cytokines IL-10, IL-2 or IL-4 had no effect (Sarraf *et al.*, 1997). Potentially these alterations in leptin concentration are an important factor in the pro-inflammatory actions attributed to this adipokine.

1.1.2.1 Immunological actions of leptin

Leptin plays a vital role in numerous immunological functions. Thus, *ob*/*ob* mice are protected in several models of inflammation such as autoimmune encephalomyelitis (Matarese *et al.*, 2001), collagen induced arthritis (Busso *et al.*, 2002) or colitis (Siegmund *et al.*, 2002). Furthermore, *ob*/*ob* mice and their counterpart with a gene defect in the leptin receptor (*db*/*db* in mice or *fa*/*fa* in rats) have a higher susceptibility to endotoxaemia (Yang *et al.*, 1997) and show decreased IL-6 and TNF-α serum concentrations after LPS treatment (Loffreda *et al.*, 1998). These animals are also protected against toxic effects of TNF-α (Takahashi *et al.*, 1999). Additionally, an impaired T cell proliferation was observed (Lord *et al.*, 1998). Together, these findings led to the general view of leptin as a pro-inflammatory adipokine. In direct *in vitro* studies of leptin functions several immunologically relevant actions of this adipokine have been discovered. For example, anti-apoptotic effects of leptin were demonstrated for virtually all immune cells, including eosinophils (Conus *et al.*, 2005), NK cells (Zhao *et al.*, 2003), T cells and thymus (Fujita *et al.*, 2002; Mansour *et al.*, 2006), dendritic cells (DCs) (Mattioli *et al.*, 2009), neutrophils (Bruno *et al.*, 2005) and monocytes (Najib and Sanchez-Margalet, 2002). Furthermore, leptin can act as a potent chemoattractant for neutrophils, monocytes and macrophages (Caldefie-Chezet *et al.*, 2003; Montecucco *et al.*, 2006; Gruen *et al.*, 2007) and can also increase phagocytic activity of macrophages (Gainsford *et al.*, 1996). Mitogenic functions of leptin were observed in peripheral blood mononuclear cells (PBMCs) (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001) and NK cells (Zhao *et al.*, 2003). Some reports also show increased T cell proliferation after leptin treatment (Lord *et al.*, 1998; Martin-Romero *et al.*, 2000), although this does not apply to all classes of T cells since an inhibitory effect of leptin on T_{reg} (T_h17) proliferation was observed (De Rosa *et al.*, 2007). Leptin was

further shown to induce cell surface expression of activation markers. For example, increased CD11b, CD25, CD36, CD38 or CD69 expression was demonstrated after leptin treatment in monocytes, T cells and neutrophils (Santos-Alvarez *et al.*, 1999; Martin-Romero *et al.*, 2000; Zarkesh-Esfahani *et al.*, 2004; Konstantinidis *et al.*, 2009).

However, some effects of leptin on expression of cell surface markers do not appear to be direct but rather indirect consequences of other leptin-mediated effects. Thus, in isolated neutrophils, Zarkesh-Esfahani *et al.* (2004) observed that leptin had no effect on CD11b expression, an essential activation marker involved in neutrophil migration and phagocytosis (Hofman *et al.*, 2000). Yet, when monocytes were added to the neutrophil culture, leptin enhanced neutrophilic CD11b expression indirectly through induction of TNF-α secretion in the monocytes (Zarkesh-Esfahani *et al.*, 2004). Indeed, in a number of studies, leptin was found to be a potent modulator of cytokine and chemokine expression in both immune cells and other cell types, mainly driving a pro-inflammatory immune response. Accordingly, in PBMCs and DCs leptin treatment increased TNF-α and IL-6 mRNA and intracellular or secreted protein expression (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001; Mattioli *et al.*, 2005). In general, leptin promotes cellular immune responses and supports a T_h1 cytokine expression pattern. Thus, leptin enhances IL-2 secretion in lymphocytes and NK cells, induces IFN-γ secretion and suppresses IL-4 in PBMCs and T cells specifically (Lord *et al.*, 1998; Martin-Romero *et al.*, 2000; Zhao *et al.*, 2003). Furthermore, leptin-induced MCP-1 or IL-8 expression was demonstrated in fibroblasts (Tong *et al.*, 2008), endothelial and hepatic stellate cells (Bouloumie *et al.*, 1999; Aleffi *et al.*, 2005).

The multitude of immunological functions influenced by leptin would suggest a central role for this adipokine in inflammatory immune responses. However, except for

observations in leptin or leptin receptor-deficient rodents, only a limited number of studies investigated the effect of leptin in the context of an additional immune challenge with inflammatory mediators such as LPS. For example, in work on murine peritoneal macrophages, leptin treatment on its own induced no secretion of any of the investigated cytokines (TNF-α, IL-6, IL-10 and IL-12) (Loffreda *et al.*, 1998). Yet, when cells were pre-incubated with leptin before LPS stimulation, the LPS-induced cytokine response was enhanced for TNF-α, IL-6 and IL-12 compared to LPS treatment alone. At the same time, although LPS induced the secretion of IL-10 and IL-1β, no enhancing effect of leptin was observed for these cytokines (Loffreda *et al.*, 1998). Likewise, in a study of murine Kupffer cells, again leptin alone had no effect on TNF-α secretion but enhanced the LPS-induced TNF-α production (Shen *et al.*, 2005). The LPS enhancing effect of leptin is not exclusive to cytokines but was also demonstrated for CD40 mRNA and cell surface expression in murine primary DCs (Lam *et al.*, 2007). In humans, leptin was shown to enhance the cell surface expression of the activation marker CD69 in LPS treated PBMCs (Santos-Alvarez *et al.*, 1999) and to increase the LPS-induced pro-inflammatory cytokine expression in PBMCs and primary DCs (Santos-Alvarez *et al.*, 1999; Mattioli *et al.*, 2005).

Together, these studies reveal a complex pattern of immune regulatory actions of leptin. Not only does leptin have immunological functions itself, in addition it also potentiates the immune response to inflammatory mediators. It is currently not understood why in some settings leptin alone induces pro-inflammatory cytokines or why in others it only acts in enhancing LPS-induced cytokine production. Likely, different study approaches, cell types or differential activation of intracellular signalling pathways play a role. However, continuing research is needed to further evaluate the role of leptin in LPS

immune responses. Also, it should be noted that only few reports on leptin and LPS stimulation investigated an immunological role of this adipokine in humans. Moreover, these studies exclusively use LPS from enterogenic bacteria, thus limiting any conclusions for other bacterial strains such as oral pathogens.

1.1.2.2 Distribution and intracellular signalling pathways of the leptin receptor

The leptin receptor is encoded by the *db* gene (Tartaglia *et al.*, 1995; Chen *et al.*, 1996). Mice or rats with mutations in this gene (*db*/*db* mice or *fa*/*fa* rats) show a similar phenotype as the *ob*/*ob* mice and develop severe obesity and diabetes (Chua *et al.*, 1996a; Chua *et al.*, 1996b; Phillips *et al.*, 1996; Yamashita *et al.*, 1997; Yamashita *et al.*, 1998). Mutations in the leptin receptor can be observed in humans as well, although only one case with three affected individuals has been reported so far (Clement *et al.*, 1998). These subjects developed severe early-onset obesity, however in contrast to leptin deficicient subjects, only mild irregularities in glucose homeostasis were noted (Clement *et al.*, 1998). Leptin receptors are detected virtually on all cell types, organs and tissues. In the immune system, leptin receptors are expressed on T cells (Siegmund *et al.*, 2004), PBMCs (Tsiotra *et al.*, 2000; Zarkesh-Esfahani *et al.*, 2001; Sanchez-Margalet *et al.*, 2002), neutrophils (Caldefie-Chezet *et al.*, 2003), NK cells (Zhao *et al.*, 2003), hematopoietic cells (Gainsford *et al.*, 1996) and lymphoid tissues (Fujita *et al.*, 2002). As expected, leptin receptors are also expressed on the blood brain barrier, the hypothalamus and cerebellum (Mercer *et al.*, 1996; Bjorbaek *et al.*, 1998) and on adipocytes (Kielar *et al.*, 1998). Furthermore, receptor expression was confirmed in tissues and organs such as skeletal muscle (Guerra *et al.*, 2007), intervertebral discs (Zhao *et al.*, 2008), the pancreas (Emilsson *et al.*, 1997), salivary glands (Bohlender *et al.*, 2003) and the endometrium (Quinton *et al.*, 2003).

The ubiquitous expression of leptin receptors emphasizes the multifunctional role of this adipokine in both metabolic and immunologic systems. This is further supported when considering that the leptin receptor is expressed in at least 6 isoforms (ObRa-f) through alternative splicing (Cioffi *et al.*, 1996; Lee *et al.*, 1996; Wang *et al.*, 1996) which all have specific functions involving leptin transport, signalling or intracellular processing. For example, ObRa-e are associated with leptin transport. Thus, the main function of ObRa is the transport of leptin across cell membranes (Hileman *et al.*, 2000) and the blood brain barrier (Kastin *et al.*, 1999). ObRb-ObRd play a role in leptin endocytosis and intracellular trafficking (Tu *et al.*, 2007) and ObRe is a soluble leptin receptor which transports leptin in blood circulation. This isoform was also shown to inhibit the transport of leptin across the blood brain barrier and to act as an antagonist for ObRa and ObRb (Tu *et al.*, 2008), hence having a regulatory function on leptin bioavailability. For both mice and humans it was observed that in lean individuals most leptin circulates in its bound form (e.g. transported by ObRe), whereas leptin binding sites of ObRe are saturated during obesity and most leptin circulates free (Houseknecht *et al.*, 1996; Sinha *et al.*, 1996b; Diamond *et al.*, 1997). Thus, ObRe is inversely correlated to serum leptin concentrations and BMI (Diamond *et al.*, 1997). Additionally, altered levels of circulating ObRe have been observed in some clinical conditions of human diseases. For example, reduced ObRe and higher leptin concentrations were detected in non-alcoholic fatty liver disease (Huang *et al.*, 2008). A different situation was noted in inflammation during liver cirrhosis: both ObRe and bound leptin concentrations increased, leaving the concentration of free leptin unaltered (Ockenga *et al.*, 2007). Thus, ObRe regulates leptin bioavailability at both the cellular level and in circulation, potentially having an impact in disease pathologies.

Every leptin receptor isoform (except for ObRe) has intracellular signalling capacities and forms homodimers, binding two leptin molecules in a 1:1 ratio (Devos *et al.*, 1997; Fong *et al.*, 1998). *In vitro*, it was also possible to create a ObRa/b heterodimer (White and Tartaglia, 1999), however this heterodimer could not display functional signalling (Bahrenberg *et al.*, 2002). Furthermore, the leptin receptor shows sequence homology to the IL-6 cytokine receptor superfamily and shares functional signalling capacities (Taga *et al.*, 1989; Tartaglia *et al.*, 1995; Baumann *et al.*, 1996; Peters *et al.*, 1998). However, only ObRb (the "long" receptor) consists of the intracellular elements for activation of a whole range of different signalling pathways (Baumann *et al.*, 1996; Bjorbaek *et al.*, 1997).

Figure 1.1 gives an overview of the possible main ObRb intracellular signalling pathways. One of the key signalling pathways is the JAK/STAT pathway. JAK2 and subsequently the phosphorylation of STAT3 has been demonstrated by numerous groups in different cell types (O'Rourke *et al.*, 2001; Sanchez-Margalet and Martin-Romero, 2001; Cui *et al.*, 2006; Cao *et al.*, 2007; Tong *et al.*, 2008), although some describe activation of STAT1 or STAT5 as well (Tanabe *et al.*, 1997; Wang *et al.*, 1997; Lam *et al.*, 2007). A second major leptin signalling pathway is the PI3K/Akt pathway. This signalling cascade can get activated through the MAPKs ERK1/2 (Dreyer *et al.*, 2003; Ktori *et al.*, 2003; Aleffi *et al.*, 2005; Bruno *et al.*, 2005; Maroni *et al.*, 2005; Cui *et al.*, 2006; Gruen *et al.*, 2007) or through the IRS pathway (Zhao *et al.*, 2000; Niswender *et al.*, 2001). The IRS pathway itself is either induced through JAK2 (Attoub *et al.*, 2000; O'Rourke *et al.*, 2001; Tong *et al.*, 2008) or independently of JAK2 signalling (Mansour *et al.*, 2006). Finally, leptin ObRb can also signal through the p38 MAPK pathway (van den Brink *et al.*, 2000; Zarkesh-Esfahani *et al.*, 2001; Maroni *et*

al., 2005; Cui *et al.*, 2006; Montecucco *et al.*, 2006; Cao *et al.*, 2007). Activation of these and other signalling pathways induces the specific metabolic effects of leptin, such as its regulatory role in glucose and energy homeostasis or its function as a satiety hormone (Fruhbeck, 2006). In an immunological relevant setting, leptin signalling pathways induce transcription factors such as STAT3, NF-κB or AP-1 in immune cells and other cell types such as fibroblasts or endothelial cells, leading to the subsequent production of pro-inflammatory cytokines and chemokines (Bouloumie *et al.*, 1999; Aleffi *et al.*, 2005; Tang *et al.*, 2007b; Tong *et al.*, 2008; Mattioli *et al.*, 2009).

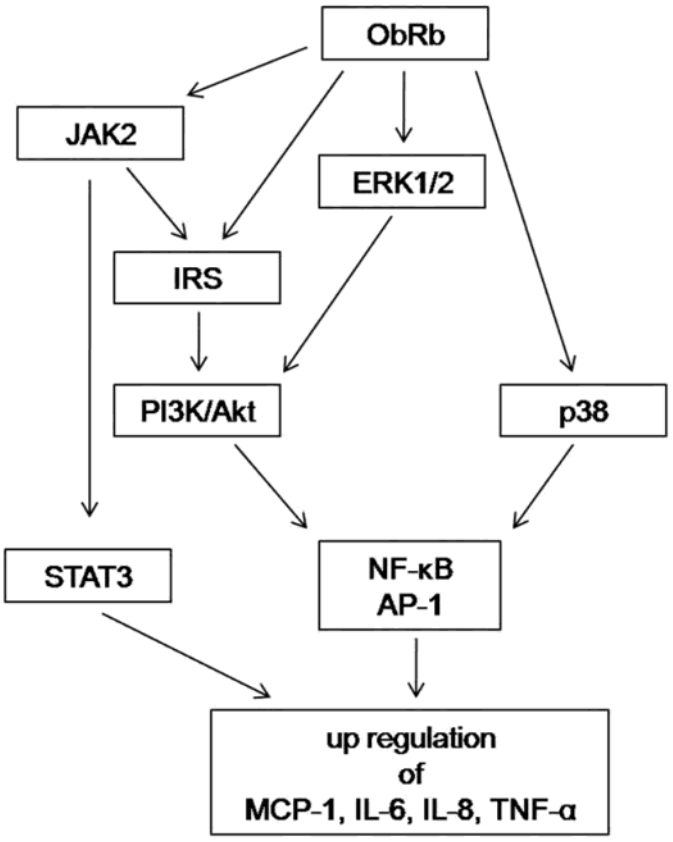

Figure 1.1 Leptin receptor ObRb intracellular signalling pathways

The flowchart gives an overview of the main leptin receptor ObRb intracellular signalling pathways relevant for the activation of pro-inflammatory transcription factors. In immune cells, the leptin receptor ObRb activates three main intracellular signalling pathways: the JAK2, the PI3K and the p38 pathway. Leptin signalling through JAK2 leads to direct activation of the transcription factor STAT3. In addition, activation of the JAK2 signalling pathway induces IRS, which consequently activates the PI3K/Akt signalling pathway. IRS can also be activated directly by ObRb signalling in a JAK2 independent pathway. Furthermore, ObRb signalling can lead to PI3K/Akt signalling via ERK 1/2. Activation of the PI3K/Akt and of the p38 signalling pathway then induces the transcription factors NF-κB and AP-1. Finally, the activation of STAT3, NF-κB and AP-1 leads to an upregulation of pro-inflammatory cytokines and chemokines such as IL-6, TNF-α, IL-8 or MCP-1.

As mentioned above, the shorter leptin receptor isoforms have some signalling capacity as well. For example, leptin can activate PI3K and MAPK pathways through ObRa, inducing cellular glucose uptake and phosphatase activity (Kellerer *et al.*, 1997; Bjorbaek *et al.*, 2001). However, although JAK2 phosphorylation is observed, the subsequent STAT pathway is not activated since only ObRb has the necessary intracellular domain for STAT signalling (Bjorbaek *et al.*, 1997; Kellerer *et al.*, 1997; Bjorbaek *et al.*, 2001). Leptin activates most signalling pathways simultaneously, yet not all are always required to induce a specific effect. Thus, leptin-induced intracellular TNF-α protein expression in monocytes requires activation of p38 MAPK but not of ERK MAPK (Zarkesh-Esfahani *et al.*, 2001). Likewise, an anti-apoptotic effect of leptin in a serum free culture of PBMCs is induced through ERK MAPK, but not through PI3K (Najib and Sanchez-Margalet, 2002). On the other hand, leptin-induced chemotaxis of monocytes requires simultaneously JAK/STAT, MAPK, and PI3K pathway activation (Gruen *et al.*, 2007). Interestingly, the concurrent activation of leptin or LPS-induced signalling pathways was revealed as one mechanism for the LPS enhancing effect of leptin. Thus, Lam *et al.* (2007) demonstrate that both leptin and LPS individually induce JAK2/STAT and PI3K/Akt pathway phosphorylation events, however the phosphorylation status of these signalling pathways was markedly increased in the combination of LPS and leptin.

1.1.3 Functions of adiponectin in the metabolism and immune system

Adiponectin is a 30 kDa protein which is mainly produced by adipocytes (Scherer *et al.*, 1995; Hu *et al.*, 1996; Maeda *et al.*, 1996; Nakano *et al.*, 1996). Other sources include the stromal-vascular fraction of adipose tissue (Fain *et al.*, 2008), salivary glands (Katsiougiannis *et al.*, 2006), the pituitary (Rodriguez-Pacheco *et al.*, 2007),

cardiomyocytes (Pineiro *et al.*, 2005), skeletal muscle (Staiger *et al.*, 2003; Delaigle *et al.*, 2004) and osteoblasts (Berner *et al.*, 2004). The physiological relevance of these other adiponectin sources is not clear. However, it is conceivable that local adiponectin production is important in some sites. Thus, it was demonstrated that adiponectin, unlike leptin, cannot cross the blood-brain barrier (Spranger *et al.*, 2006) and therefore the local production in the pituitary is likely the only source for central adiponectin signalling. Adiponectin has various functions in metabolism, for example the stimulation of food intake and decreased energy expenditure (Kubota *et al.*, 2007) or the regulation of fatty acid and glucose metabolism (Bruce *et al.*, 2005). Adiponectin knock-out mice develop insulin resistance and glucose intolerance (Kubota *et al.*, 2002; Maeda *et al.*, 2002) and adiponectin is recognized as an insulin sensitizer and an anti-diabetic agent (Berg *et al.*, 2001; Yamauchi *et al.*, 2001; Combs *et al.*, 2004; Fu *et al.*, 2005). Furthermore, adiponectin also plays a role in the regulation of bone resorption (Yamaguchi *et al.*, 2008), although findings so far are conflicting. Thus, a negative correlation of serum adiponectin concentration with bone mineral density was described (Richards *et al.*, 2007; Basurto *et al.*, 2009) yet adiponectin was also shown to stimulate osteoblast proliferation *in vitro* (Berner *et al.*, 2004; Luo *et al.*, 2005). Possibly the balance between osteoblast and osteoclast activity is regulated through different local or circulating adiponectin concentrations in a comparable manner as discussed for leptin (Martin *et al.*, 2007).

Adiponectin circulates the serum of healthy individuals at a range between 2-17 µg/ml (Arita *et al.*, 1999), which makes it one of the most abundant proteins in human plasma (Stumvoll and Haring, 2002). Although most groups report a gender difference for serum adiponectin with men having lower concentrations (average 7 µg/ml) than

women (average 10 µg/ml) (Arita *et al.*, 1999), this gender difference appears to diminish with increasing BMI (Putz *et al.*, 2004; Hicks *et al.*, 2007). In contrast to leptin, adiponectin is negatively correlated with the BMI (Arita *et al.*, 1999; Merl *et al.*, 2005) and several studies report increased adiponectin concentrations after weight loss (Coughlin *et al.*, 2007; Engl *et al.*, 2007; Madsen *et al.*, 2008). Adiponectin is found in serum at different oligomeric states: a trimer (low molecular weight, LMW), hexamer (middle molecular weight, MMW) and a multimer (high molecular weight, HMW) (Hada *et al.*, 2007; Radjainia *et al.*, 2008; Schraw *et al.*, 2008) (Figure 1.2). These isoforms are directly secreted from the adipocytes and do not form or change in serum (Schraw *et al.*, 2008). Additionally, adiponectin can be converted in plasma into globular adiponectin through proteolytic cleavage (Fruebis *et al.*, 2001; Waki *et al.*, 2005). Post-translational modifications such as hydroxylation or glycosylation are needed for the assembly of higher molecular weight forms (hexamers and multimers) of adiponectin (Richards *et al.*, 2006; Wang *et al.*, 2006). Structural studies further revealed disulfide bonds in the formation of adiponectin isoforms (Tsao *et al.*, 2003; Liu *et al.*, 2008). Interestingly, the proportions of isoform concentrations change with different conditions and gender. For example, obese individuals have less HMW than lean people (Schraw *et al.*, 2008) and a shift from LMW to HMW was observed during weight loss (Engl *et al.*, 2007). Furthermore, females have more HMW than men (Schraw *et al.*, 2008) and both HMW and MMW, but not LMW show a negative association with levels of female sex steroid hormones (Leung *et al.*, 2009).

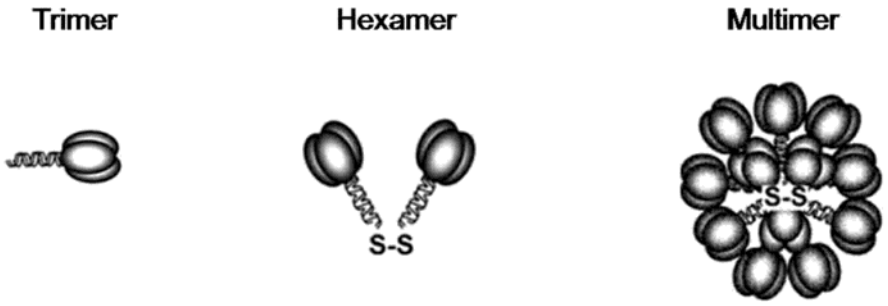

Figure 1.2 Structures of adiponectin isoforms

The pictures show the structures of the three main adiponectin isoforms. The trimer is composed of three single adiponectin molecules, forming a triple helix with a globular domain. Two of these trimers then associate with disulfide bonds, forming the hexamer. The association of several hexamers then results in the multimer isoform of adiponectin (from Kadowaki and Yamauchi, 2005).

Few studies have investigated the nutritional and circadian effects on adiponectin. However, the findings indicate that these environmental factors have exactly the opposite effect on adiponectin concentrations than on leptin. Hence, adiponectin concentrations were shown to decrease during the night (Gavrila *et al.*, 2003) and after a high fat meal (Esposito *et al.*, 2003). In addition and again in contrast to leptin, adiponectin concentrations are downregulated by a number of pro-inflammatory cytokines such as IL-6, TNF-α or IL-18 (Bruun *et al.*, 2003; Fasshauer *et al.*, 2003; Degawa-Yamauchi *et al.*, 2005; Chandrasekar *et al.*, 2008; Zappala and Rechler, 2009). Thus, in the context of its role in metabolism and of regulatory events of adiponectin concentrations, it appears that this adipokine is the matching part to leptin. However, regarding immunological activities of adiponectin, this is not always the case. Unlike leptin, which is mainly associated with inflammatory properties, adiponectin can be attributed both pro and anti-inflammatory roles, depending on the immediate conditions.

1.1.3.1 Immunological actions of adiponectin

A number of *in vitro* studies report potent immune-regulatory functions of adiponectin in several cell types, in which the adipokine is generally inducing anti-inflammatory effects. Thus, adiponectin can bind to pro-inflammatory chemokines such as MCP-1 or RANTES (Masaie *et al.*, 2007) and in macrophages it also has direct suppressive effects on chemokine production (Saijo *et al.*, 2005; Okamoto *et al.*, 2008). In macrophages and other myeloid cells adiponectin was shown to inhibit phagocytosis and to induce the production of IL-10 and IL-1Ra (Yokota *et al.*, 2000; Kumada *et al.*, 2004; Wolf *et al.*, 2004). Furthermore, adiponectin can suppress leptin-induced TNF-α production at both mRNA and protein level in macrophages (Zhao *et al.*, 2005), although it was demonstrated that adiponectin itself induces the secretion of TNF-α and IL-6 (Tsatsanis

et al., 2005). However in this study, Tsatsanis *et al.* (2005) speculate that due to its high concentration in the circulation of lean individuals, adiponectin promotes tolerance to pro-inflammatory stimuli, including its own. In endothelial cells, several reports demonstrate an anti-inflammatory role for adiponectin. In these cells, the adipokine inhibits TNF-α induced NF-κB activation and IL-8 production (Ouchi *et al.*, 2000; Kobashi *et al.*, 2005; Hattori *et al.*, 2008). Support for an anti-inflammatory role of adiponectin is also obtained from adiponectin knock-out mice which display high adipose tissue TNF-α mRNA expression and high circulating TNF-α concentrations (Maeda *et al.*, 2002).

An interesting feature of adiponectin is its ability to bind LPS (Peake *et al.*, 2006). This potentially neutralizes LPS and induces an anti-inflammatory effect. For example, although pure LPS injections have no different effects on cytokine concentrations in adiponectin knock-out mice than in wild-type (Pini *et al.*, 2006), these mice have a higher mortality rate, LPS levels and pro-inflammatory cytokine concentrations after surgery-induced polymicrobial sepsis (Uji *et al.*, 2009). Furthermore, adiponectin concentrations show a negative correlation to LPS concentrations in wild-type mice during surgery-induced polymicrobial sepsis, indicating a protective LPS neutralizing effect of adiponectin during acute inflammation (Tsuchihashi *et al.*, 2006). Additionally, a number of studies report suppressive effects of adiponectin on LPS-induced NF-κB activation and pro-inflammatory cytokine production (Yokota *et al.*, 2000; Wulster-Radcliffe *et al.*, 2004; Ajuwon and Spurlock, 2005; Tsatsanis *et al.*, 2005; Yamaguchi *et al.*, 2005; Park *et al.*, 2008) and on LPS-induced osteoclast generation (Yamaguchi *et al.*, 2007).

However, sometimes adiponectin appears to enhance LPS-induced effects, for example increasing phagocytosis and chemokine production in macrophages (Saijo *et al.*, 2005). The conflicting results of adiponectin as a pro- and anti-inflammatory adipokine can most likely be attributed to the effects of its different isoforms. For example, in monocytes NF-κB activation was only observed with HMW adiponectin but not with MMW or LMW (Haugen and Drevon, 2007) and in muscle homogenates, only LMW induced AMP-activated protein kinase, whereas MMW and HMW had no effect (Tsao *et al.*, 2003). A study using endothelial cells shows that both HMW and globular adiponectin can induce NF-κB, but HMW is a less potent activator than globular adiponectin (Tomizawa *et al.*, 2008). However, in the same study the analysis of TNF-α induced NF-κB activation indicated that HMW inhibits NF-κB much faster than globular adiponectin (Tomizawa *et al.*, 2008). Thus, the different adiponectin isoforms appear to have different functions. Neumeier *et al.* (2006) clearly demonstrate both pro and anti-inflammatory actions of different adiponectin isoforms during LPS stimulation in human monocytes and macrophages. In this study, LPS-induced activation of NF-κB and IL-6 production was suppressed by LMW adiponectin only, HMW had no effect. Furthermore, only LMW but not HMW adiponectin induced IL-10 production, revealing an anti-inflammatory effect for adiponectin trimers. In contrast, only HMW but not LMW on its own induced IL-6 production, associating HMW forms of adiponectin with potentially pro-inflammatory actions (Neumeier *et al.*, 2006).

Finally, due to its LPS binding properties, some of the observed pro or anti-inflammatory effects of adiponectin are possibly attributed to LPS contamination. Turner *et al.* (2009) reveal LPS contamination in a majority of commercially available recombinant adiponectin preparations. Furthermore, the adiponectin induced TNF-α

expression or the suppressive effect of adiponectin on LPS-induced TNF-α expression could be mimicked by LPS of the same concentration as the determined contamination and adiponectin effects were abolished after addition of a LPS inhibitor (Turner *et al.*, 2009). Considered together, results so far indicate both pro-and anti-inflammatory roles for adiponectin. However, only a limited number of studies addressed the different adiponectin isoform functions and often the LPS binding-effect of adiponectin is not considered as a potent interfering factor for any findings. Furthermore, any detailed signalling studies of adiponectin or its isoforms are rare since receptors for adiponectin were only discovered recently. Thus, the role of adiponectin as a pro- and/or anti-inflammatory adipokine remains to be established.

1.1.3.2 Distribution and intracellular signalling pathways of the adiponectin receptor

Three receptors have been identified for adiponectin. AdipoR1 and AdipoR2 expression was detected on skeletal muscle (Yamauchi *et al.*, 2003; Staiger *et al.*, 2004), pancreatic β-cells (Kharroubi *et al.*, 2003), liver (Yamauchi *et al.*, 2003), the pituitary (Rodriguez-Pacheco *et al.*, 2007), fat tissue (Bluher *et al.*, 2007), fibroblasts (Tang *et al.*, 2007a), osteoblasts (Berner *et al.*, 2004) and immune cells (Chinetti *et al.*, 2004; Alberti *et al.*, 2007; Pang and Narendran, 2008). The third adiponectin receptor, T-cadherin, is a member of the cadherin family of transmembrane proteins which are generally associated with intercellular adhesion (Philippova *et al.*, 2009). However, T-cadherin is an exception and has intracellular signalling capacities (Philippova *et al.*, 2009). T-cadherin expression is detected on endothelial and muscle cells (Hug *et al.*, 2004).

Thus, adiponectin receptors are equally ubiquitously expressed as the leptin receptors, emphasizing an important role for this adipokine in various metabolic and immunologic

functions. However, although most cells and tissues express both AdipoR1 and AdipoR2, they are not evenly distributed and their expression is differentially regulated. For example, AdipoR1 is mainly found on skeletal muscle, AdipoR2 mainly in the liver (Yamauchi *et al.*, 2003). On monocytes, both AdipoR1 and AdipoR2 are expressed, however during differentiation into macrophages, AdipoR1 expression decreases, while AdipoR2 expression remains stable (Chinetti *et al.*, 2004). Furthermore, adiponectin receptor expression is downregulated through insulin (Tsuchida *et al.*, 2004) and during obesity (Alberti *et al.*, 2007). It is conceivable that the differential receptor expression and regulation plays an important role in the effects of the different adiponectin isoforms. Thus, AdipoR1 and AdipoR2 are recognized as the main receptors for all isoforms (Yamauchi *et al.*, 2003; Yamauchi *et al.*, 2007), whereas the receptor T-cadherin recognizes HMW and MMW only (Hug *et al.*, 2004).

Knowledge on adiponectin signalling is still limited. Two adaptor proteins (APPL1 and RACK1) for intracellular signalling of AdipoR1 and AdipoR2 were identified (Mao *et al.*, 2006; Xu *et al.*, 2009) and a recent study reports a role for protein kinase CK2 in AdipoR1 signalling (Heiker *et al.*, 2009). In the context of combined LPS and adiponectin stimulation, it is interesting to note that in macrophages, adiponectin was shown to activate IRAK-M (Zacharioudaki *et al.*, 2009), an inhibitor of TLR signalling (Kobayashi *et al.*, 2002). This possibly provides another explanation for any anti-inflammatory effects of adiponectin which are seen in conjunction with LPS signalling. A number of studies demonstrate adiponectin-induced activation of ERK1/2 and p38 MAPK signalling pathways (Wulster-Radcliffe *et al.*, 2004; Yoon *et al.*, 2006; Kamio *et al.*, 2008), yet only few reports specifically address which adiponectin receptor is involved. In a work by Tang *et al.* (2007a) the authors describe an enhancing effect of

adiponectin on IL-6 expression in synovial fibroblasts. However, although both AdipoR1and AdipoR2 are expressed on these cells, they demonstrate that the effect is exclusively mediated via AdipoR1 and not AdipoR2 (Tang *et al.*, 2007a). In another study Lee *et al.* (2008) show adiponectin-induced activation of ERK1/2 through AdipoR1 and AdipoR2 in vascular smooth muscle or endothelial cells and in hepatocytes. In addition, at the same time they reveal a suppressive effect of adiponectin signalling through T-cadherin on ERK1/2 activation. Thus, it is likely that not only the adiponectin isoforms determine a metabolic or immunologic response, but also the type of adiponectin receptor as well.

1.1.4 Other adipokines

Since the discovery of leptin and adiponectin, the number of newly identified adipokines is continuously growing. Novel adipokines often are proteins which were previously recognized as products from other cells and tissues. For example visfatin, chemerin or apelin were first identified as products from lymphocytes, the skin or the stomach, respectively (Samal *et al.*, 1994; Nagpal *et al.*, 1997; Tatemoto *et al.*, 1998) and only later descibed as adipokines (Boucher *et al.*, 2005; Fukuhara *et al.*, 2005; Bozaoglu *et al.*, 2007). Other adipokines, such as resistin or omentin, were directly detected as new proteins, secreted by the adipose tissue (Steppan *et al.*, 2001; Yang *et al.*, 2006). Adipokines such as the cytokines and chemokines or steroid hormones generally are well characterized in their metabolic and immunological functions (Landers and Spelsberg, 1991; Charo and Ransohoff, 2006; Dinarello, 2007). However, to date little information is available on most of the newly identified adipokines and their role in immunological immune responses often remains unclear. Apart from leptin

and adiponectin, visfatin and resistin are likely the best characterized amongst the novel adipokines.

1.1.4.1 Visfatin

Visfatin is a 52 kDa protein that was originally identified as a novel pre-B-cell colony-enhancing factor (PBEF) in a lymphocyte cDNA library (Samal *et al.*, 1994) and later recognized as an enzyme of the NAD^+ biosynthesis pathway (Rongvaux *et al.*, 2002) and as a secretory product from the adipose tissue (Fukuhara *et al.*, 2005). Although it can be secreted from adipocytes (Tanaka *et al.*, 2007), main sources for visfatin also include immune cells such as macrophages, neutrophils and lymphocytes, the bone marrow, liver and muscles (Samal *et al.*, 1994; Jia *et al.*, 2004; Curat *et al.*, 2006). Visfatin in circulation has a positive correlation with the BMI and the concentration range found in healthy individuals is 14-15 ng/ml, with no apparent sexual dimorphism (Berndt *et al.*, 2005). The few studies that have investigated regulation of visfatin expression suggest that visfatin is suppressed by IL-6, TNF-α and insulin (Kralisch *et al.*, 2005; Haider *et al.*, 2006; MacLaren *et al.*, 2007) and increased by glucose (Haider *et al.*, 2006).

The regulation of visfatin by insulin and glucose is of particular interest since in the original discovery of visfatin as an adipokine, Fukuhara *et al.* (2005) describe it as an insulin-mimetic, with potential theraupeutic properties as an anti-diabetic agent. However, after contradictory results from other groups (Berndt *et al.*, 2005; Zhang *et al.*, 2006; Oki *et al.*, 2007), serious doubt was cast on the findings from Fukuhara *et al.* and the study was retracted (Fukuhara *et al.*, 2007). In the meantime, visfatin was recognized as a regulator of insulin secretion from pancreatic β-cells in its role as an enzyme in the NAD^+ biosynthesis pathway and not as an insulin-mimetic (Revollo *et*

al., 2007). Furthermore, visfatin is implicated with the pathology of some inflammatory diseases such as rheumatoid arthritis or inflammatory bowel disease (Otero *et al.*, 2006; Moschen *et al.*, 2007) and a positive correlation of serum visfatin concentrations with inflammatory markers was detected (Oki *et al.*, 2007). *In vitro* studies revealed several immune-regulatory properties for this adipokine. Visfatin induces pro-inflammatory cytokines such as TNF-α, IL-6 or IL-1β in monocytes and fibroblasts (Brentano *et al.*, 2007; Moschen *et al.*, 2007) and has anti-apoptotic effects in macrophages and neutrophils (Jia *et al.*, 2004; Li *et al.*, 2008). In addition, visfatin works as a chemoattractant in monocytes and B cells (Moschen *et al.*, 2007). Thus, in a number of ways the pro-inflammatory functions of visfatin appear to be closely related to leptin and potentially the two adipokines act synergistically in the recruitment of immune cells to adipose tissue or in the induction of pro-inflammatory cytokines. However, a number of possible immunological functions of visfatin such as its effect on immune cell proliferation or its role in conjunction with LPS still need to be investigated.

1.1.4.2 Resistin

Resistin was named after the discovery of its insulin resistance inducing properties in mice (Steppan *et al.*, 2001). It is exclusively secreted from adipocytes in mice and was thought to provide the link between obesity and diabetes (Steppan *et al.*, 2001). Accordingly, resistin shows a strong positive correlation to BMI and impairs insulin-induced glucose uptake (Steppan *et al.*, 2001). However, despite the promising findings in mice, it soon became clear that the situation in humans is different. In contrast to mice, in humans resistin is almost exclusively secreted from the stromal-vascular fraction of the adipose tissue (Curat *et al.*, 2006) and not from adipocytes (Fain *et al.*, 2003). Furthermore, circulating resistin concentrations (5-6 ng/ml) show no correlation

to insulin sensitivity in humans and only a weak correlation with BMI (Utzschneider *et al.*, 2005). Thus, a number of studies indicate that in humans, higher levels of circulating resistin concentrations are not associated with diabetes (Yaturu *et al.*, 2006; Dominguez Coello *et al.*, 2008; Heidemann *et al.*, 2008; Chen *et al.*, 2009).

Nonetheless, a role for resistin in human diabetes is still discussed as an attributer to the overall inflammatory diabetic state and thereby indirectly to insulin resistance. Although resistin shows no direct correlations to BMI or insulin sensitivity, it is positively correlated to pro-inflammatory cytokines such as TNF-α or IL-6 (Hui-Bing *et al.*, 2006; Yaturu *et al.*, 2006). Furthermore, *in vitro* studies reveal potent pro-inflammatory properties for resistin. Thus, resistin induces TNF-α and IL-6 expression at both the mRNA and protein level in human PBMCs and macrophages through activation of NF-κB (Bokarewa *et al.*, 2005; Silswal *et al.*, 2005). Interestingly, pro-inflammatory cytokines and also LPS increase the resistin expression, potentially inducing a positive feedback-loop and further exaggerating any inflammatory conditions (Kaser *et al.*, 2003; Anderson *et al.*, 2007).

1.2 Myeloid cells

1.2.1 Myeloid cell differentiation

The development of lymphoid and myeloid lineages from haematopoietic stem cells (HSC) in the bone marrow is a complex process and still an intense area of ongoing research. Knowledge on immune cell origin and differentiation is vital for the design of new therapeutics to treat leukeamic disorders and for a better understanding of the function of different immune cell subsets during inflammation (Gordon and Taylor, 2005). Although heterogeneity of lymphoid and myeloid cells has been recognized for

some time (Wiktor-Jedrzejczak and Gordon, 1996; Georgopoulos, 1997), distinct progenitors from HSC for lymphoid and myeloid lineages were only discovered recently. Thus, a common lymphoid progenitor (CLP) gives directly rise to T, B and NK cells (Kondo *et al.*, 1997), whereas cells of the myeloid lineage first pass through a common myeloid progenitor (CMP) which itself gives rise to a granulocyte/macrophage progenitor (GMP) and a megakaryocyte/erythrocyte progenitor (MEP) (Akashi *et al.*, 2000). The GMP again gives rise to the myeloblast, from which polymorphonuclear leukocytes (PMN) originate, and to the macrophage/dendritic cell progenitor (MDP) (Fogg *et al.*, 2006). The MDP is accountable for the direct development of monocytes and so-called "resident" macrophages and dendritic cells. These resident macrophages and DCs are part of a normal body homeostasis and include cells such as Langerhans cells, microglia and dendritic cells of spleen and lymph nodes (also known as conventional DCs) (Auffray *et al.*, 2009). In contrast, so-called "inflammatory" macrophages and dendritic cells differentiate from circulating blood monocytes in an inflammatory environment in response to inflammatory stimuli (Auffray *et al.*, 2009). In addition, circulating blood monocytes were also shown to differentiate into osteoclasts (Udagawa *et al.*, 1990; Matsuzaki *et al.*, 1998). Furthermore, the GMP is thought to give rise to a common dendritic cell progenitor (CDP) which exclusively generates resident dendritic cells (Onai *et al.*, 2007). However, Auffray *et al.* (2009) argue that CDP and MDP are likely the same cells. Cell lineages and subsets are characterized through expression of cell surface markers. For example, the myeloblast and the MDP are distinguished through absence or presence, respectively, of the chemokine receptor CX_3CR1 (Fogg *et al.*, 2006). Thus, Auffray *et al.* (2009) note that both CDP and MDP share the same phenotype in cell surface receptor expression and suggest that differential cell culture conditions provide an explanation for conflicting

results. However, both groups acknowledge the need for more research to clarify findings.

In the regulation of haematopoiesis in general and in myeloid cell differentiation in particular, an essential role for the transcription factor PU.1 (Spi-1) was discovered. PU.1 is a member of the Ets family, a group of transcription factors which are involved in cell maturation, growth and differentiation (Oikawa and Yamada, 2003). Thus, PU.1 knock-out mice have defects in both lymphoid and myeloid lineages and do not produce lymphoid or myeloid progenitors (Scott *et al.*, 1994; Iwasaki *et al.*, 2005). In addition, when PU.1 expression is enforced in HSC through transfections with a PU.1 vector, these cells only develop into the myeloid lineage (Nerlov and Graf, 1998). Furthermore, PU.1 expression is upregulated in macrophage differentiation (Shibata *et al.*, 2001; Bonfield *et al.*, 2003) and important for the expression of typical macrophage cell surface receptors such as CD11b or the granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF) receptors which are associated with phagocytosis and cell growth (Pahl *et al.*, 1993; Zhang *et al.*, 1994a; Hohaus *et al.*, 1995; Baek *et al.*, 2009). Another important regulator of haematopoiesis and myeloid cell differentiation is the adipokine leptin. Thus, Claycombe *et al.* (2008) report 70% less absolute numbers of B cells, 40 % less absolute numbers of granulocytes and 25 % absolute numbers of monocytes in *ob*/*ob* mice compared to wild-type controls. In addition, they also observed a near normalisation of cell numbers after treatment of the *ob*/*ob* mice with leptin (Claycombe *et al.*, 2008). The mechanisms of regulatory functions of leptin in haematopiesis are not identified yet, however possibly its anti-apoptotic effects play a role in sustaining lymphoid and myeloid progenitor cell populations (Lam *et al.*, 2006; Claycombe *et al.*, 2008).

1.2.2 Monocyte subsets and immunological functions

Just as lymphocytes are comprised of different subsets, monocytes display considerable heterogeneity as well. Circulating blood monocytes account for approximately 10 % of all leukocytes. Of these, more than 80 % are known as "classic" monocytes due to features originally associated with monocytes (Gordon and Taylor, 2005). Thus, classic monocytes travel to the site of infection following a gradient of the chemokine MCP-1 (Matsushima *et al.*, 1989) where they differentiate into inflammatory macrophages and DCs. During inflammation, MCP-1 is secreted by several cell types, including endothelial cells (Rollins *et al.*, 1990), smooth muscle cells (Grimm *et al.*, 1996) or adipocytes (Christiansen *et al.*, 2005), but also by monocytes and macrophages themselves (Leonard *et al.*, 1993). Furthermore, upon stimulation with pathogen-associated molecular patterns (PAMPs), classic monocytes secrete several pro-inflammatory cytokines or chemokines, such as TNF-α, IL-β, IL-6 or IL-8 and MCP-1 (Bailly *et al.*, 1990; Dentener *et al.*, 1993; Leonard *et al.*, 1993). This induces the recruitment and activation of other immune cells such as granulocytes or T cells and production of prostaglandins and matrix metalloproteinases (MMPs) (Pugin *et al.*, 1999; Nakao *et al.*, 2002; Dinarello, 2007). Hence, classic monocytes are vital in mounting an efficient immune response against several different bacterial, fungal or protozoan pathogens (Serbina *et al.*, 2008).

Next to the classic monocytes, a number of smaller subpopulations was identified which each account for less than 10 % of the total number of monocytes during homeostasis (Grage-Griebenow *et al.*, 2001). The different monocyte populations are characterized through differential expression of cell surface markers but also through distinct roles in homeostatic or inflammatory conditions. Thus, classic monocytes express high levels of

CD14 but are negative for CD16 (FcγRIII). In contrast, another well recognized monocyte subset is positive for CD16 yet displays low CD14 expression (Grage-Griebenow *et al.*, 2001). In addition, $CD14^{low}CD16^{+}$ monocytes show increased TLR2 expression and TNF-α production in response to LPS when compared to $CD14^{+}CD16^{-}$ monocytes (Belge *et al.*, 2002). Circulating cell numbers of $CD14^{low}CD16^{+}$ monocytes increase during sepsis and acute or chronic infections such as rheumatoid arthritis, AIDS or asthma (Fingerle *et al.*, 1993; Rivier *et al.*, 1995; Thieblemont *et al.*, 1995; Nockher and Scherberich, 1998; Iwahashi *et al.*, 2004). Thus, this monocyte subset is likely to be a major contributer of TNF-α during inflammation and potentially plays an important role in the pathogenesis of several inflammatory diseases (Belge *et al.*, 2002). However, although $CD14^{low}CD16^{+}$ monocytes show an inflammatory phenotype during disease, in contrast to $CD14^{+}CD16^{-}$ monocytes this subset has the potential to differentiate into conventional DCs during normal body homeostasis (Geissmann *et al.*, 2003). Interestingly, Sunderkötter *et al.* (2004) demonstrate that $CD14^{low}CD16^{+}$ monocytes are more mature since they actually develop from the $CD14^{+}CD16^{-}$ subset which possibly explains their ability to differentiate into DCs even under non-inflammatory conditions.

Proliferation studies further support the finding on monocyte maturation. Mature monocytes are generally believed to have less proliferating potential (van Furth *et al.*, 1979) and the $CD14^{low}CD16^{+}$ subset exhibits reduced proliferating capacity in comparison to $CD14^{+}CD16^{-}$ monocytes (Clanchy *et al.*, 2006). In addition, in the same study the authors also identify a novel monocyte subset which features high CD14 expression while still having proliferating capacity and is thus believed to be less mature (Clanchy *et al.*, 2006). This proliferating monocyte subset is thought to

differentiate into macrophages and contribute to a rapid cell accumulation at an inflammatory site (Clanchy *et al.*, 2006), however its true functions yet have to be investigated. Apart from direct inflammatory responses to PAMPs and acting as precursors for macrophages and DCs during inflammation, monocytes constantly survey their environment during homeostasis. In this context, a unique monocyte subset with "patrolling" behaviour was identified recently in mice (Auffray *et al.*, 2007). These monocytes crawl along the endothelial wall, often turning in loops and travelling against the blood flow but only covering short distances and rarely leaving the vessel (Auffray *et al.*, 2007). However, upon infection, the "patrolling" monocytes rapidly exit the endothelial vessels and migrate to the site of infection. Here, they provide the very first immune response to pathogens, even before PMN or classic monocytes start to arrive (Auffray *et al.*, 2007). In summary, the multitude of monocyte functions and heterogeneity emphasizes the important role of this cell type in the immune system. Any manipulations of monocyte differentiation or maturation through adipokines such as leptin or adiponectin potentially changes immune responses to PAMPs and thus may alter the pathogenesis of inflammatory diseases.

1.2.3 Toll-like receptors and LPS signalling

To date, 12 TLRs have been identified in mammals (Akira *et al.*, 2006). These TLRs recognise and respond to a wide range of PAMPs such as viral RNA or DNA, lipoteichoic acid (LTA), peptidoglycans or flagellin (reviewed in Akira and Takeda, 2004). In addition, it was observed that TLR4 knock-out mice are resistant to any biological effects of LPS (Poltorak *et al.*, 1998; Takeuchi *et al.*, 1999) and hence TLR4 was recognized as the main receptor for LPS (Du *et al.*, 1999). LPS is a component of the outer membrane of the Gram negative bacterial cell wall, composed of a

polysaccharide (O-region), followed by a core oligosaccharide and finally a hydrophobic domain (Lipid A) (Rietschel *et al.*, 1994; Miller *et al.*, 2005). The Lipid A part of LPS is highly efficient in stimulating immune responses through recognition by TLRs and induces gene expression and secretion of pro-inflammatory cytokines and chemokines (Miller *et al.*, 2005). TLRs are not only expressed in leukocytes (Muzio *et al.*, 2000), but also in non-immune cells such as fibroblasts, epithelial cells, keratinocytes, endothelial cells (Miller and Modlin, 2007) or adipocytes (Kopp *et al.*, 2009). Thus, a whole variety of cell types can recognize PAMPs and trigger an immune response, even if leukocytes are not present at the first point of contact with pathogens.

TLRs belong to the family of type I transmembrane receptors which also include the IL-1 receptors (Medzhitov, 2001). They exist as dimers (Ozinsky *et al.*, 2000) and are composed of a leucine rich repeat and an evolutionary conserved TIR domain (for Toll/IL-1 receptor), which is responsible for signal transduction to adapter proteins (Medzhitov, 2001). The TIR domain is also a part of the main adapter proteins for TLR signalling: MyD88, MAL, TRIF and TRAM (O'Neill and Bowie, 2007). Although TLR4 is the main receptor for LPS, this TLR alone is not sufficient for LPS induced immune responses and several other proteins are needed to induce LPS signalling. In plasma, LPS first forms a complex with the LPS-binding protein (LBP) (Schumann *et al.*, 1990). Next, this complex is bound by either soluble or membrane bound CD14 (Wright *et al.*, 1990). MD-2 then associates with TLR4 to recognize the CD14/LPS complex (Shimazu *et al.*, 1999). The actual TLR4 signalling relayed through the adaptor proteins can then take two routes, described as the MyD88 dependent and MyD88 independent pathway. These two pathways correspond to an early or delayed response to LPS as defined by a MyD88 knock-out model (Akira *et al.*, 2000). In this study, LPS

stimulation of bone marrow derived DCs from MyD88 knock-out mice induced DC maturation as confirmed by upregulation of several cell surface markers and enhanced capacity to stimulate T cells, although the response was delayed compared to wild-type mice. In contrast, TLR4 deficient mice showed no response to LPS, confirming the Myd88 independent pathway (Akira *et al.*, 2000). In the MyD88 dependent pathway, MAL is the next adaptor protein in TLR4 signalling (Fitzgerald *et al.*, 2001; Horng *et al.*, 2002). The MyD88 independent pathway utilizes TRIF (Yamamoto *et al.*, 2003a) and TRAM (Yamamoto *et al.*, 2003b) as adaptor proteins for TLR4 signalling. The adaptor proteins of either pathway finally activate TRAF6 (Cao *et al.*, 1996; Sato *et al.*, 2003) which induces a phosphorylation cascade that results in the subsequent activation of pro-inflammatory signalling pathways such as NF-κB or MAPK.

In contrast to TLR4, a whole range of structurally diverse ligands such as lipoproteins, LTA, LPS, peptidoglycans or zymosan have been reported for TLR2 (Zahringer *et al.*, 2008). Furthermore, TLR2 does not appear as a homodimer like TLR4, but associates with TLR1 or TLR6 to form a heterodimer (Ozinsky *et al.*, 2000). The high diversity of different TLR2 ligands is possibly explained through the heterodimerization. A study in HEK cells transfected with different TLR2 combinations shows that depending on the combination (either TLR1/2 or TLR6/2), a different PAMP is recognized (Triantafilou *et al.*, 2006). The TLR2 signalling pathway is similar to TLR4 signalling, although strictly MyD88 dependent (O'Neill and Bowie, 2007). Additionally, both CD14 and MD-2 seem to be required accessory proteins for TLR2 signalling (Dziarski *et al.*, 2001; Manukyan *et al.*, 2005; Nilsen *et al.*, 2008). Thus, stimulation of TLR2 and CD14 transfected HEK cells with highly purified Lipid A from several LPS sources did not

induce a response in an NF-κB reporter system. However, when cells were additionally transfected with MD-2, a strong NF-κB activation was observed (Dziarski *et al.*, 2001).

While the Lipid A region of *Escherichia coli* (*E. coli)* LPS is generally perceived as a conserved structure (Berezow *et al.*, 2009), Lipid A from the periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) is highly diverse and structurally different from *E. coli* Lipid A (Ogawa, 1993; Kumada *et al.*, 1995). Several studies have demonstrated that, in contrast to the TLR4 ligand *E. coli* LPS, LPS from *P. gingivalis* can signal via TLR2 (Zhou *et al.*, 2005; Hajishengallis *et al.*, 2006; Triantafilou *et al.*, 2007; Zhou and Amar, 2007), TLR4 (Chen *et al.*, 2006; Reife *et al.*, 2006) or even both TLRs (Darveau *et al.*, 2004). Additionally, apart from employing both TLRs, *P. gingivalis* LPS was described as an antagonist for LPS signalling through TLR4 (Ogawa *et al.*, 1994; Yoshimura *et al.*, 2002; Chen *et al.*, 2006; Coats *et al.*, 2007; Triantafilou *et al.*, 2007). A hypothesis for different LPS signalling suggests the actual shape of the LPS as a possible determining factor for the signalling pathway. A more conical LPS shape, like the one from *E. coli* induces TLR4, while the more cylindrical LPS shape from *P. gingivalis* induces TLR2 signalling (Netea *et al.*, 2002). However, some controversy whether or not *P. gingivalis* LPS signals via TLR2 or TLR4 still exists. In a study of commercially available LPS preparations known for TLR2 signalling, no TLR2 response was induced after repurification of the LPS preparation (Hirschfeld *et al.*, 2000). Instead, another study isolated a new component that is purified together with Lipid A as the actual TLR2 ligand (Hashimoto *et al.*, 2004). Therefore, the authors of these reports argue that rather than LPS itself, some contaminants in the LPS preparations are responsible for TLR2 signalling.

Nonetheless, *P. gingivalis* LPS signalling through TLR2 is potentially biologically relevant and could affect periodontal disease pathogenesis. Thus, a number of reports reveal differential immune responses to TLR2 or TLR4 ligands. For example, in contrast to the TLR4 ligand *E. coli* LPS, the TLR2 ligand *P. gingivalis* LPS was found to be only a weak inducer of the pro-inflammatory cytokines and chemokines IL-1β, IFN-γ, MCP-5 and CXCL5 (Hirschfeld *et al.*, 2001; Barksby *et al.*, 2009). It was also demonstrated that *P. gingivalis* LPS primes dendritic cells for inducing a T_h-2 response, whereas *E. coli* LPS favors a T_h-1 response (Jotwani *et al.*, 2003). Together with the antagonistic actions of *P. gingivalis* on TLR4 signalling from other pathogens, this TLR2-induced differential cytokine and chemokine expression pattern could play a role in the strategy of this periodontal pathogen to evade immune responses and thus contribute to disease pathogenesis (Yoshimura *et al.*, 2002; Bostanci *et al.*, 2007). In addition, differential TLR signalling might also impact on the potential of leptin or adiponectin to modulate LPS-induced immune responses. However, so far no studies have investigated the role of these adipokines in TLR2 signalling.

1.2.3.1 Regulation of TLR expression

Apart from different TLR signalling, the proportions of TLR expression potentially modulate host immune responses to pathogens and hence impact on inflammatory disease pathogenesis. Thus, a number of studies report significant changes of TLR2 and TLR4 expression under certain conditions and with various stimuli. For example, increased TLR2 or TLR4 expression was observed at both mRNA and protein level in human sepsis and tuberculosis and is thought to be associated with disease pathogenesis (Marsik *et al.*, 2003; Armstrong *et al.*, 2004; Prabha *et al.*, 2008; Schaaf *et al.*, 2009). Additonally, *in vitro* studies demonstrate an upregulation of TLR expression by IL-1-α,

IL-10, GM-CSF or by LPS in myeloid cells (Muzio *et al.*, 2000; Flo *et al.*, 2001; Kurt-Jones *et al.*, 2002; Marsik *et al.*, 2003). The results are not always uniform and some stimuli induce a downregulation and not an increase of TLR expression. Thus, Flo *et al.* (2001) show a decrease in TLR2 cell surface expression on primary human monocytes in response to IFN-γ or TNF-α. This result is somewhat conflicting in comparison to the enhancing effect of LPS on TLR2 expression since the pro-inflammatory cytokines IFN-γ and TNF-α are generally induced by LPS. However, LPS activates a whole spectrum of cytokines and chemokines and eventually influences TLR expression on a more complex level than single cytokines, yet detailed reports on the regulation of TLR expression are limited and several mechanisms incompletely understood.

Changes in TLR expression are regulated at both protein and mRNA level and a recent study also indicates post-transcriptional regulation through microRNAs (Chen *et al.*, 2007). The regulation of TLR cell surface expression involves complicated mechanisms of TLR translocation from cytoplasm to the cell surface and movement of TLRs within the cell membrane. Most information on the process of TLR trafficking is only just emerging. Thus, the presence of MD-2 was found to be essential for TLR4 glycosylation and its subsequent translocation to the cell surface (Nagai *et al.*, 2002; Ohnishi *et al.*, 2003). Also, the two recently discovered proteins PRAT4A and PRAT4B are required (Konno *et al.*, 2006; Takahashi *et al.*, 2007). In addition, a lateral diffusion mechanism within the cell membrane was demonstrated for both TLR4 and TLR2 upon LPS stimulation (Triantafilou *et al.*, 2004). Furthermore, the whole TLR4/LPS complex is endocytosed after stimulation and transported through the ESCRT machinery (endosomal sorting complex required for transportation, reviewed in Williams and

Urbe, 2007) to endosomes for degradation and subsequent human leukocyte antigen (HLA) class II mediated antigen presentation (Husebye *et al.*, 2006).

At the mRNA level, the transcription factors PU.1 and Sp1 play an important role in regulation of TLR expression. Reporter assays and siRNA studies reveal a direct role for PU.1 in regulation of the TLR4 promoter activity and TLR4 mRNA expression. Thus, PU.1 binds to a consensus-binding site of the Ets family of transcription factors in the proximal human TLR4 promoter region (Rehli *et al.*, 2000) and LPS treatment can induce the association of PU.1 with the TLR4 promoter (Pedchenko *et al.*, 2005). Additionally, epigenetic suppression of PU.1 results in reduced TLR4 mRNA expression (Joo *et al.*, 2008). Putative evidence also indicates a PU.1 binding site in the human TLR2 promoter region (Haehnel *et al.*, 2002; Lin and Rikihisa, 2004). Some evidence suggests that PU.1 auto regulates its own expression through an upstream regulatory promoter element (Chen *et al.*, 1995; Okuno *et al.*, 2005). Furthermore, GM-CSF is thought to play an important role in PU.1 expression. Hence, a mouse GM-CSF knock-out model and patients with GM-CSF deficiency display decreased PU.1 expression, which is normalised again after restoring GM-CSF (Shibata *et al.*, 2001; Bonfield *et al.*, 2003). Fittingly, the GM-CSF knock-out model also exhibited decreased TLR2 mRNA expression which was normalised after restoring GM-CSF expression (Shibata *et al.*, 2001), implicating a fundamental role of GM-CSF in regulation of TLR expression through PU.1. The transcription factor PU.1 has several phosphorylation sites and a main mechanism for regulation of PU.1 transcriptional activity is the alteration of its phosphorylation state. Depending on the cell type and function studied, phosphorylation of different sites is essential for increased PU.1 activity. For example, Pongubala *et al.* (1993) revealed that PU.1 phosphorylation at Ser 148 was needed for

recruitment of a B-cell transcription factor whereas Celada *et al.* (1996) demonstrated a critical role for PU.1 phosphorylation at Ser 41 and Ser 45 in macrophage proliferation. Furthermore, a transfection study in a mouse tumour cell line suggests the PI3K/Akt pathway has a role in induction of the PU.1 Ser 41 phosphorylation (Rieske and Pongubala, 2001) and Lodie *et al.* (1997) report PU.1 Ser 148 phosphorylation after LPS stimulation in murine macrophages. In addition, a protein which inhibits PU.1 transcriptional activity has been discovered (Hirose *et al.*, 2003).

An important transcription factor in TLR2 mRNA expression is Sp1. The TLR2 promoter has a Sp1 binding site (Haehnel *et al.*, 2002) and regulation of transcriptional activity of Sp1 through phosphorylation has been extensively studied (reviewed in Chu and Ferro, 2005). In contrast to TLR2, the TLR4 promoter lacks the Sp1 binding site (Rehli *et al.*, 2000), suggesting a differential regulation of the two TLRs at the mRNA level. It is interesting to note a considerable species difference between the human and murine TLR2 promoter. Although murine and human TLR4 promoter share approximately 70 % sequence homology, only approximately 10 % sequence homology were observed between human and murine TLR2 promoter (Haehnel *et al.*, 2002). This suggests that the TLR2 promoter region was not conserved during evolution and that regulation of TLR2 expression may vary in different species. Finally, it should be mentioned that the adipokine leptin appears to have some regulatory function on several TLR mRNA expression patterns, although this was demonstrated for adipocytes only (Batra *et al.*, 2007). For example, in *ob*/*ob* and *db*/*db* mice, Batra *et al.* (2007) observed increased TLR mRNA expression in comparison to the wild type controls. In addition, a study in murine hepatic stellate cells demonstrates leptin-induced phosphorylation of Sp1 (Lin *et al.*, 2006), suggesting a possible mechanism for leptin in the regulation of

TLR2 expression. However, no investigations on leptin and regulation of TLR expression in myeloid cells were conducted yet and it is unknown if this adipokine is involved.

1.3 Periodontal disease and diabetes

1.3.1 The role of the immune system in the pathogenesis of diabetes

The International Diabetes Federation (IDF) estimates that diabetes affects more than 246 million people worldwide in 2007 and a worldwide increase to 380 million people is projected by 2025 (Gan, 2006). Diabetes (diabetes mellitus) is characterized by a state of chronic hyperglycaemia due to insufficient insulin production or insulin resistance (Alberti and Zimmet, 1998). Two major types of diabetes with distinct pathogenesis can be distinguished. Type 1 diabetes mellitus (T1DM) has a strong genetic background associated with HLA haplotypes and accounts for 5 to 10 % of all diabetes mellitus cases (Daneman, 2006). In T1DM the insulin producing pancreatic β-cells are destroyed in a T cell mediated autoimmune attack (Daneman, 2006). In T2DM, the pancreatic β-cells still produce insulin but their number is reduced and insulin production is insufficient to overcome insulin resistance (Kahn *et al.*, 2006). Insufficient insulin or the inability of cells to react to insulin then leads to a state of chronic hyperglycaemia and results in long term complications such as neuropathy, nephropathy, retinopathy, other micro-and macrovascular diseases (Southerland *et al.*, 2005).

Furthermore, periodontal disease is another complication of diabetes (Southerland *et al.*, 2005). The leading cause for developing insulin resistance is obesity (Kahn *et al.*, 2006). Obesity is accompanied by higher levels of free fatty acids and inflammatory mediators which can inhibit insulin signalling pathways and impair cellular glucose

uptake (Guilherme *et al.*, 2008). Particularly, the observed increase of inflammatory mediators led to the notion that obesity, insulin resistance and T2DM are now recognised as inflammatory diseases (Duncan *et al.*, 2003; Dandona *et al.*, 2004). Thus, several studies report increased concentrations of C-reactive protein (CRP), MCP-1, TNF-α and IL-6 during obesity and diabetes (Pickup *et al.*, 2000; Freeman *et al.*, 2002; Han *et al.*, 2002) and increased infiltration of macrophages into adipose tissue was observed (Weisberg *et al.*, 2003; Kamei *et al.*, 2006; Lumeng *et al.*, 2007). Additionally, a study in TNF-α knock-out mice discovered that these mice are protected from insulin resistance in diet-induced obesity (Uysal *et al.*, 1997). Furthermore, polymorphisms in the promoter regions of TNF-α and IL-6 have been proposed as possible contributors to insulin sensitivity or resistance and to the risk of developing diabetes (Fernandez-Real *et al.*, 1997; Day *et al.*, 1998; Fernandez-Real *et al.*, 2000; Kubaszek *et al.*, 2003a; Kubaszek *et al.*, 2003b). However, a number of studies found no associations between cytokine polymorphisms and an increased risk for diabetes (Sheu *et al.*, 2001; Valenti *et al.*, 2002; Danielsson *et al.*, 2005) and recent meta-analyses suggest that rather than single nucleotide polymorphisms, a combination of several genes and other factors such as the increased level of free fatty acids in obesity are more likely to play a role (Qi *et al.*, 2006; Feng *et al.*, 2009).

In addition, the hyperglycaemic state itself contributes to increased inflammation and inflammatory products in diabetes. Chronic exposure to high glucose concentrations results in unspecific reactions of glucose with proteins, creating AGEs (Schmidt *et al.*, 1992). These altered proteins activate ERK and NF-κB signalling pathways (Yan *et al.*, 1994; Lander *et al.*, 1997; Kislinger *et al.*, 1999) through special receptors (RAGEs) on several cell types (Neeper *et al.*, 1992). For example, in endothelial cells, AGEs

signalling was shown to induce TNF-α secretion (Rashid *et al.*, 2004), and in macrophages AGEs improve adhesion properties and act as a chemoattractant (Schmidt *et al.*, 1993; Mamputu and Renier, 2004). Furthermore, blocking of RAGEs and thereby the inflammatory response to AGEs, resolved impaired wound healing in diabetic mice (Goova *et al.*, 2001) and decreased alveolar bone loss in human T1DM patients (Lalla *et al.*, 2000). One of the best well known AGEs in diabetes is glycated haemoglobin (HbA1c). HbA1c has an approximate half life of three months and is used as a measurement of long term monitoring glycaemic control (Nathan *et al.*, 2007). Increased pro-inflammatory mediators also appear to derive from dysregulation in TLR signalling and expression. Thus, TLR signalling can be activated by dietary fatty acids (Lee *et al.*, 2004; Shi *et al.*, 2006; Milanski *et al.*, 2009). In addition, T2DM patients have higher circulating LPS concentrations (Creely *et al.*, 2007) and increased TLR and CD14 expression on adipocytes and monocytes from diabetics has been observed (Patino *et al.*, 2000; Fogelstrand *et al.*, 2004; Creely *et al.*, 2007; Devaraj *et al.*, 2008). Interestingly, insulin infusions in T2DM down regulate TLR expression, possibly through suppressive effects on PU.1 activity (Ghanim *et al.*, 2008). The decreased TLR expression is accompanied by a reduction in inflammation and has direct effects on insulin resistance. It was observed that TLR4 knock-out mice have reduced levels of IL-6 and TNF-α and are protected from diet-induced obesity and insulin resistance (Poggi *et al.*, 2007; Tsukumo *et al.*, 2007). Furthermore, blocking of TLR2 signalling with antibodies inhibits fatty acid-induced IL-6 production and reduces insulin-resistance in muscle-cells (Senn, 2006). Hence, the dysregulation of TLRs in diabetes contributes to the overall inflammatory state and is likely to affect immune responses to PAMPs such as LPS.

1.3.1.1 The role of leptin and adiponectin in diabetes

Secretion of pro-inflammatory cytokines and chemokines by the adipose tissue further enhances the inflammatory diabetic state and contributes to insulin resistance. In addition, other adipokines have been shown to have a regulatory role in insulin metabolism and signalling. For example, increased baseline leptin concentrations and leptin or leptin receptor polymorphisms are associated with an increased risk of developing diabetes (McNeely *et al.*, 1999; Nannipieri *et al.*, 2006; Han *et al.*, 2008; Welsh *et al.*, 2009). Kieffer and Habener (2000) propose that leptin interacts with insulin in an "adipoinsular axis" and they introduce the concept that both hormones regulate each other in a bidirectional feedback loop with insulin increasing the production of leptin and leptin in turn reducing insulin secretion and gene expression. Furthermore, leptin and insulin signalling pathways share common mediators. Insulin signalling induces phosphorylation of IRS, a protein that regulates enzymes involved in the localization of glucose transporters to the cell surface (Stumvoll *et al.*, 2005). However, a number of studies also describe induction of the PI3K/Akt pathway through IRS by leptin (see 1.1.2.2), revealing a crosstalk between insulin and leptin signalling. In addition to its direct involvement in the regulation of insulin and glucose homeostasis, increased leptin concentrations in diabetes likely induce an overproduction of pro-inflammatory mediators and further contribute to inflammation and insulin resistance. In contrast to leptin, adiponectin concentrations are lower in T2DM and a negative correlation exists between adiponectin and insulin (Hotta *et al.*, 2000) and the increased risk of developing T2DM (Nakashima *et al.*, 2006; Snijder *et al.*, 2006; Mojiminiyi *et al.*, 2007; Heidemann *et al.*, 2008; Jalovaara *et al.*, 2008). Futhermore, several polymorphisms in the adiponectin gene are associated with T2DM (Menzaghi *et al.*, 2002; Hivert *et al.*, 2008; Vimaleswaran *et al.*, 2008) and results are highly

reproducible (Vasseur *et al.*, 2006). A regulatory relationship exists between adiponectin and insulin as has been observed with leptin. Thus, insulin induces adiponectin secretion from adipocytes and in turn, adiponectin enhances insulin sensitivity (Combs *et al.*, 2004). Although it is known that the positive effect of adiponectin on insulin sensitivity is achieved through enhanced lipid clearance and suppression of insulin-induced endogenous glucose production in the liver (Combs *et al.*, 2004), possibly anti-inflammatory effects of adiponectin contribute as well. However, no data are available to date and *in vitro* studies report conflicting results on pro-and anti-inflammatory properties of adiponectin (see 1.1.3.1).

1.3.2 The role of the immune system in the pathogenesis of periodontal disease

Innate immunity

Periodontal disease is an inflammation of the gingival and periodontal tissues induced by an overreaction of the immune system towards bacterial plaque. In gingivitis, the inflammation is reversible and does not reach supporting structures of the periodontium. In periodontitis, the continued inflammation irreversibly destroys connective tissue and bone and eventually results in tooth loss (Pihlstrom *et al.*, 2005). Dixon *et al.* (2004) introduced the concept of a "molecular dialogue" between bacteria and the host which leads to a healthy equilibrium. Disruption of this "established state" can lead to disease. Thus, Dixon *et al.* (2004) proposed that in health, a "wall" of neutrophils effectively defends the periodontium against oral bacteria and LPS or other inflammatory mediators released by bacteria. These neutrophils migrate out of the blood into the periodontal tissues along a gradient composed of chemoattractant IL-8 and cell adhesion molecules such as E-selectin and ICAM produced by the junctional epithelium and

endothelial cells in response to the bacterial challenge. Disruption of this gradient through oral bacteria (such as *P. gingivalis*), environmental factors or underlying inflammatory diseases results in a misdirection of neutrophil migration and a disruption of the protective "wall" (Dixon *et al.*, 2004). The bacterial load and levels of inflammatory mediators rise and increased IL-8 but also MCP-1 expression of the periodontal tissues (Tonetti *et al.*, 1994; Kusumoto *et al.*, 2004) leads to recruitment of more neutrophils and of monocytes and macrophages. The constant expression of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and MMPs finally induces a breakdown of connective tissue, pocket formation, activation of osteoclasts and bone destruction (Graves and Cochran, 2003; Graves, 2008).

An important element and indicator for inflammation in periodontal disease or health is gingival crevicular fluid (GCF). GCF is a fluid that originates from the bloodstream and emerges between the surface of the tooth and the junctional epithelium. It contains a mixture of several molecules such as cytokines, enzymes and antibodies, as well as leukocytes and bacteria (Delima and Van Dyke, 2003). It contains inflammatory mediators in periodontal disease which are reduced after successful treatment of the disease (Toker *et al.*, 2008). The periodontal pathogen *P. gingivalis* is one of the key bacteria associated with periodontal disease (Lamont and Jenkinson, 1998; Socransky *et al.*, 1998). *P. gingivalis* is almost absent in periodontal health, yet is found in increased numbers in periodontal disease where it plays an important role in inflammation and bone destruction through production of several virulence factors such as LPS, chemokines or proteinases (Holt *et al.*, 1999). Furthermore, *P. gingivalis* can also act as an antagonist (Coats *et al.*, 2007), thereby disrupting the normal host response to other bacteria.

Innate immune responses to bacteria and bacterial products clearly play a crucial role in periodontal disease. Periodontal tissues express a whole array of TLRs and the expression pattern appears to differ in periodontal disease compared to health (Asai *et al.*, 2001; Wang *et al.*, 2001; Mori *et al.*, 2003; Ren *et al.*, 2005; Uehara *et al.*, 2007). For example, Mori *et al.* (2003) describe increased TLR2 and TLR4 expression in gingival tissues in advanced stages of periodontal disease. Furthermore, a recent study reports upregulation of TLR4 expression on circulating and gingival tissue B cells in periodontal disease (Shin *et al.*, 2009). Additionally, in an experimental model of periodontitis, TLR4 knock-out mice exhibited less bone loss than wildtype mice after a bacterial periodontal challenge (Hou *et al.*, 2000). Finally, the composition of myeloid cell subsets in circulation appears to be altered in periodontal disease. For example, it was shown that PBMCs from periodontitis patients can spontaneously generate osteoclasts (Tjoa *et al.*, 2008) and that the percentage of $CD14^{low}CD16^{+}$ monocytes is increased (Nagasawa *et al.*, 2004). Interestingly, the circulating $CD14^{low}CD16^{+}$ monocyte subset exhibits increased TLR2 expression (Belge *et al.*, 2002). Together with its potent TNF-α production (Belge *et al.*, 2002), this could impact on immune responses to known TLR2 ligands such as *P. gingivalis* LPS. In addition, any alteration of monocyte immune responses through adipokines such as leptin or adiponectin may further potentiate inflammation and emphasizes the importance of investigating the capacity of adipokines in modulating TLR signalling and expression.

Adaptive immunity

While innate immunity is a crucial factor in the initiation of periodontal disease, it is now recognised that established periodontal disease is also linked to adaptive immune responses. In particular, shifts in T_h1/T_h2 cytokine profiles appear to play an important

role in the establishment of "stable" or "progressive" periodontal lesions (Seymour and Gemmell, 2001). A predominant T_h1 cytokine profile (IFN-γ, IL-12, TGF-β) activates cellular immunity, inducing macrophages, NK cells, neutrophils and cytotoxic T cells. A predominant T_h2 cytokine profile (IL-4, IL-10) activates humoral immune responses, mediated by B cell antibody production. It is thought that in a "stable" periodontal lesion, T_h1 cytokines such as IFN-γ and IL-12 increase phagocytosis and neutrophil activity which prevents excessive tissue destruction and contains the infection (Henderson *et al.*, 2009). In contrast, a shift towards a T_h2 cytokine profile with increased B cell activation is observed in "progressive" lesions (Berglundh and Donati, 2005; Kinane and Mark Bartold, 2007). Activated B cells produce higher concentrations of IL-1β and IL-6, which promote connective tissue and bone destruction (Henderson *et al.*, 2009). B cell production of autoantibodies against collagen (Rajapakse and Dolby, 2004) further enhance breakdown of periodontal tissues. Regulatory T cell subsets potentially play a role in facilitating the shift from a T_h1 to a T_h2 cytokine profile or, in other words from a "stable" to a "progressive" periodontal lesion. Regulatory T cells can be detected in inflammatory sites of gingivitis and periodontitis, however cell numbers increase in periodontal disease in proportion with B cell numbers (Nakajima *et al.*, 2005). Importantly, regulatory T cells produce TGF-β (Andersson *et al.*, 2008), a key cytokine in driving T_h17 differentiation (Bettelli *et al.*, 2006). IL-17, the main secretory product of T_h17 cells, has regulatory functions in bone homeostasis (Yu *et al.*, 2007) and is elevated in periodontal disease (Vernal *et al.*, 2005; Lester *et al.*, 2007; Ohyama *et al.*, 2009). IL-17 acts synergistically with TNF-α and IL-1β (Katz *et al.*, 2001) and also induces these and other pro-inflammatory cytokines in myeloid cells (Beklen *et al.*, 2007), thereby linking adaptive immune responses with innate immunity.

Periodontal disease should therefore be considered as a complex multi-factorial inflammatory process and more research is needed to identify regulatory mechanisms.

1.3.2.1 The role of leptin and adiponectin in periodontal disease

Although both leptin and adiponectin potentially play a vital role in periodontal disease via their immune-regulatory properties or their known activity in the regulation of bone metabolism, few studies have investigated these adipokines in the context of periodontal disease. In the periodontium, leptin was reported to have a "protective" effect since leptin concentrations in GCF and gingiva were higher in healthy sites and declined with increased tissue destruction (Johnson and Serio, 2001; Bozkurt *et al.*, 2006; Karthikeyan and Pradeep, 2007). The effect of adiponectin in the periodontium is unknown and no published data are available regarding GCF adiponectin concentrations. In contrast to GCF, serum leptin concentrations were positively correlated with the extent of periodontal tissue destruction (Karthikeyan and Pradeep, 2007) whereas serum adiponectin concentrations do not appear to be altered in periodontal disease (Furugen *et al.*, 2008; Saito *et al.*, 2008). Reports on leptin or adiponectin concentrations in other inflammatory diseases with an inflammatory pathogenesis vary. For example, in rheumatoid arthritis, studies show increased serum leptin and adiponectin concentrations with increased severity of disease (Senolt *et al.*, 2006; Targonska-Stepniak *et al.*, 2008). Yet another study detects no significant differences in serum leptin and rheumatoid arthritis disease activity (Wislowska *et al.*, 2007) and a negative correlation between CRP and adiponectin concentration in acute coronary syndrome was described (Otake *et al.*, 2008). Together, these reports suggest that, at least in some conditions, leptin or adiponectin play a role in inflammatory disease pathogenesis. However, no studies are available to date on the effect of these adipokines in

modulating immune responses to periodontal pathogens and thus any mechanisms and potential therapeutic targets for periodontal disease treatment remain to be elucidated.

1.3.2.2 Diabetes as a risk factor for periodontal disease

Several risk factors such as smoking, poor oral health care or medication for underlying systemic diseases have been established for periodontal disease (Pihlstrom *et al.*, 2005). Furthermore, genetic predispositions possibly play a role as well, although the precise genetic elements have not yet been defined (Taylor *et al.*, 2004; Kinane *et al.*, 2005; Yoshie *et al.*, 2007; Nikolopoulos *et al.*, 2008). In addition, diabetes is now widely recognised as a major risk factor for periodontal disease (King, 2008). A number of studies describe an increased risk for severe periodontal disease with increased HbA1c (Tsai *et al.*, 2002; Campus *et al.*, 2005; Karima *et al.*, 2005; Jansson *et al.*, 2006) and a positive correlation between HbA1c and GCF IL-1β concentration was detected (Engebretson *et al.*, 2004). Also, both IL-6 and IL-1β concentrations in periodontal tissues are increased in periodontal disease in diabetics in comparison to periodontal disease in systemically healthy individuals (Duarte *et al.*, 2007). Although a meta-analyis indicates the same extent of periodontal disease between diabetics and systemically healthy individuals (Khader *et al.*, 2006), the authors still reach the conclusion that diabetics have worse oral hygiene and an increased risk for more severe periodontal disease than non-diabetics (Khader *et al.*, 2006). Thus, it appears that somehow the inflammatory oral environment is altered in diabetes which likely affects immune responses to periodontal pathogens and consequently periodontal health.

However, the connection for an increased risk of severe periodontal disease in diabetes is still not completely understood. For example, apparently no differences in the oral microflora between diabetics and non-diabetics with periodontal disease are observed

(Yuan *et al.*, 2001; Lalla *et al.*, 2006). Nonetheless, a number of other possible relationships potentially provide an explanation. Thus, due to the hyperglycaemic state, physiological mechanisms such as vascular repair and collagen metabolism are altered not only systemically but also in the gingival tissues (Brennan, 1989; Tepper *et al.*, 2002; Bhatwadekar *et al.*, 2008). This interferes with the transportation of nutrients and leukocyte migration, resulting in impaired tissue turnover and delayed wound healing in the periodontium (Goova *et al.*, 2001; Ryan *et al.*, 2003). In addition, Southerland *et al.* (2006) hypothesize that the combined challenge of LPS from periodontal pathogens such as *P. gingivalis* and the accumulation of glycated proteins potentially cause an exaggerated immune response. Higher circulating concentrations of inflammatory mediators are observed in diabetics (Pickup *et al.*, 2000; Freeman *et al.*, 2002; Han *et al.*, 2002) and indeed a positive correlation between serum TNF-α and severity of periodontal disease was observed (Engebretson *et al.*, 2007). Also, monocytes and neutrophils from diabetics generally appear to be in a higher activated state and more sensitive towards LPS stimulation (Salvi *et al.*, 1997; Karima *et al.*, 2005).

It is interesting to note that some studies actually report improvement of HbA1c, insulin resistance and serum TNF-α (Iwamoto *et al.*, 2001; Kiran *et al.*, 2005) in diabetics after treatment for periodontal disease, although a meta-analysis did not detect a significant change in HbA1c concentrations after periodontal treatment in diabetics (Janket *et al.*, 2005). Furthermore, in otherwise systemically healthy individuals, increased leukocyte counts and glucose intolerance were detected in severe periodontal disease (Saito *et al.*, 2004; Nibali *et al.*, 2007) and periodontal treatment decreased serum CRP and IL-6 concentrations (D'Aiuto *et al.*, 2004). Hence, the connection between diabetes and periodontal disease potentially is not a one-way but rather a bi-directional relationship

in which an improvement of the diabetic status would impact on periodontal health and vice versa. Also, the effect of periodontal treatment on inflammatory mediators in circulation emphasizes the importance of an immunological connection between the two diseases and the potential for any immune modulatory agents, such as the adipokines leptin and adiponectin, to have a relevant part in this relationship.

1.3.2.3 The role of leptin and adiponectin in periodontal disease and diabetes

The role of adipokines in periodontal disease as pro- or anti-inflammatory mediators is not well understood. As mentioned above, adipokine concentrations are altered during diabetes, making them an interesting target for a link between diabetes and severe periodontal disease, yet evidence so far is limited. An experimental model of periodontitis shows higher serum TNF-α concentrations and an earlier onset of insulin resistance in *fa/fa* rats with periodontal disease than in lean controls with periodontal disease (Watanabe *et al.*, 2008). A higher serum TNF-α concentration was also observed in diabetic *db/db* mice with a *P. gingivalis* skin wound infection (Naguib *et al.*, 2004). However, both these studies use the *fa/fa* rats or *db/db* mice for their known ability to develop obesity and diabetes and they fail to acknowledge a role for leptin in the immune response in their experimental setup. Thus, it is not possible to draw any conclusions for a role of leptin in a connection between diabetes and periodontal disease. The few reports on adiponectin in periodontal disease and diabetes vary. Matsumoto *et al.* (2009) describe increased serum adiponectin concentrations after periodontal disease treatment in T2DM yet a study by Iwamoto *et al.* (2003) finds no effect of treatment on this adipokine. However, the aim the study by Iwamoto *et al.* (2003) was to investigate inflammatory systemic conditions and periodontal disease as a risk factor for atherosclerosis and thus not all included patients had T2DM. This likely

affects the overall result of periodontal treatment on adiponectin concentrations. Finally, a study in T1DM patients reported higher adiponectin concentrations in diabetics with periodontal disease than in systemically healthy controls with periodontal disease (Lalla *et al.*, 2006). However, again the aim of the study was not to investigate a role of adiponectin in diabetes and adiponectin measurements were only taken as an indicator for insulin sensitivity. Nonetheless, together these reports suggest a potential role for leptin and adiponectin in the relationship between diabetes and periodontal disease. Yet clearly, the lack of studies with a true aim to investigate these two adipokines makes it difficult to draw any conclusions and thus further investigations are needed to clarify the role of leptin and adiponectin in periodontal disease and diabetes.

1.4 Aims

1. Investigation of the effect of leptin on human monocyte immune responses to *P. gingivalis* LPS.

2. To determine the effect of leptin on human monocyte TLR expression.

3. To evaluate a regulatory role of leptin in human monocyte TLR expression, cell maturation and differentiation.

4. To assess circulating and local levels of leptin and adiponectin in T2DM and periodontal disease before and after periodontal treatment in humans.

The following experiments were conducted to investigate the aims of the present study:

1. Stimulation of THP-1 and primary human monocytes with leptin and LPS from *E. coli* or *P. gingivalis* and measurement of TNF-α mRNA and protein expression (Chapter 3).

2. Stimulation of THP-1 and primary human monocytes with leptin or LPS and analysis of cell surface and mRNA expression of TLRs (Chapter 4).

3. Stimulation of THP-1 and primary human monocytes with leptin and analysis of PU.1 and GM-CSF protein and mRNA expression, PU.1 activation status and changes in THP-1 monocyte morphology (Chapter 5).

4. Determination of serum leptin and adiponectin and GCF adiponectin concentrations in T2DM patients with and without periodontal disease before and after treatment for periodontitis (Chapter 6).

Chapter 2 Materials and Methods

Chapter 2 Materials and Methods

2.1 Cell culture

Unless otherwise stated, all reagents and buffers were purchased from Sigma-Aldrich (Poole, UK), all plasticware from Greiner Bio One (Stonehouse, UK).

2.1.1 THP-1 monocytes

The human monocyte cell line THP-1 was originally derived from the blood of a one-year-old boy with acute monocytic leukaemia (Tsuchiya *et al.*, 1980) and is now commercially available. During culture, cells are non-adherent and in a pro-monocyte state. THP-1 monocytes are well recognised as a monocyte cell model in immunology research and display a near-diploid karyotype (Odero *et al.*, 2000). Importantly, THP-1 monocytes express the normal monocytic range of TLRs and adipokine receptors, making them a suitable cell model to address the aims of the present study.

2.1.1.1 Recovery of THP-1 monocytes

THP-1 monocytes were purchased from the European Collection of Cell Cultures (Salisbury, UK) as frozen vials. Cells were thawed quickly at 37 °C in a water bath and $2 – 4 \times 10^6$ cells were transferred to a 75 cm^2 tissue culture flask. Slowly, cell culture medium (RPMI-1640 medium, supplemented with FCS (10 % v/v), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) were added and cells were placed overnight in an incubator set to 37 °C, 5 % CO_2 (Sanyo, MCO-17/20 AIC). The next day, medium was replaced completely. Cells were centrifuged for 5 min at 2000 rpm, 20 °C in a Sigma 3K10 centrifuge (SLS, Nottingham, UK). Supernatant was

discarded and cells resuspended in fresh cell culture medium. Cell culture was continued as described in 2.1.1.2.

2.1.1.2 Cell culture of THP-1 monocytes

THP-1 monocytes were maintained in culture at a concentration of 3 – 8 x 10^5 cells/ml in cell culture medium at 37 °C, 5 % CO_2. Medium was changed three times a week and cells were passaged at regular intervals to assure optimum cell concentration. Cells were counted under a microscope using a haemocytometer (Bright-Line, improved Neubauer, Hausser Scientific, PA, USA) and cells were passaged at least one time after thawing before being used for an experiment. All experiments were conducted from cells in passages from 2-9.

2.1.1.3 Cell viability

Cell viability during routine culture was monitored with trypan blue exclusion. Trypan blue is a blue dye that will only pass through a damaged cell membrane, thus only staining dead cells (Freshney and Liss, 1987). 10 µl of cell suspension was diluted 1:1 (v/v) with trypan blue. The number of blue-stained (dead) cells and unstained (viable) cells was counted on a haemocytometer. The number of viable cells was taken as 100 % and the percentage of dead cells was subtracted. Cell viability was found to be > 99 % during routine cell culture.

2.1.1.4 Vitamin D_3 treatment of THP-1 monocytes

Unless otherwise stated, THP-1 monocytes (1 x 10^6 cells/ml) were treated with 0.1 µM Vitamin D_3 (1α, 25-Dihydroxy-Vitamin D_3, Calbiochem, Merck Chemicals, Nottingham, UK) for 48 h prior to use in stimulation experiments. Also, unless

otherwise stated, all experiments were conducted in duplicate cultures, on three independent occasions.

The treatment with Vitamin D_3 induces the cells to differentiate along the myeloid lineage to mature monocyte-like cells which resemble the natural phenotype of primary human monocytes (Kitchens *et al.*, 1992; Schwende *et al.*, 1996). The cells become adherent and also increase the expression of the monocyte marker CD14. The increase of CD14 expression was confirmed by flow cytometry (Figure 2.1), see section 2.4.

Before Vitamin D_3 treatment, THP-1 cells only show a slight increase in fluorescence intensity in comparison to an isotype control (Figure 2.1). However, a drastic increase in fluorescence intensity can be observed after Vitamin D_3 treatment, revealing a strong upregulation of CD14 expression on the cell surface of THP-1 cells. These data confirm results described in a recent study by Foster *et al.* (2005).

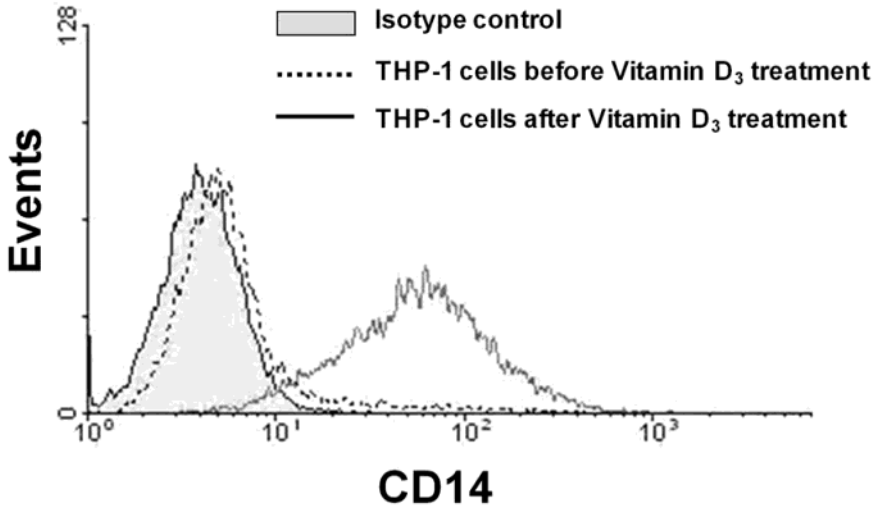

Figure 2.1 Effect of Vitamin D_3 treatment on CD14 expression of THP-1 cells

THP-1 cells were treated with Vitamin D_3 for 48 h. Untreated cells were included as a control. Cells were collected and CD14 expression on the cell surface was analysed with flow cytometry. The graph is representative of two independent experiments.

2.1.1.5 Freezing of THP-1 monocytes

To ensure continuous culture, superfluous THP-1 monocytes were frozen in liquid nitrogen. 2 – 4 x 10^6 cells/ml were resuspended in freezing medium (RPMI-1640 medium supplemented with FCS (20 % v/v), L-glutamine (2 mM) and 10 % glycerol). Cells were transferred to cryovials and placed in a freezing container (Nalgene, Cryo 1 °C, Hereford, UK) with propan-2-ol (VWR International, Poole, UK) at -80 °C in a Sanyo Ultra Low freezer (MDF-U30865) over night. The following day, cells were stored in liquid nitrogen.

2.1.2 THP-1-Blue-CD14 and HEK-Blue-4 cell lines

Both THP-1-Blue-CD14 and HEK-Blue-4 cell lines are genetically engineered reporter cell lines for activation of pro-inflammatory transcription factors such as NF-κB or AP-1. Through a vector, these cell lines are stably transfected with an alkaline phosphatase gene (SEAP, Secreted form of Embryonic Alkaline Phosphatase) under the control of a reporter inducible by NF-κB, AP-1 or other pro-inflammatory transcription factors. Upon activation, alkaline phosphatase is secreted and can be detected with a SEAP reporter assay in cell culture supernatants (see 2.2.1.1).

The THP-1-Blue-CD14 cells are derived from THP-1 monocytes (see above) and are non-adherent during culture. They express both functional TLR4 and TLR2 and all other receptors known in THP-1 monocytes. In addition to the alkaline phosphatase gene the THP-1-Blue-CD14 cells are also transfected with resistance genes for the selection antibiotics Zeocin and Blasticidin and overexpress CD14. The overexpression of CD14 induces increased LPS sensitivity. The expression of antibiotic resistance

genes is necessary to ensure that only cells containing the vector with the SEAP system continue to replicate during cell culture.

The HEK-Blue-4 cell line is engineered from HEK293 cells (Human Embryonic Kidney cells) and is adherent during culture. Comparable to the THP-1-Blue-CD14 cells the HEK-Blue-4 cells are also transfected with resistance genes for selection antibiotics in addition to the SEAP gene. Furthermore, these cells are transfected with TLR4 and the necessary co-receptors MD2 and CD14 to ensure effective LPS signalling.

2.1.2.1 THP-1-Blue-CD14 cell culture

THP-1-Blue-CD14 cells were purchased from Invivogen (via Autogen Bioclear, Calne, UK) as a frozen vial with 3 – 5 x 10^6 cells in passage 4. Upon receipt, cells were thawed as described in 2.1.1.1 and centrifuged for 5 min at 3000 rpm, 20 °C. Cells were resuspended in cell culture medium (without any antibiotics) and transferred to a 25 cm^2 flask containing the same medium (equilibrated for at least 15 min at 5 % CO_2 prior to incubation). Cells were incubated over night at 37 °C, 5 % CO_2. Medium was changed the next day and culture was continued in cell culture medium (with antibiotic supplements as before, but without selection antibiotics) as described above (see 2.1.1.2). After five days of culture, selection antibiotics Zeocin (200 µg/ml) and Blasticidin (10 µg/ml) (Invivogen, via Autogen Bioclear) were added to the culture medium. Cells were passaged at least one time (see 2.1.1.2) before use in experiments.

Superfluous THP-1-Blue-CD14 cells were frozen in liquid nitrogen as described in 2.1.1.5.

2.1.2.2 HEK-Blue-4 cell culture

HEK-Blue-4 cells were obtained from Invivogen (via Autogen Bioclear) as a growing culture in HEK culture medium (Dulbecco's modified Eagles medium, Gibco, Invitrogen, Groningen, CH, supplemented with 200 mM L-glutamine, high glucose (4.5 g/ml), 10 % FCS (v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), 1 x Normocin and 1 x HEK-Blue-4 Selection, Invivogen, via Autogen Bioclear). Cells were cultured at 37 °C, 5 % CO_2.

Cells were passaged when 60 – 80 % confluency was reached. Cells were rinsed with PBS and then trypsinized for approximately 3 min with a Trypsin-EDTA dilution (0.05 % Trypsin in EDTA 4Na, diluted 1:3 with PBS). Medium was added to quench the trypsinization and cells were centrifuged for 5 min at 1500 rpm, 20 °C. Cells were resuspended in HEK culture medium, counted as described above (see 2.1.1.3) and either seeded at 0.5 – 1 x 10^6 cells/well of a 6-well tissue culture plate for experiments or centrifuged again, resuspended to 0.5 – 1 x 10^7 cells/ml in HEK freezing medium (HEK culture medium supplemented with 10 % DMSO, without any antibiotics) and frozen in liquid nitrogen as described in 2.1.1.5.

2.1.3 Isolation and culture of primary human monocytes

Primary human monocytes were isolated from a buffy coat of healthy blood donors. Each buffy coat is derived from an individual donor and therefore is different in each experiment. Blood was obtained from the National Blood Service (Newcastle upon Tyne, UK). Two different methods were applied for monocyte isolation. In the adherent cell method, monocytes are isolated by their ability to adhere to tissue culture

plates. The second method uses antibodies with magnetic beads for positive or negative selection of monocytes.

2.1.3.1 Adherent cell method

Blood was diluted 1:1 in PBS/2 mM EDTA and overlayed 1:1 on a Histopaque gradient (Histopaque-1077). After a centrifuge step for 20 min at 800 g, 20 °C, the buffy coat layer was collected and diluted in 40 ml PBS/2 mM EDTA. Leukocytes were centrifuged for 10 min at 800 g, 20 °C, supernatant was discarded and the cell pellet was resuspended in 3 ml ACK lysis buffer (150 mM NH_4Cl, w/v, BDH Laboratory supplies, Poole, UK; 10 mM $KHCO_3$, w/v, BDH Laboratory supplies; 0.1 mM EDTA, pH 7.3, w/v) for 3 min to lyse erythrocytes. 10 ml PBS was added and leukocytes were centrifuged for 5 min at 800 g, 20 °C. The lysis step was repeated up to three times until no erythrocytes were further visible. Leukocytes were resuspended in cell culture medium (see 2.1.1.2), transferred to three 6-well tissue culture plates (4 ml cell suspension/well) and incubated over night at 37 °C, 5 % CO_2. The next day, non-adherent cells were removed by washing each well two times with 1 ml cell culture medium. Adherent cells (monocytes) were collected, resuspended in cell culture medium and centrifuged for 5 min at 2000 rpm, 20 °C. The cells were resuspended in cell culture medium, counted on a haemocytometer and 4 x 10^6 cells were transferred to each well of a 6-well tissue culture plate. Plates were incubated over night at 37 °C, 5 % CO_2 and then used for stimulation experiments.

2.1.3.2 Magnetic bead method

Blood was diluted 1:1 in isolation buffer (PBS/1 mM EDTA, supplemented with 2 % FCS) and overlayed 1:1 on a Histopaque gradient at room temperature. After a centrifuge step for 20 min at 800 g, 20 °C, the buffy coat layer was collected, diluted

into 40 ml of isolation buffer and centrifuged for 7 min at 600 g, 4 °C. The cell pellet was resuspended in 50 ml isolation buffer and centrifuged for 7 min at 250 g, 4 °C. The cell pellet was resuspended once more in 50 ml isolation buffer and passed through a 30 µm cell mesh. Leukocytes were counted and resuspended to 1 x 10^8 cells/ml for positive selection or to 5 x 10^7 cells/ml for negative selection of monocytes in RoboSep buffer (StemCell Technologies, Grenoble, France). Monocyte isolation was then accomplished using a positive or negative CD14 selection kit (StemCell Technologies) on the fully automated cell separator RoboSep (StemCell Technologies) following the manufacturer's instructions. On the RoboSep, the cell suspension was incubated with antibody cocktails (positive selection: monoclonal CD14; negative selection: monoclonal CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A, F_cR blocker) and magnetic beads. The beads with the attached cells were automatically collected with a magnet and purified monocytes were resuspended in RoboSep buffer.

After isolation, monocytes were counted on a haemocytometer, resuspended in cell culture medium and 4 x 10^6 cells were transferred to each well of a 6-well tissue culture plate. Plates were incubated over night at 37 °C, 5 % CO_2 and then used for stimulation experiments.

2.1.3.3 Mycoplasma testing

Mycoplasma contamination of cell cultures can yield erroneous experimental results (Rottem and Barile, 1993). To ensure cell cultures were free of mycoplasma, a commercially available test (MycoAlert, Lonza, Slough, UK) for mycoplasma screening was carried out according to the manufacturer's instructions. This assay uses ATP produced by the activity of certain mycoplasmal enzymes in a bioluminescent reaction

with luciferin and measures the emitted light. The intensity of emitted light is linear to ATP concentrations in samples.

100 µl of cell culture supernatant and positive or negative controls were transferred to a luminometer plate. 100 µl of MycoAlert reagent was added to each well and the luminescence was measured after 5 min for 1 s on a luminometer (Microlumat Plus LB 96V, Berthold Technology, Hertfordshire, UK). Following the addition of 100 µl of MycoAlert substrate to each well the luminescence was measured again after 10 min. The ratio of the second to the first reading was calculated. A result ≤ 1 indicated no mycoplasma contamination of the sample.

Table 2.1 shows results of the luminometer readings at the two timepoints. The ratio of the two readings for the THP-1 cell culture supernatant was < 1. This implies that the tested sample of THP-1 monocytes in culture was mycoplasma-free.

Luminescence reading	Positive control [hv]	Negative control [hv]	Supernatant of THP-1 monocytes [hv]
Reading I	4	4	3
Reading II	311	1	1
$\frac{\text{Reading II}}{\text{Reading I}}$	77.75	0.25	0.33

Table 2.1 Mycoplasma testing of THP-1 monocytes

Cell culture supernatants of THP-1 monocytes were tested for mycoplasma contamination in a bioluminescence reaction. A positive and a negative control were included. Table shows luminometer readings of emitted light at two timepoints. Data are derived from one experiment.

2.2 Reagents

2.2.1 LPS

LPS from *E. coli* 0111.B4 was purchased from Invivogen (via Autogen Bioclear) as a TLR4 ligand. This LPS was extracted with a phenol-water mixture by the manufacturer and may also contain other bacterial components such as lipoproteins. LPS from *P. gingivalis*, strain W50, was a kind gift from Dr. M. Rangarajan (QMW college, School of Medicine and Dentistry, London, UK). It was unknown if this LPS was a TLR2 or TLR4 ligand. LPS working concentrations were prepared in cell culture medium.

2.2.1.1 SEAP (Secreted Embryonic Alkaline Phosphatase) reporter assay

LPS and endotoxin testing stimulation experiments in HEK-Blue-4 and THP-1-Blue-CD14 cells were analysed with a SEAP reporter assay. After activation of the SEAP reporter gene by pro-inflammatory transcription factors such as NF-κB or AP-1, alkaline phosphatase is secreted from the cells. SEAP then catalyses a specific substrate in the detection medium and a blue colour develops in the enzymatic reaction. The intensity of the blue colour is equivalent to the activity of SEAP.

20 µl of sample was applied to each well of a 96-well tissue culture plate. HEK-blue-4 cells were diluted to 1-1.25 x 10^5 cells/ml in HEK-blue detection medium (Invivogen, via Autogen Bioclear) and 200 µl of the cell suspension was added to each sample (~2.5 x 10^4 cells/well). The plate was incubated over night at 37 ºC, 5 % CO_2 and the absorption measured on a spectrophotometer (Titertek Multiskan MCC/340, Thermo Fisher Scientific, Loughborough) at 620 nm the next day.

A similar principle was applied for experiments with THP-1-Blue-CD14 cells. To increase the sensitivity of the assay, THP-1-Blue-CD14 monocytes were converted to macrophages as recommended by the manufacturer. THP-1-blue-CD14 cells were diluted to 1-2 x 10^6 cells/ml in growth medium without selection antibiotics, supplemented with 50 ng/ml PMA (Phorbol myristate acetate). 100 µl of the suspension was added to each well of a 96-well tissue culture plate. The medium was changed after 18 h (without PMA) and cells were incubated for two days at 37 °C, 5 % CO_2 to fully differentiate. The medium was changed twice on the second day and cells were incubated for further 5 days, with changing the medium twice daily to allow for excess removal of SEAP induced by PMA stimulation.

Cells were washed with PBS and 100 µl of HEK-blue detection medium was added to each well. After addition of 10 µl sample/well, cells were incubated over night and the absorption measured on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek, Leicestershire, UK) at 620 nm the next day.

2.2.1.2 Screening for TLR signalling

Although *P. gingivalis* LPS is a known TLR2 ligand (Zhou *et al.*, 2005; Hajishengallis *et al.*, 2006; Triantafilou *et al.*, 2007; Zhou and Amar, 2007), several studies also showed it can signal through TLR4 (Chen *et al.*, 2006; Reife *et al.*, 2006). It was therefore necessary to confirm the obtained *P. gingivalis* LPS as a TLR2 ligand.

TLR2 signalling of LPS from *P. gingivalis* was confirmed with two separate experiments. In the first experiment, TLR4 signalling capacity of LPS from *P. gingivalis* was tested with a SEAP reporter assay in the HEK-Blue-4 cell line. Samples were added to HEK-blue-4 cells and processed as described in the SEAP reporter assay

(2.2.1.1). Cells were stimulated with 100 ng/ml *P. gingivalis* LPS and a range of different concentrations of LPS from *E. coli* 0111.B4 was used as a positive control. Endotoxin free water was included as a negative control.

Figure 2.2 shows increasing SEAP activity (represented as the OD reading of the blue colour intensity) with increasing *E. coli* LPS concentrations, with the highest activity at 100 ng/ml. Even at 0.1 ng/ml, *E. coli* LPS-induced SEAP activity was clearly higher than the negative control. In contrast, 100 ng/ml of *P. gingivalis* LPS did not differ noticeable from the negative control. This result indicates that at the concentration used in all subsequent stimulation experiments (100 ng/ml), LPS from *P. gingivalis* W50 does not induce TLR4 signalling. In contrast, *E. coli* LPS induces TLR4 signalling in a dose-responsive manner.

In the second experiment, a sample of LPS from *P. gingivalis* W50 was sent to Invivogen (Grenoble, France) and tested by the company in the HEK-293-hTLR2 cell line, using the same principle as for the HEK-Blue-4 cell line. The HEK-293-hTLR2 cell line only expresses a functional TLR2 and no TLR4, therefore any observed SEAP activity would be due to TLR2 signalling.

HEK-293-hTLR2 cells were stimulated with *P. gingivalis* LPS in a range of different concentrations as indicated. The TLR2 specific chemical compound Pam2CSK4 was included as positive control for TLR2 signalling. In addition, as a control to test if components in the sample would interfere with the reporter system, *P. gingivalis* LPS signalling was tested in HEK-blue cells which do not express any TLRs (HEK-TLR^{-}). HEK-TLR^{-} cells were stimulated with 100 ng/ml *P. gingivalis* LPS or stimulated with

TNF-α as a positive control. Unstimulated cells were used as a negative control. Concentration for the TNF-α stimulation was not disclosed by the company.

LPS from *P. gingivalis* W50 and Pam2CSK4 both induced activation of TLR2 in the HEK-293-hTLR2 reporter system in a dose-responsive manner (Figure 2.3). However, Pam2CSK4 was a more potent inducer of TLR2 than *P. gingivalis* LPS. The highest activation of TLR2 signalling was seen at 100 ng/ml. While TLR2 activation was still detectable for Pam2CSK4 at the lowest concentration (0.01 ng/ml), the last detectable TLR2 activation was seen at 1 ng/ml for *P. gingivalis* LPS.

P. gingivalis LPS did not induce a response in the SEAP reporter system in HEK-TLR$^-$ cells (Figure 2.4). In contrast, TNF-α strongly induced activation of SEAP. It can be concluded that there were no components in the *P. gingivalis* LPS sample which could have interfered with the SEAP reporter system and therefore might have resulted in false positive or false negative results. The TNF-α-induced activation of SEAP demonstrates that HEK-TLR$^-$ cells are responsive and that the SEAP reporter system is functioning correctly.

In summary, the results of the SEAP reporter assay experiments confirm that the LPS from *P. gingivalis* W50 signals through TLR2 and not through TLR4, whereas *E. coli* LPS was confirmed as a TLR4 ligand.

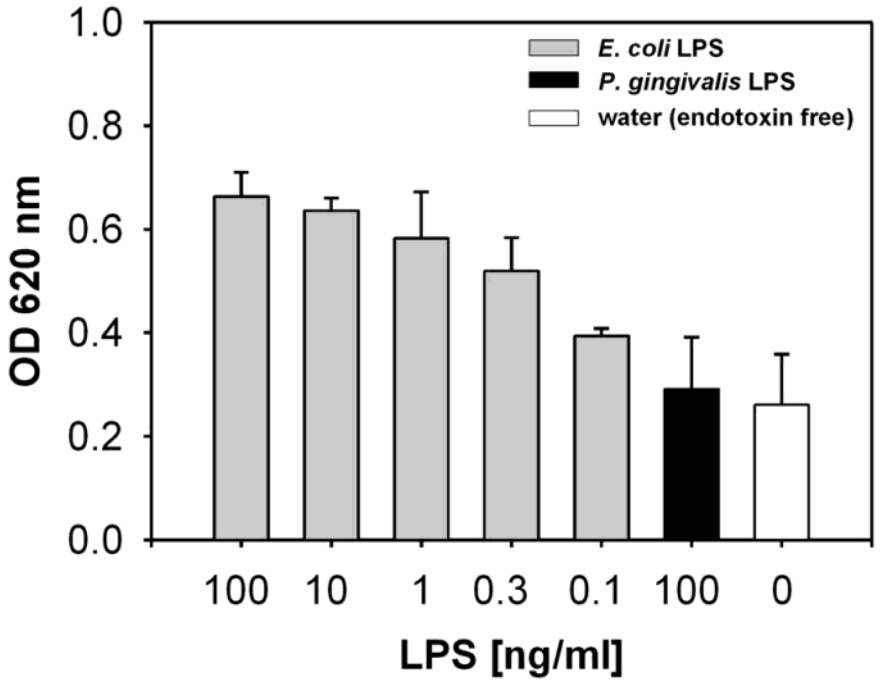

Figure 2.2 Activation of TLR4 signalling in HEK-Blue-4 cells

HEK-Blue-4 cells were stimulated with LPS from *E. coli* 0111.B4 or LPS from *P. gingivalis* W50 at the concentrations indicated. Activation of TLR4 signalling was analysed with the SEAP reporter assay. The graph represents mean + SD from triplicate cultures of one experiment.

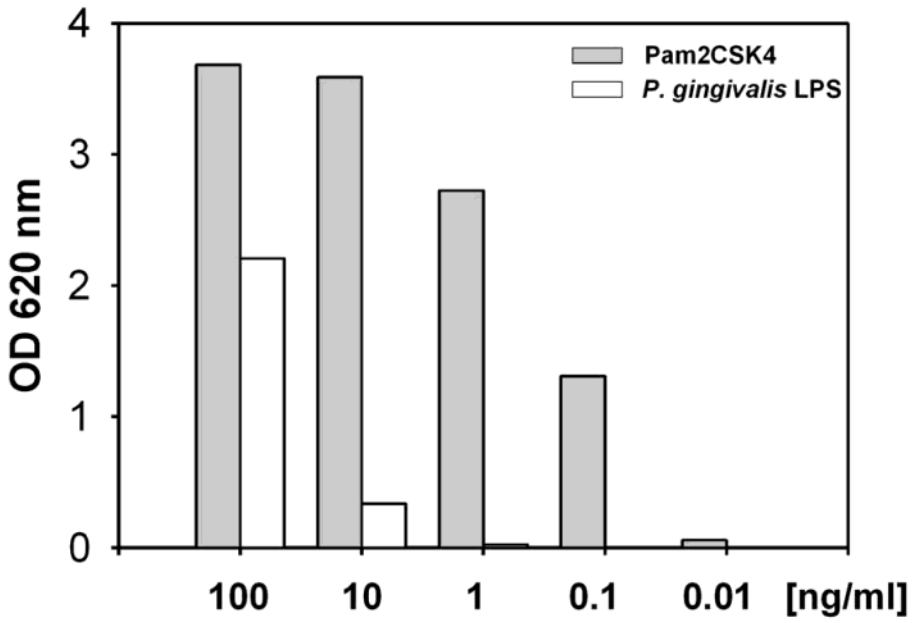

Figure 2.3 Activation of TLR2 signalling in HEK-293-hTLR2 cells

HEK-293-hTLR2 cells were stimulated with LPS from *P. gingivalis* W50 or with Pam2CSK4 at the concentrations indicated. Activation of TLR2 signalling was analysed with the SEAP reporter assay. The graph represents data from one experiment.

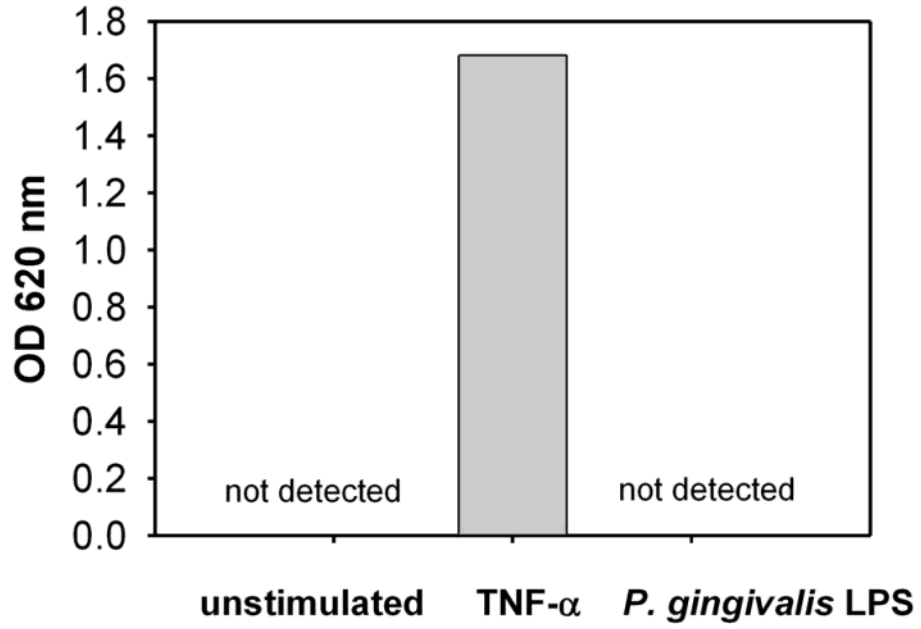

Figure 2.4 Testing for interference of the sample in the reporter system

HEK-TLR$^-$ cells were stimulated with LPS from *P. gingivalis* W50 (100 ng/ml) or with TNF-α (concentration unknown). Activation of the reporter gene system was analysed with the SEAP reporter assay. The graph represents data from one experiment.

2.2.2 Leptin

Human recombinant leptin (expressed in *E. coli* K12) was purchased from R&D Systems (Abingdon, UK) and reconstituted to a stock solution of 1 mg/ml with 20 mM sterile Tris/HCl (pH 8.0, w/v) according to the manufacturer's instructions. Working dilutions were prepared in cell culture medium. Reconstituted leptin was stored at -80 °C. Leptin concentrations used in experiments were chosen based on previous studies applying a comparable experimental setup (Loffreda *et al.*, 1998; Zarkesh-Esfahani *et al.*, 2001).

2.2.2.1 Testing for endotoxin contamination

Using recombinant proteins produced in bacteria such as *E. coli* always involves a risk of contamination with LPS. To determine the endotoxin concentration of human recombinant leptin, a commercially available endotoxin assay (Limulus amebocyte lysate, LAL, QCL-1000, Lonza) was carried out according to the manufacturer's instructions. The LAL assay takes advantage of an enzymatic reaction with endotoxin from gram negative bacteria which is occurring naturally in amebocytes of the horseshoe crab (*Limulus polyphemus*). In this assay, endotoxin from Gram-negative bacteria induces the activation of an enzyme in the LAL. The rate of activation is determined by the endotoxin concentration. The activated enzyme then catalyzes a reaction with a substrate, inducing a colour change in the assay solution. The observed colour change is equivalent to the amount of endotoxin present.

A 4-point standard curve of LPS from *E. coli* 0111.B4 (supplied with assay) was prepared with the following concentrations: 1.0 EU/ml, 0.5 EU/ml, 0.25 EU/ml and 0.1 EU/ml (Figure 2.5). 10 EU (endotoxin unit) is equivalent to 1 ng standard reference

LPS according to the Food and Drug administration (FDA). Recombinant human leptin was tested at a concentration of 100 µg/ml. Endotoxin free water was included as a negative control. 50 µl of standard or sample was transferred to a 96-well plate pre-equilibrated to 37 ºC. Standards were assayed in triplicates, samples in duplicates. 50 µl of LAL reagent was added and the assay was incubated for 10 min. After addition of 100 µl substrate and a further incubation for 10 min, the enzymatic reaction was stopped with 50 µl of 10 % SDS. The absorption was read at 405 nm on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek).

The EU concentration of 100 µg/ml human recombinant leptin was calculated from the standard curve (Table 2.2). 100 µg/ml of human recombinant leptin was found to contain 0.53 EU/ml. This converts into 0.053 ng/ml LPS. The highest leptin concentration used for stimulation experiments in the present study was 1 µg/ml. This leptin concentration would contain 0.0005 ng/ml LPS.

The recombinant human leptin is expressed in *E. coli* K12. To determine if low concentrations of *E. coli* K12 LPS could still trigger an immune response in THP-1 monocytes and thus interfere with any leptin-induced effects, a stimulation experiment of THP-1 monocytes with a range of different *E. coli* K12 LPS concentrations was conducted. THP-1 monocytes (0.5×10^6) were stimulated with different *E. coli* K12 LPS (Autogen Bioclear) concentrations as indicated (Figure 2.6) for 2 h. Unstimulated cells served as control. Cell culture supernatants were collected and analysed for TNF-α expression with ELISA (see 2.3).

E. coli K12 LPS induced TNF-α protein expression in THP-1 monocytes in a dose responsive manner (Figure 2.6). The highest TNF-α concentration (807 pg/ml) in the

cell culture supernatants was observed at 100 ng/ml *E. coli* K12 LPS. TNF-α protein concentrations then steadily declined with decreasing *E. coli* K12 LPS concentrations. The lowest detectable TNF-α protein concentration (83 pg/ml) was observed at 0.5 ng/ml *E. coli* K12 LPS. No TNF-α was detected in supernatants of cells stimulated with any lower concentrations of *E. coli* K12 LPS or in the control. The *E. coli* K12 LPS contamination which was detected in the human recombinant leptin with the LAL endotoxin assay was 0.0005 ng/ml. This LPS concentration is a 1000 times lower than the lowest *E. coli* K12 LPS concentration (0.5 ng/ml) which still induced a detectable TNF-α protein expression. Furthermore, it should be noted that in all subsequent experiments in the present study single stimulations with human recombinant leptin never induced a detectable TNF-α expression in cell culture supernatants. Any observed effect of leptin is therefore unlikely due to LPS contamination.

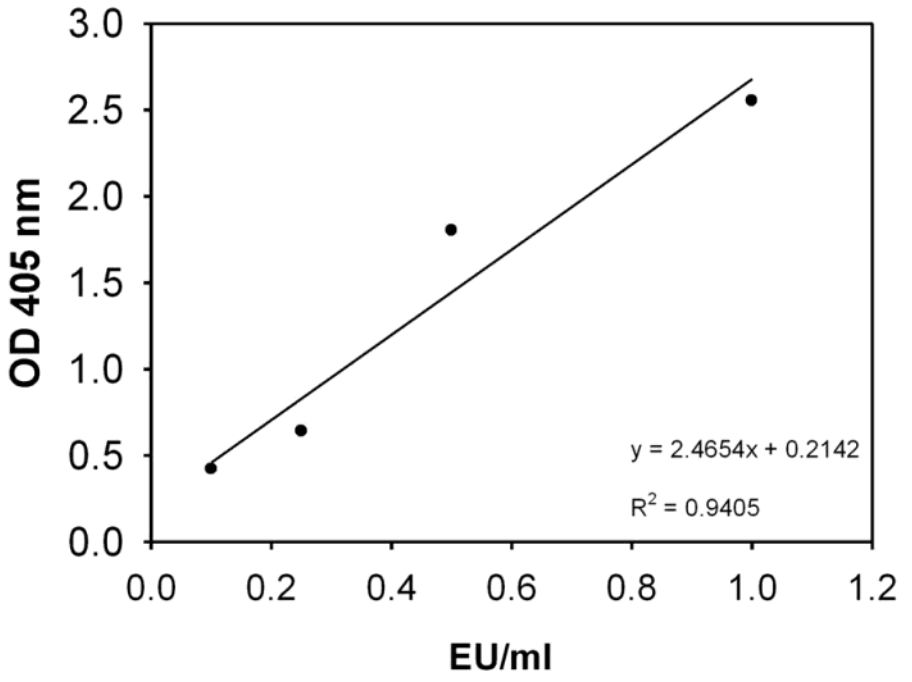

Figure 2.5 Standard curve of the LAL endotoxin assay

The absorbance at 405 nm of 1.0 EU/ml, 0.5 EU/ml, 0.25 EU/ml and 0.1 EU/ml was plotted against EU/ml. A regression line was plotted to create the 4-point standard curve for the LAL endotoxin assay. EU: endotoxin unit.

Leptin	OD 405 nm	EU/ml	LPS [ng/ml]
100 µg/ml	1.513	0.53	0.053
1 µg/ml	–	–	0.0005

Table 2.2 Determination of endotoxin contamination of human recombinant leptin

The EU concentration of 100 µg/ml human recombinant leptin was calculated from the standard curve of the LAL endotoxin assay. The EU was then converted into LPS concentration and the LPS concentration contained in 1 µg/ml of human recombinant leptin was calculated. Table shows data from one experiment. EU: endotoxin unit.

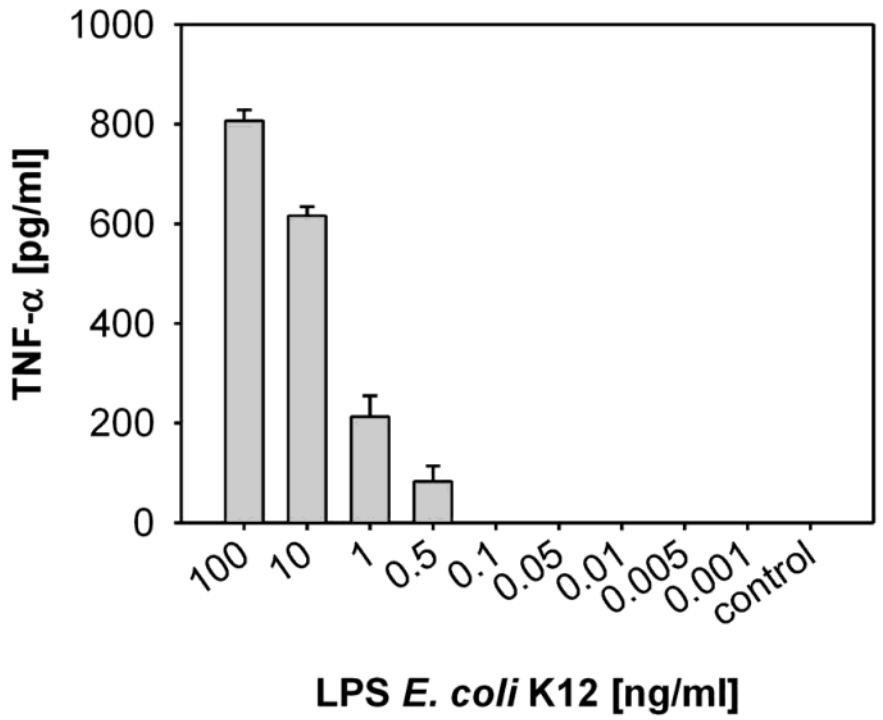

Figure 2.6 *E. coli* K12 LPS-induced TNF-α expression in THP-1 monocytes

THP-1 monocytes were stimulated with LPS from *E. coli* K12 for 2 h at the concentrations indicated. Supernatants were collected and analysed for TNF-α expression. The graph represents mean + SD from one experiment with quadruplicate cultures.

2.2.2.2 Analysis of leptin potency to activate intracellular signalling pathways

The activity of human recombinant leptin was tested by the manufacturer in human leptin receptor transfected BaF3 mouse pro-B cells. However, this could not be validated in the present study. As an alternative, the potency of leptin to activate intracellular signalling pathways was therefore tested with the SEAP reporter assay in the THP-1-Blue-CD14 cell line.

PMA treated THP-1-Blue-CD14 cells were stimulated with 100 ng/ml LPS from *P. gingivalis* W50 or 50 µg/ml human recombinant leptin. Unstimulated cells served as a control. In addition, as a negative control for leptin activity the cells were stimulated with 50 µg/ml of a heat-treated leptin sample. Heat-treatment was induced by boiling the leptin sample for 30 min at 95 °C as described by Muller *et al.* (1997) and will result in a denatured leptin protein.

Figure 2.7 shows that *P. gingivalis* LPS and leptin induce SEAP activity in the THP-1-Blue-CD14 reporter system. At the tested concentrations *P. gingivalis* LPS was 60 % more potent to induce SEAP activity than leptin. No SEAP activity was detected in unstimulated cells or in cells stimulated with the heat-treated leptin sample. In conclusion, the human recombinant leptin used in the present study can induce intracellular signalling pathways which will result in the activation of pro-inflammatory transcription factors such as NF-κB or AP-1. The effect is due to the leptin protein activity and was not observed for denatured leptin.

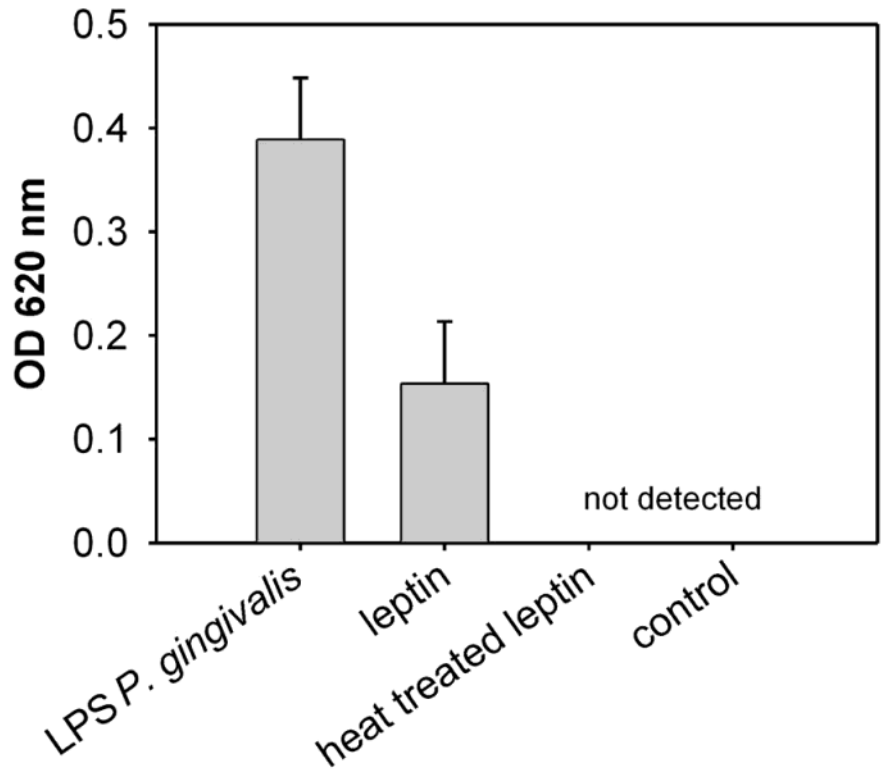

Figure 2.7 Potency of leptin to activate intracellular signalling pathways in THP-1-Blue-CD14 cells

PMA treated THP-1-Blue-CD14 cells were stimulated with LPS from *P. gingivalis* W50 (100 ng/ml), leptin (50 µg/ml) or heat-treated leptin (50 µg/ml). Activation of intracellular signalling pathways was analysed with a SEAP reporter assay. The graph represents mean + SD of duplicate cultures in one experiment.

2.2.3 Evaluation of mitogenic or cytotoxic effects of reagents

The mitogenic or cytotoxic effects of leptin and LPS were evaluated with the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK) in THP-1 monocytes. In this assay, NADH and NADPH, which are secreted in the supernatants of living cells, convert a chemical compound (Owen's reagent) to a coloured product. The conversion directly corresponds to cellular activity, providing a readout for changes in cell numbers.

2.2.3.1 Cell Titer 96 cell proliferation assay procedure

The assay was carried out according to the manufacturer's instructions. A 10-point standard curve of THP-1 monocytes with 2 x 10^6 cells/ml as the highest standard was produced using a 2-fold dilution series in fresh cell culture medium. Cell culture medium alone was used as a zero standard. Cell numbers of stimulated samples and control were equally adjusted with fresh culture medium to a cell number within the range of the standard curve. For this, cells of each stimulation or control were resuspended in 12 ml of cell culture medium. In a 96-well tissue culture plate, 100 µl of the standard or sample dilutions were transferred to each well. Standards were analysed in triplicates, samples in duplicates. After ½ h of incubation at 37 °C, 5 % CO_2, 20 µl of Owen's reagent were added to each well and cells were incubated for further 2 hours. After incubation, absorption was measured at 460 nm on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek) and a reading at 645 nm was subtracted to adjust for plate background. An example of a standard curve for the cell proliferation assay is shown in Figure 2.8.

2.2.3.2 The mitogenic and cytotoxic effect of leptin and LPS

To test the mitogenic or cytotoxic effect of leptin and LPS, stimulation experiments of THP-1 monocytes were conducted using the same stimulation timepoints, cell numbers and leptin or LPS concentrations as in all subsequent stimulation experiments in the present study. In a first experiment, THP-1 monocytes (4×10^6) were stimulated with 500 ng/ml leptin for 5 h. In a second experiment, THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 24 h. Unstimulated cells served as controls. All conditions were set up in triplicate cell cultures. Cells were collected and processed in the Cell Titer 96 cell proliferation assay as described in 2.2.3.1.

After 5 h of leptin stimulation, no significant change in the cell number of THP-1 monocytes was detected in comparison to the control ($p = 0.2$, Figure 2.9). The cell number was 6.56×10^6 cells/ml for leptin stimulated THP-1 monocytes and 6.99×10^6 cells/ml for the control. These data show that after 5 h, human recombinant leptin neither has a mitogenic nor a cytotoxic effect in THP-1 monocytes.

After 24 h of leptin stimulation, no significant change in the cell number of THP-1 monocytes was detected in comparison to the control ($p = 0.4$, Figure 2.10). The cell number was 4.18×10^6 cells/ml for leptin stimulated THP-1 monocytes and 7.18×10^6 cells/ml for the control. These data show that after 24 h, human recombinant leptin neither has a mitogenic nor a cytotoxic effect in THP-1 monocytes. In contrast, cell numbers of THP-1 monocytes were significantly lower ($p < 0.05$) in comparison to control after 24 h of *E. coli* LPS stimulation (Figure 2.10). The cell number of *E. coli* LPS stimulated cells was 26 % lower than the cell number of unstimulated cells, reaching a concentration of 5.32×10^6 cells/ml. This result could indicate that after 24 h

E. coli LPS has a cytotoxic effect on the THP-1 monocytes. However, LPS-stimulation of THP-1 monocytes or stimulation with other pro-inflammatory agents such as PMA induces cell differentiation and growth cycle arrest (Suzuki *et al.*, 2009). Therefore, it is possible that the unstimulated THP-1 monocytes continued to proliferate while LPS-stimulated cells changed from proliferation to differentiation. This is further supported by the finding that even after 24 h of *E. coli* LPS stimulation the cell number was still higher than at the start of the experimental setup (5.32×10^6 versus 4×10^6 cells/ml).

In summary, results of the Cell Titer 96 cell proliferation assay show that leptin has no proliferative or cytotoxic effects on THP-1 monocytes after 5 or 24 hours. Any leptin-induced changes in cytokine or cell surface receptor expression are therefore not due to changes in cell numbers. In addition, although cell numbers are lower after 24 h LPS-stimulation in THP-1 monocytes in comparison to control, this likely indicates more a decline in the proliferative capacity rather than LPS-induced cell death.

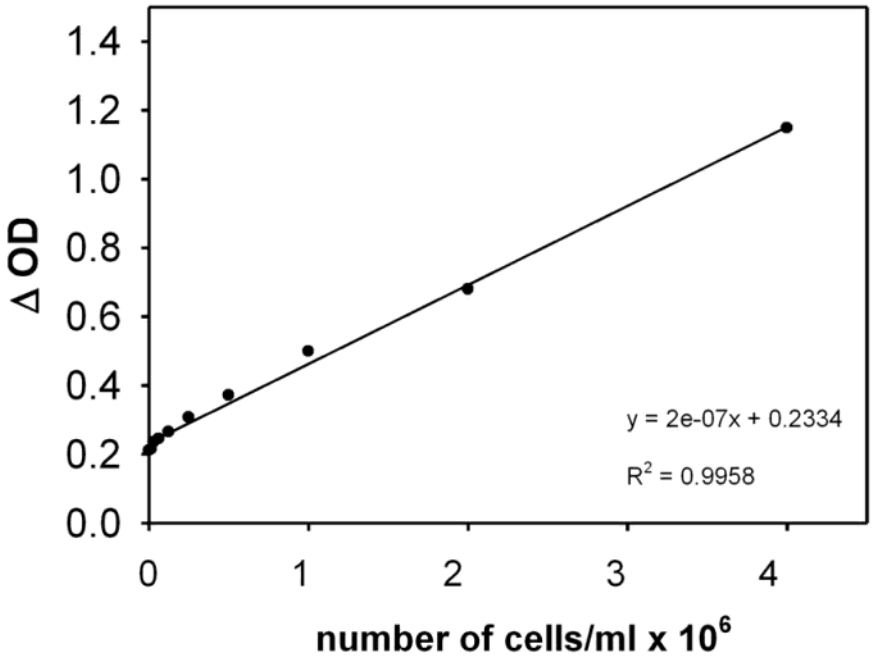

Figure 2.8 Standard curve of the Cell Titer 96 cell proliferation assay

The Δ OD of the different cell numbers in the standard curve was plotted against the number of cells. A regression line was plotted to create the 10-point standard curve for the Cell Titer 96 cell proliferation assay. Δ OD: OD 460 nm – OD 645 nm.

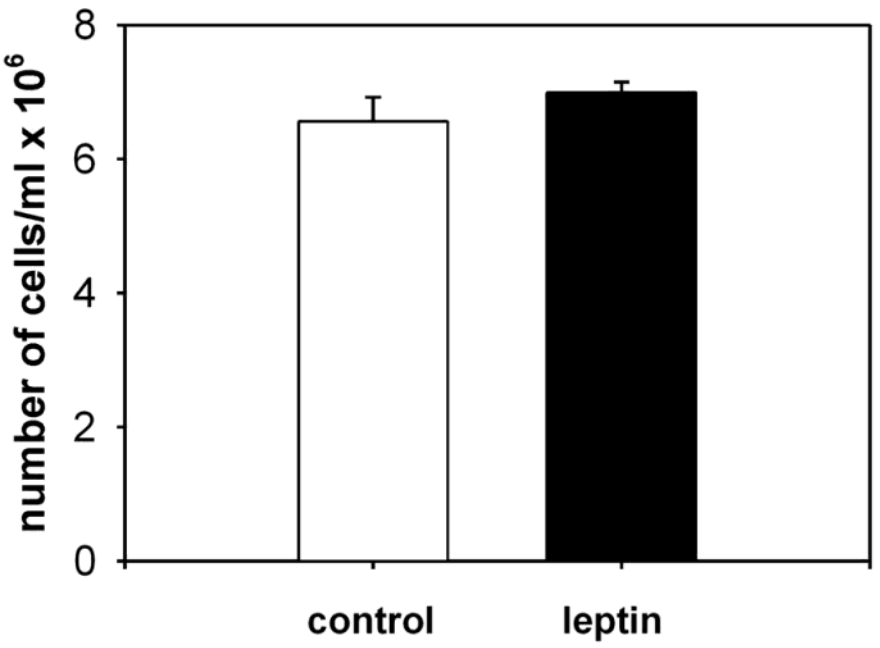

Figure 2.9 The effect of leptin on cell proliferation in THP-1 monocytes after 5 h

THP-1 monocytes were stimulated with leptin (500 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 5 h. The graph represents median + IQR from one experiment with three independent cultures. Cell numbers are readjusted for the 12 ml dilution. Statistics: Mann-Whitney *U* test: not significant.

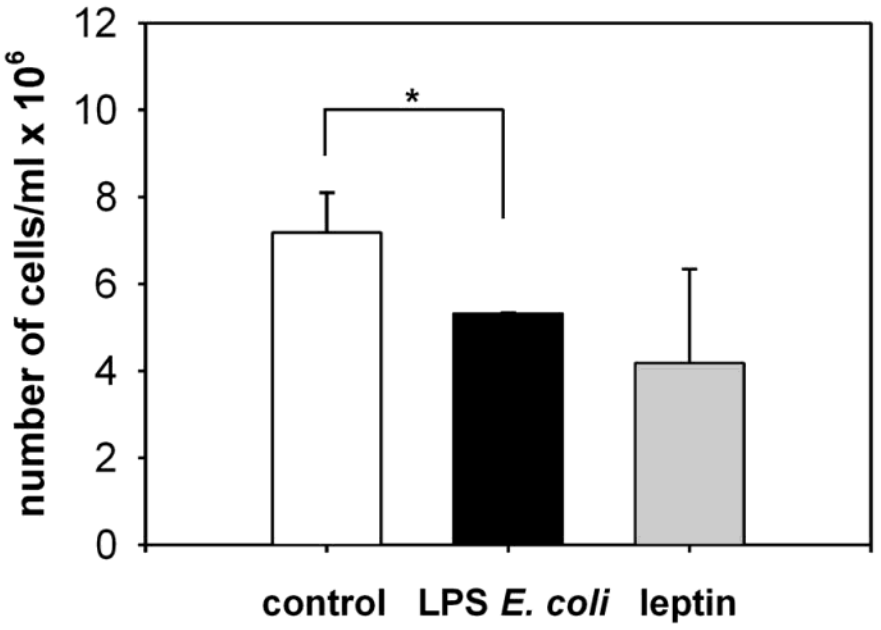

Figure 2.10 The effect of leptin or LPS on cell proliferation in THP-1 monocytes after 24 h

THP-1 monocytes were stimulated with leptin (1000 ng/ml) or LPS from *E. coli* 0111.B4 (100 ng/ml). Cell proliferation was analysed with the Cell Titer 96 proliferation assay after 24 h. The graph represents median + IQR from one experiment with three independent cultures. Cell numbers are readjusted for the 12 ml dilution. Statistics: Mann-Whitney *U* test: *$p < 0.05$.

2.3 Enzyme-linked immunosorbent assays (ELISA)

Cytokine and adipokine concentrations in cell culture supernatants (retained from cell collections for other experiments and frozen at -20 °C until further processing), serum and GCF samples were determined with commercially available ELISA kits (Duoset, R&D systems). The assays were carried out according to the manufacturer's instructions. All assays were performed at room temperature. Details of antibody working concentrations and standard curve detection ranges for each ELISA are listed in Table 2.3.

ELISA	Capture antibody	Detection antibody	Standard curve range [pg/ml]
Adiponectin	2 µg/ml	2 µg/ml	4000 – 62.5
GM-CSF	2 µg/ml	0.5 µg/ml	1000 – 15.625
Leptin	4 µg/ml	12.5 ng/ml	2000 – 31.25
TNF-α	4 µg/ml	75 ng/ml	1000 – 15.625

Table 2.3 Antibody working concentrations and standard curve detection ranges for ELISAs

The table lists the capture and detection antibody working concentrations and the ranges of the standard curves for the adiponectin, GM-CSF, leptin and TNF-α ELISA.

A 96-well ELISA plate (Greiner Bio One) was coated with 100 µl capture antibody diluted in PBS and incubated overnight. The following day, the plate was washed three times with wash buffer (0.05 % Tween 20 in PBS, v/v) and nonspecific binding was blocked with 300 µl reagent diluent (RD, 1 % BSA in PBS, w/v) for one hour. After repeating the washing step, 100 µl standards (in triplicates, serial dilution prepared in RD) or sample (in duplicates) were applied. A triplicate RD sample was included as a negative control. Standard and samples were incubated for two hours. The plate was washed again and 100 µl detection antibody diluted in RD was applied. After incubation for two hours and subsequent washing, the assay was incubated with 100 µl horse radish peroxidase (diluted in RD) for 20 minutes, kept away from direct light. The plate was washed again and 100 µl of a 1:1 dilution of TMB and H_2O_2 (Substrate reagent kit, R&D systems) was applied. The plate was incubated avoiding direct light and the reaction was stopped with 50 µl 2 N H_2SO_4 after 20 minutes. Absorbance was read at 450 nm on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek). A reading at 550 nm was subtracted to correct for plate background. Protein concentrations of samples were calculated of the standards with the supplied software (KC4 KinetiCalc, BioTek) for the spectrophotometer using a 4-parameter logistic curve fit. Examples of a standard curve for each ELISA are shown in Figure 2.11-Figure 2.14.

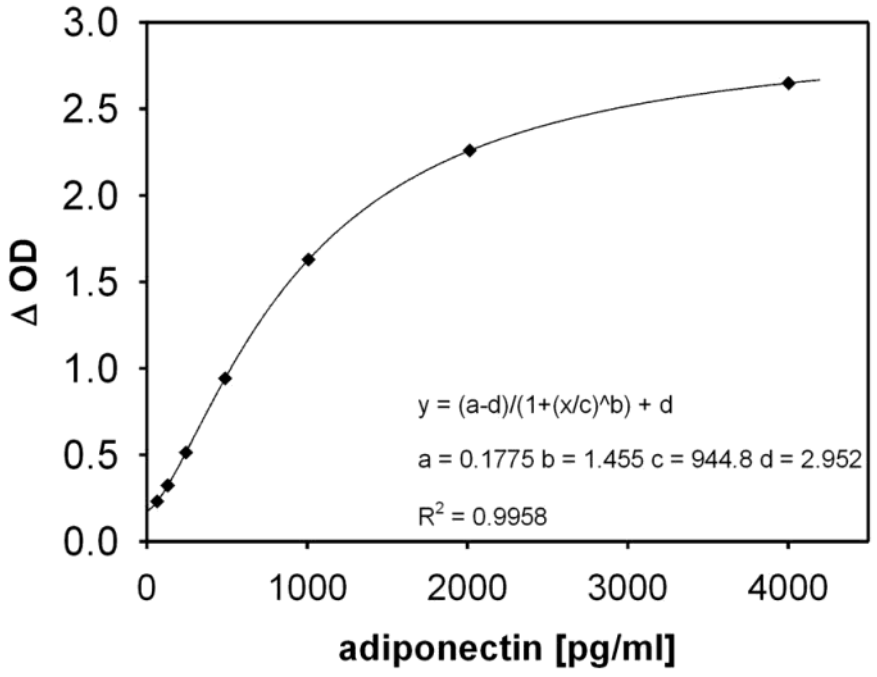

Figure 2.11 Standard curve of the adiponectin ELISA

The Δ OD of the different adiponectin concentrations was plotted against the adiponectin concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the adiponectin ELISA. Δ OD: OD 450 nm – OD 550 nm.

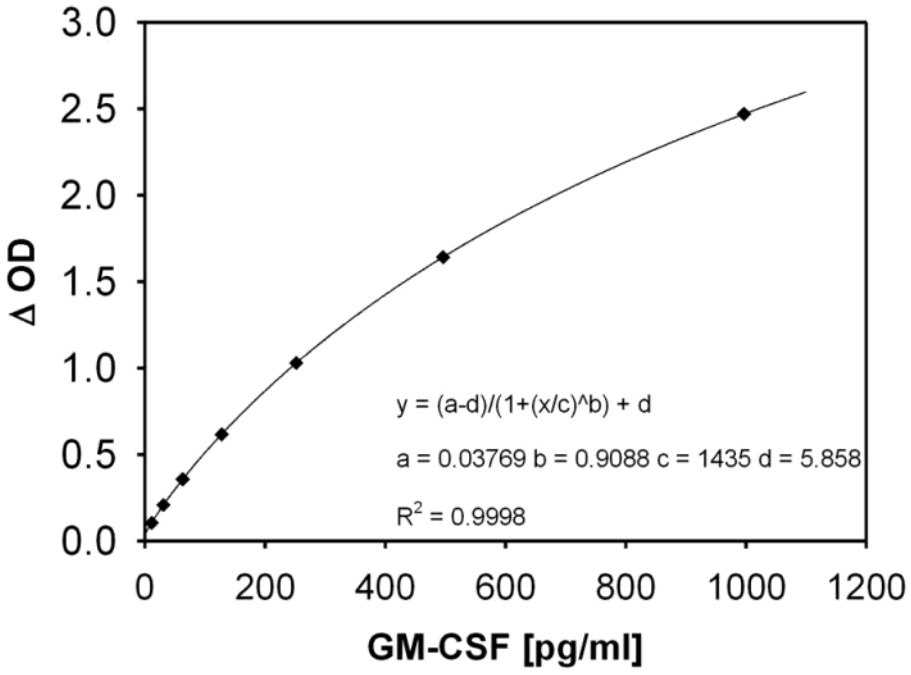

Figure 2.12 Standard curve of the GM-CSF ELISA

The Δ OD of the different GM-CSF concentrations was plotted against the GM-CSF concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the GM-CSF ELISA. Δ OD: OD 450 nm – OD 550 nm.

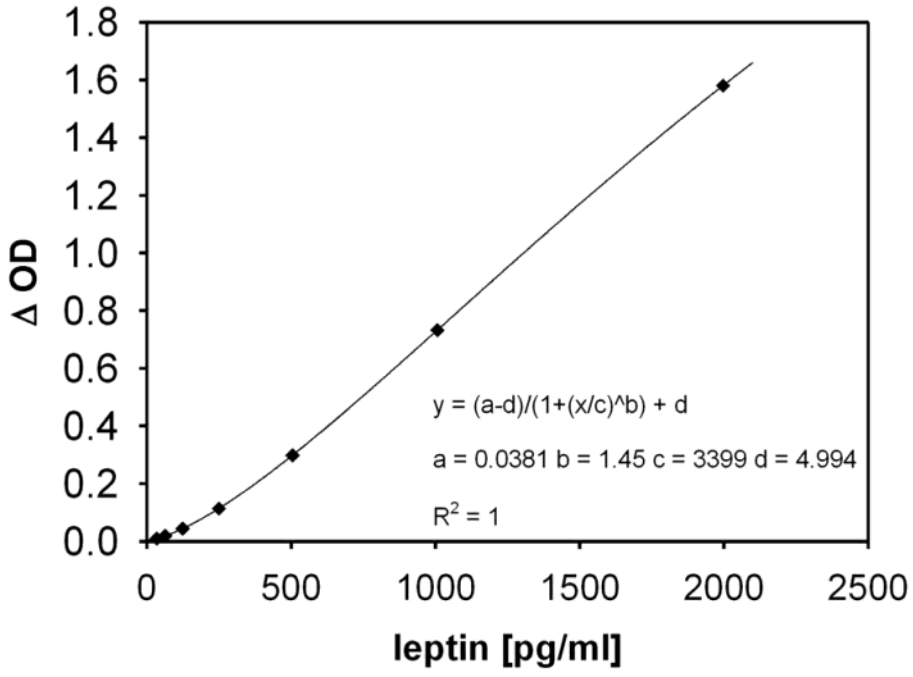

Figure 2.13 Standard curve of the leptin ELISA

The Δ OD of the different leptin concentrations was plotted against the leptin concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the leptin ELISA. Δ OD: OD 450 nm – OD 550 nm.

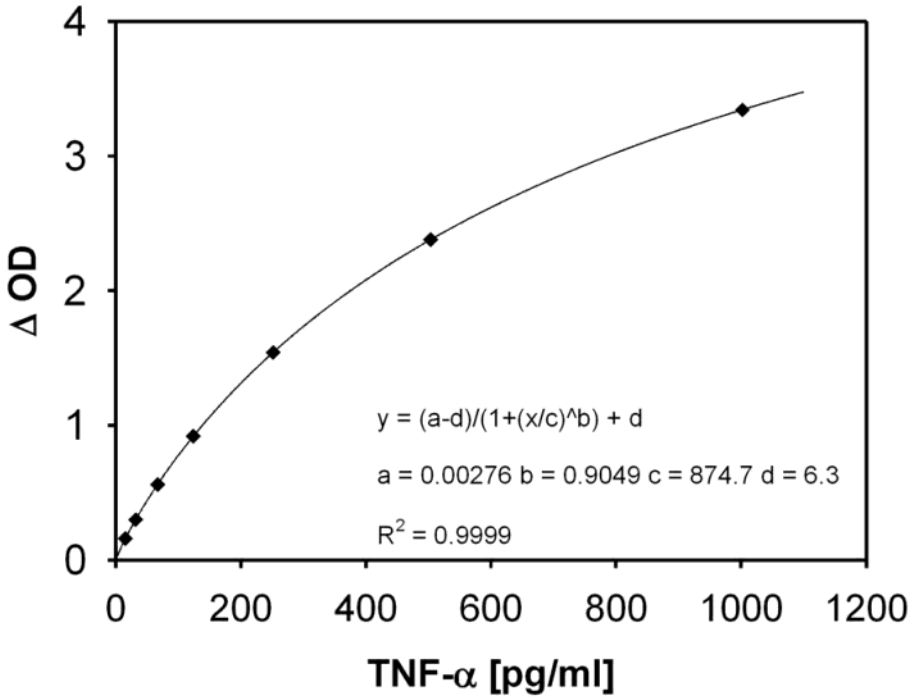

Figure 2.14 Standard curve of the TNF-α ELISA

The Δ OD of the different TNF-α concentrations was plotted against the TNF-α concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the TNF-α ELISA. Δ OD: OD 450 nm – OD 550 nm.

2.3.1 Validation of leptin and adiponectin ELISAs for human samples

All ELISAs used in the present study were certified by the manufacturer to analyse cytokine or adipokine concentrations in cell culture supernatants. Through personal communication (Zoë Webb, Technical Correspondent, R&D Systems), it was established that the leptin ELISA detects both free and soluble receptor bound leptin and that the adiponectin ELISA detects all adiponectin isoforms. However, the leptin and adipokine ELISAs were not certified for the use of human samples. A number of components in human samples such as sample matrix, rheumatoid factor or complement can interfere with the accuracy of an ELISA (Davies, 2001). Therefore, spike/recovery and linearity experiments were carried out to validate the leptin and adiponectin ELISAs for the use of human serum samples. In spike/recovery assays, a known amount of ELISA standard is "spiked" into a sample. The resulting concentration ("recovery" of the spiked sample) indicates whether a component in the sample interferes in the ELISA. In addition, spiked or unspiked samples were also serially diluted to test for linearity. This will give another indication if a sample component is interfering with accurate detection and will also determine the linear dilution range to compare samples with different dilutions.

In addition to the validation, intra-and inter-assay variation and the sensitivity of the leptin and adiponectin ELISAs for human serum and GCF samples were determined. The validation of the adiponectin ELISA for human serum samples was not repeated for GCF due to the small working volume that was available ($\leq$ 200 µl). However, intra-and inter-assay variation of GCF samples provide another indication for assay accuracy and reproducibility.

2.3.1.1 Spike/recovery assay

Leptin and adiponectin are found in the ng and µg range in human serum. Therefore, serum samples were diluted in RD to yield a value within the range of the standard curve. Throughout the study, a 1:16, 1:20 or 1:50 dilution of serum samples was used for detection of leptin, a 1:4444.44 dilution for detection of serum adiponectin. For detection of adiponectin in GCF, samples were diluted 1:3. The dilutions used in the spike/recovery assay were treated as the new "neat" samples. For validation of the leptin ELISA, a 1:16 dilution of human serum was spiked with 800 pg/ml human recombinant leptin. For validation of the adiponectin ELISA, a 1:4444.44 dilution of human serum was spiked with 1200 pg/ml human recombinant adiponectin. RD was spiked with the same amount of recombinant leptin or adiponectin to create a spiked control. The spiked sample, the neat sample and the spiked control were then diluted 1:2, 1:4 and 1:8 in RD in a 2-fold dilution series. Leptin and adiponectin concentrations of all samples were determined with ELISAs as described above. Results were adjusted for the dilutions of the 2-fold dilution series. Recovery of the spiked sample, the control spike and the serial dilutions was calculated as follows:

spiked sample recovery: $\frac{spiked\ sample - neat\ sample}{amount\ spiked}$ x 100

spiked control recovery: $\frac{spiked\ control}{amount\ spiked}$ x 100

example calculation for recovery of dilutions: $\frac{spiked\ sample}{1{:}2\ spiked\ sample}$ x 100

A recovery between 80-120 % is generally accepted as a good indication that the assay is suitable for use with the tested sample (R&D systems).

Results of the spike/recovery assays for leptin and adiponectin are shown in Table 2.4. The spiked sample recovery for leptin and adiponectin was 93 % and 97 %, respectively. This result indicates that no factors in the tested samples interfere with the accuracy of the leptin and adiponectin ELISAs. The recovery of the spiked control was 96 % and 88 % for leptin and adiponectin, respectively. This ensures that the assay procedure itself worked accurately. In addition, the serial dilutions of the spiked sample and control all were within the accepted 80-120 % range. This again gives more confidence in the accuracy of the leptin and adiponectin ELISA for the use of human samples and indicates a linear relationship between the different dilutions. Only the 1:2 dilution of the serial dilution series for the neat samples was within the 80-120 % recovery range. At lower dilutions, the recovery dropped down to 61 % for leptin or was not detectable any more for adiponectin. However, at the lower dilutions of the samples, the OD readings were at the low end or even below the lowest point of the standard curve and therefore not longer accurate measurements. In conclusion, the validation of the leptin and adiponectin ELISAs successfully established the use of these ELISAs for analysis of human serum samples.

sample	leptin [pg/ml]	% recovery	adiponectin [pg/ml]	% recovery
spiked sample	1045.3	93	1414.25	97
1:2 spiked sample	1139.25	109	1284.60	91
1:4 spiked sample	1128.45	108	1298.00	92
1:8 spiked sample	1231.2	118	1291.20	91
neat sample	300.28	–	246.28	–
1:2 neat sample	290.48	97	237.20	96
1:4 neat sample	248.3	83	168.31	68
1:8 neat sample	182.43	61	n.d.	n.d.
spiked control	765.22	96	1050.86	88
1:2 spiked control	754.58	99	979.21	93
1:4 spiked control	729.35	95	916.98	87
1:8 spiked control	846.81	111	892.71	85

Table 2.4 Spike/recovery and linearity of the leptin and adiponectin ELISA for human serum samples

Human serum samples were spiked with 800 pg/ml leptin or 1200 pg/ml adiponectin. RD was spiked with the same amount (spiked control). 2-fold serial dilutions were prepared and spiked samples, neat samples and spiked controls were analysed in leptin and adiponectin ELISAs. Results show the obtained adipokine concentrations corrected for the serial dilution factor and the % recovery. n.d. not detected.

2.3.1.2 Intra-and inter-assay variation and assay sensitivity

Intra-assay variation for leptin and adiponectin ELISAs for serum samples was determined in 7 replicates in one assay. Intra-assay variation for the adiponectin ELISA for GCF samples was determined in 4 replicates in one assay. Inter-assay variation for leptin and adiponectin ELISAs for serum samples was determined in 4 independent assays. The inter-assay variation for the adiponectin ELISA for GCF samples was determined in 3 independent assays. Results are shown in Table 2.5 and Table 2.6. The intra-and inter-assay variation was calculated as follows:

$$\frac{SD}{mean} \text{ x } 100$$

Intra- and inter-assay variation for the leptin ELISA for human serum samples was 3 % and 6 %, respectively. Intra- and inter-assay variations for the adiponectin ELISA for human serum samples were both 4 %. Intra- and inter-assay variation for the adiponectin ELISA for human GCF samples was 4 % and 7 %, respectively. In summary, for all ELISAs and sample types, the intra-and inter-assay variation was less than 10 %. This indicates a high reproducibility for the leptin and adiponectin ELISAs for human serum and GCF samples and increases the confidence for using these ELISAs to analyse serum and GCF.

The assay sensitivity for the leptin and adiponectin ELISA was determined by adding 2.5 standard deviations to the mean OD value of the negative control and calculating the corresponding concentration in the 4 parameter curve fit using the “solve” function in SigmaPlot. The minimum detectable dose was 5.6 and 1 pg/ml for leptin and adiponectin, respectively.

replicate number	serum leptin [ng/ml]	serum adiponectin [µg/ml]	GCF adiponectin [ng/ml]
1	90.22	4.13	3.08
2	96.19	4.27	3.09
3	92.15	3.80	3.08
4	88.86	4.12	2.84
5	89.02	4.07	–
6	89.58	3.89	–
7	89.58	4.25	–
average	90.8	4.08	3.02
SD	2.62	0.17	0.12
Intra-assay variation [%]	3	4	4

Table 2.5 Intra-assay variation of leptin and adiponectin ELISAs for human serum and GCF samples

Human serum and GCF samples were analysed in replicates for leptin and adiponectin in one assay for each sample type and the intra-assay variation was calculated.

assay number	serum leptin [ng/ml]	serum adiponectin [µg/ml]	GCF adiponectin [ng/ml]
I	23.55	4.18	3.47
II	22.52	4.10	3.95
III	20.31	4.08	3.81
IV	22.69	3.85	–
average	22.27	4.05	3.75
SD	1.38	0.14	0.25
Inter-assay variation [%]	6	4	7

Table 2.6 Inter-assay variation of leptin and adiponectin ELISAs for human serum and GCF samples

Human serum and GCF samples were analysed for leptin and adiponectin in independent assays for each sample type and the inter-assay variation was calculated.

2.4 Flow cytometry

Cell-surface receptor expression on THP-1 and primary human monocytes was analysed with flow cytometry.

4 x 10^6 THP-1 monocytes or primary human monocytes per stimulation were cultured as described in 2.1. Cells were collected, centrifuged for 5 min at 300 g, 20 °C (Denley centrifuge, BR401, DJB labcare, Buckinghamshire, UK) and resuspended to a concentration of 0.5 x 10^6 cells/ml in FACS buffer (1 % BSA/PBS with 2 mM EDTA, w/v). 1 ml of cell suspension was transferred to a FACS tube (BD Falcon, Oxford, UK), 2 more ml of FACS buffer were added and cells were centrifuged for 5 min at 300 g, 20 °C. Cells were resuspended in 3 ml FACS buffer supplemented with 10 % human serum (v/v) and incubated under gentle agitation (R100 Rotatest shaker, Luckham, Sussex, UK) at room temperature for 15 min to block nonspecific binding. After blocking, cells were centrifuged as described above and resuspended in ice cold FACS buffer. Cells were centrifuged for 5 min at 300 g, 4 °C and the washing step with ice cold FACS buffer was repeated. Cells were resuspended in 50 µl FACS buffer with different antibodies and controls (TLR4-PE was purchased from eBioscience, Hatfield, UK, all other antibodies from Serotec, Oxford, UK) as indicated in Table 2.7 and incubated on ice, protected from direct light, under gentle agitation for 45 min. After incubation, cells were washed two times with ice cold FACS buffer as described above, finally resuspended in 300 µl FACS buffer and 10,000 events were acquired on a flow cytometer (FACScan, Becton Dickinson, Oxford, UK, also see 2.4.1). Acquired data were analysed with WinMDI 2.8 (Joe Trotter, Miscellaneous software) as described in 2.4.2.

Technical difficulties were encountered during the assessment of TLR4 expression on the cell surface of THP-1 monocytes. First attempts of analysis with FITC-conjugated antibodies (TLR4-FITC, clone HTA125, Serotec and TLR4-FITC, clone HTA125, Santa Cruz, Biotechnology, Wembley, UK) in a similar setting as for TLR2 failed to show any differences to the isotype control. This did also not change after LPS treatment. In fact, fluorescence intensity was often found to be lower than the isotype control. After discussion of this problem with experts in the field (Dr. Desa Lilic, Dr. Stuart Kendrick, Prof. Neil Sheerin, North East Immunology, regional group of the British Society for Immunology) it was decided to use a PE-conjugated TLR4 antibody as a single stain. The PE dye gives a brighter signal than the FITC dye and allowed for analysis of TLR4 expression on the cell surface. However, due the brighter signal, a strong interference between CD14-FITC and TLR4-PE was observed, which could not be compensated. Therefore, it was not possible to analyse TLR4 expression in primary human monocytes.

Antibody	Clone	IgG class	Antigen	Working concentration [µg/ml]
CD14-PE	UCHM1	IgG2a	Mouse-anti human	2.5
Isotype control-PE	MRCOX-34	IgG2a	Mouse-anti rat	2.5
TLR2-FITC	TLR2.3	IgG2a	Mouse-anti human	5
Isotype control-FITC	MRCOX-34	IgG2a	Mouse-anti rat	5
TLR4-PE	HTA 125	IgG2a	Mouse-anti human	2.5

Table 2.7 Working concentrations and specifications for flow cytometry antibodies

The table shows the clone, IgG class and the origin of flow cytometry antibodies used in the present study. Antibody working concentrations were established in titration experiments with the specific antibody and its isotype control.

2.4.1 Acquisition settings

On FSC (forward scatter) and SSC (side scatter) settings, the cell population was gated to exclude any cell debris. FSC and SSC detect light scattered by cells due to physical properties. FSC will give an estimate of the cell size while SSC detects cell granularity. Autofluorescence settings were corrected on an unstained sample in FL-1 (FITC) and FL-2 (PE). Every sample analysed in flow cytometry will have some type of autofluorescence. It is therefore important to ensure that this autofluorescence does not interfere with the actual fluorescence of the dyes which are detected in FL-1 and FL-2. Compensation was corrected on single stained samples (FITC or PE only) in FL-1 or FL-2, respectively. Although each dye has a distinct peak of maximum emission (525 nm for FITC, 575 nm for PE), the FITC and PE emission spectra ranges overlap. This overlap has to be subtracted to avoid false positive signals in FL-1 or FL-2, respectively.

2.4.2 Analysis settings

Different tools of the flow cytometry analysis programme were used to analyse the acquired data. The tools were chosen to meet the characteristic properties of each data set. The cell surface expression of TLR4 on THP-1 monocytes was determined only in single stain experiments. Therefore, FSC was chosen as a second parameter to create dot plots of TLR4 fluorescence for TLR4 analysis (Figure 2.15). Two regions were drawn using TLR4 as the main parameter. Region 1 (R1) was set at the upper endpoint of the isotype control and at the lower endpoint of the control. Region 2 (R2) was set in line with the upper endpoint of R1 and drawn wide enough to include the highest observed TLR4 fluorescence intensity of each experimental sample set. R2 was then analysed for percentage changes in acquired events for TLR4 fluorescence using the

"stats" function. The cell surface expression of TLR2 on THP-1 and primary human monocytes was determined in combination with CD14 expression. Therefore, TLR2 and CD14 were chosen as the two parameters to create dot plots and the quadrant tool was used to analyse the percentages of TLR2 expression (Figure 2.16). The quadrant tool divides each dot plot in 4 quadrants, i.e. an upper right, upper left, lower right and lower left quadrant. The lines of the quadrant were set at the endpoints of TLR2 and CD14 fluorescence intensity for the isotype control in a way so that the isotype control composed the lower left quadrant. In all samples, events for CD14 were in the upper quadrants. Therefore, the "stats" function could be used to analyse the percentage changes in acquired events for TLR2 in the upper right quadrant. The difference between isotype control and CD14 staining was too large and could not be analysed in dot plots. Therefore, histograms of CD14 fluorescence were created (Figure 2.17) and CD14 cell surface expression was analysed with the "stats" function using CD14 median fluorescence intensity. It is important to note that analysis settings were kept the same for each individual experiment as drawing of new regions or shifts of quadrants within an experiment will lead to false results.

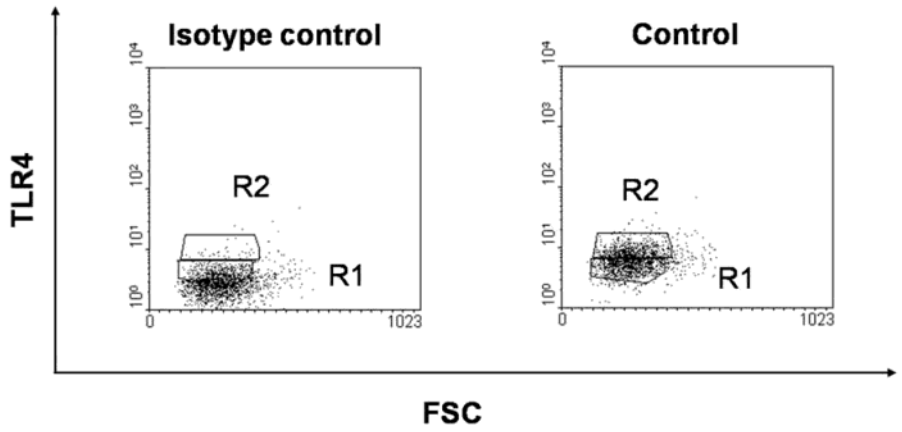

Figure 2.15 Representative examples of analysis settings for TLR4 expression on the cell surface of THP-1 monocytes

THP-1 monocytes were analysed for cell surface TLR4 expression with flow cytometry. Graphs are a representative example of settings for TLR4 analysis. R1: Region 1, R2: Region 2, FSC: Forward Scatter.

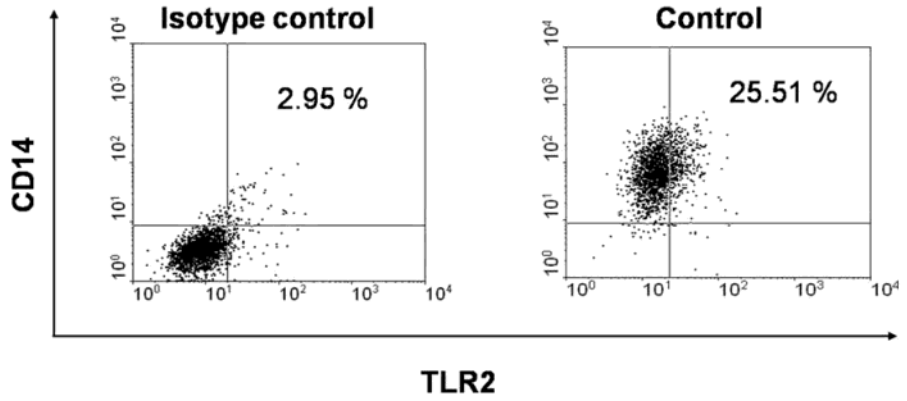

Figure 2.16 Representative examples of analysis settings for TLR2 expression on the cell surface of THP-1 monocytes

THP-1 monocytes were analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graphs are a representative example of settings for TLR2 analysis. Numbers present the percentage of events in the upper right quadrant.

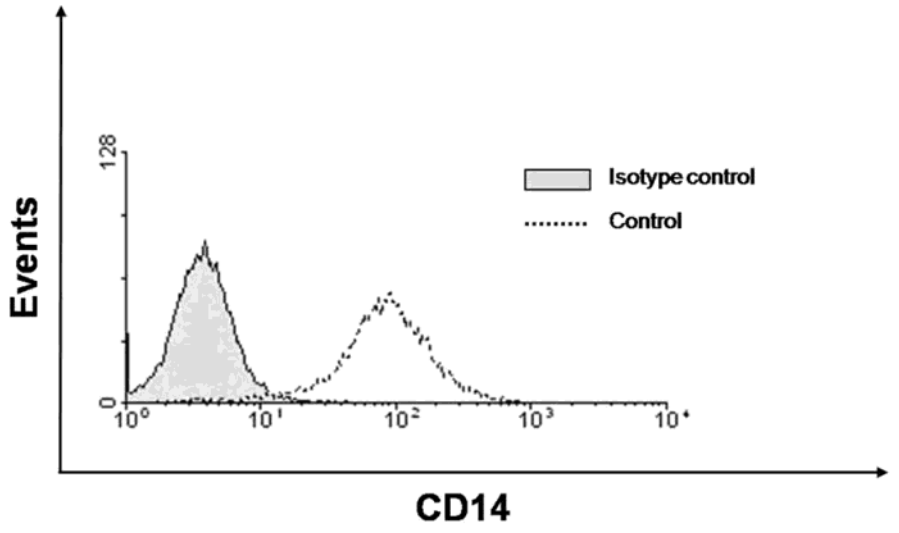

Figure 2.17 Representative examples of analysis settings for CD14 expression on the cell surface of THP-1 monocytes

THP-1 monocytes were analysed for cell surface CD14 expression with flow cytometry. Graph shows histograms as a representative example of settings for CD14 analysis.

2.5 mRNA analysis

2.5.1 RNA extraction

RNA was extracted from THP-1 monocytes with a commercially available kit (GenElute, Mammalian Total RNA Miniprep Kit, Sigma) following the manufacturer's instructions. Cells (4 x 10^6) were lysed with 350 µl of a β-mercaptoethanol/lysis solution mixture (1:100, v/v), filtered through a column and centrifuged for 2 min at 13000 rpm, room temperature (Biofuge pico, Heraeus, DJB labcare). The filtration column was discarded and the lysate was frozen at -80 °C until further processing.

Upon thawing, 350 µl of 70 % ethanol was added to the lysate and the solution was centrifuged for 15 s at > 10 rpm, room temperature, through a silica membrane column. The column was washed three times with washing solutions by centrifugation as before. Finally, the RNA was eluted with 50 µl of elution solution by centrifugation. The concentration of RNA in each sample was determined in ng/µl with a reading at 260 nm on a spectrophotometer (ND-1000, NanoDrop Technologies, Thermo Fisher Scientific). Samples were stored at -80 °C until further processing.

2.5.2 Reverse transcription of RNA

Extracted RNA from each sample was reverse transcribed into cDNA with a commercially available kit (High capacity cDNA reverse transcription kit, Applied Biosystems, Warrington, UK) following the manufacturer's instructions. A 2 X master mix of buffer, enzymes (reverse transcriptase and RNase inhibitor), dNTPs and random primers was prepared on ice as follows:

2 µl 10 X reverse transcription buffer

0.8 µl 10 X Random Primers

2 µl 25 X dNTP mix

1 µl MultiScribe Reverse Transcriptase

1 µl RNase Inhibitor

3.2 µl Nuclease-free H_2O

1 µg of RNA, adjusted to an equal volume of the master mix with nuclease-free water, was added. The reverse transcription reaction was run on a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) with the following settings:

25 °C 10 min – 37 °C 120 min – 85 °C 5 s – 15 °C ∞

cDNA was stored at 4 °C until further processing.

2.5.3 Realtime RT-PCR

In a standard PCR, DNA is detected after the exponential phase of amplification. In contrast, realtime PCR analyses DNA amplification during the exponential phase, where quantitative differences between the amounts of input DNA can still be detected. In the present study, TaqMan Probes were used in Realtime RT-PCR to quantify differences in mRNA expression levels. TaqMan Probes are a combination of a fluorescent probe and the forward and reverse primers for the gene of interest. The probe has a reporter dye (FAM-6) at the 5' end and a nonfluorescent quencher dye at the 3' end attached to it, which suppresses the reporter dye fluorescence unless

activated. During DNA amplification, the 5'-3' nuclease activity of the DNA polymerase cleaves the probe, resulting in separation of the quencher dye from the reporter dye. The now increased fluorescence can be recorded with a detection system. The probe attaches itself only to the DNA between the forward and reverse primer sequences, therefore unspecific fluorescence does not occur during the replication process. While the accumulation of PCR products is monitored over several replication cycles, a cycle threshold (Ct) value of fluorescence is noted for each cycle. The Ct value is the point at which the fluorescence signal of the accumulated PCR product exceeds a set background fluorescence value. The lower the Ct value, the more DNA of the gene of interest was in the sample and therefore the threshold is reached earlier. To determine the amount of cDNA for the target gene, cDNA of a reference gene of the same sample is amplified at the same time. The reference gene is expressed at a constant level and relative fold changes in the mRNA expression of the target gene after different stimulations can be calculated with the comparative Ct method ($2^{-\delta\delta Ct}$) (Livak and Schmittgen, 2001) as follows:

1. $\delta Ct = Ct_{(\text{target gene})} - Ct_{(\text{reference gene})}$

2. $\delta\delta Ct = \delta Ct_{(\text{stimulation})} - \delta Ct_{(\text{control})}$

3. $2^{-\delta\delta Ct}$

TaqMan Gene Expression Assays (Applied Biosystems) with a Realtime PCR kit (SensiMixdT, Quantace, London) were used for quantification of cDNA. The assays were performed according to the manufacturer's instructions. All samples were analysed in duplicates and a negative control with nuclease-free water was added.

A master mix of SensiMix with either the TaqMan Probe (GM-CSF: Hs00171266_m1; TLR2: Hs00152932_m1; TLR4: Hs00152939_m1; TNF-α: Hs00174128_m1) for the target gene or a probe for RNA polymerase II (Hs00172187_m1) as the reference gene was prepared on ice as follows:

12.5 µl 2 X SensiMix

1.25 µl TaqMan Probe

8.75 µl Nuclease-free H_2O

2.5 µl of cDNA for each sample were added and the assay was run on a Realtime thermal cycler (ABI Prism, 7000 Sequence Detection System, Applied Biosystems) at the following cycle:

50 °C 2 min – 95 °C 10 min – 40 X | 95 °C 15 s – 60 °C 1 min |

Relative fold changes between stimulations were calculated with the comparative Ct method ($2^{-\delta\delta Ct}$).

2.6 Immunofluorescence analysis

5 x 10^5 THP-1 or primary human monocytes were seeded in 500 µl cell culture medium in a 24-well tissue culture plate on glass cover slips (∅ 13 mm, VWR International). Cells were stimulated after two days. Supernatants were removed and cells were fixed for 5 min with ice cold methanol. Coverslips were transferred to a new 24-well tissue culture plate and washed three times with PBS. Nonspecific binding was blocked for 1 h with wash buffer (PBS/0.05 % Tween-20), supplemented with 1 % BSA. After blocking, cells were incubated with 2 µg/ml of the primary antibody PU.1 (PU.1 (H-

135) rabbit polyclonal, Santa Cruz, Biotechnology) in PBS overnight at 4 °C. The following day, cover slips were washed three times with wash buffer and incubated with a secondary, FITC conjugated antibody (sheep-anti-rabbit IgG-FITC, 1:200 in PBS) for 1 h. An unstained sample and a sample stained with the secondary antibody only were included as a negative control. Cover slips were washed three times with wash buffer and once with PBS, transferred to microscope slides and mounted with a hard set DAPI mounting medium (Vector Laboratories, Peterborough, UK). An additional cover slip was placed on top. Cell images of THP-1 monocytes were acquired on a confocal laser scanning microscope (TCS SP2 UV, Leica, Heidelberg, Germany) within three days with the help of Dr. Trevor Booth (Bio-Imaging Lab Manager, Clinical and Laboratory Sciences, Newcastle University, Newcastle upon Tyne, UK). Cell images of primary human monocytes were acquired on a standard fluorescence microscope (BX61, Olympus, Essex, UK) within three days with the help of Dr. Jared Thornton (Research Associate, Oral Biology Research Lab, Newcastle University, Newcastle upon Tyne, UK).

Microscope settings were based on samples stained with the secondary antibody only and were kept the same throughout each individual experiment. Figure 2.18 shows an example of the microscope settings. The obtained images are similar between the unstained sample (Figure 2.18a) and the secondary antibody stain only (Figure 2.18b). The DAPI stain clearly confirms the presence of cells, while almost no fluorescence can be observed in the unstained sample or the sample with the secondary antibody stain only. These results confirm that there is no interference from autofluorescence or from nonspecific binding of the secondary antibody.

Additionally, to obtain a numerical output of differences in staining intensities, for THP-1 monocytes FITC pixel intensities of the 5 brightest cells per slide were evaluated with the histogram function of the image processing and analysis software ImageJ (National Institute of Health, USA).

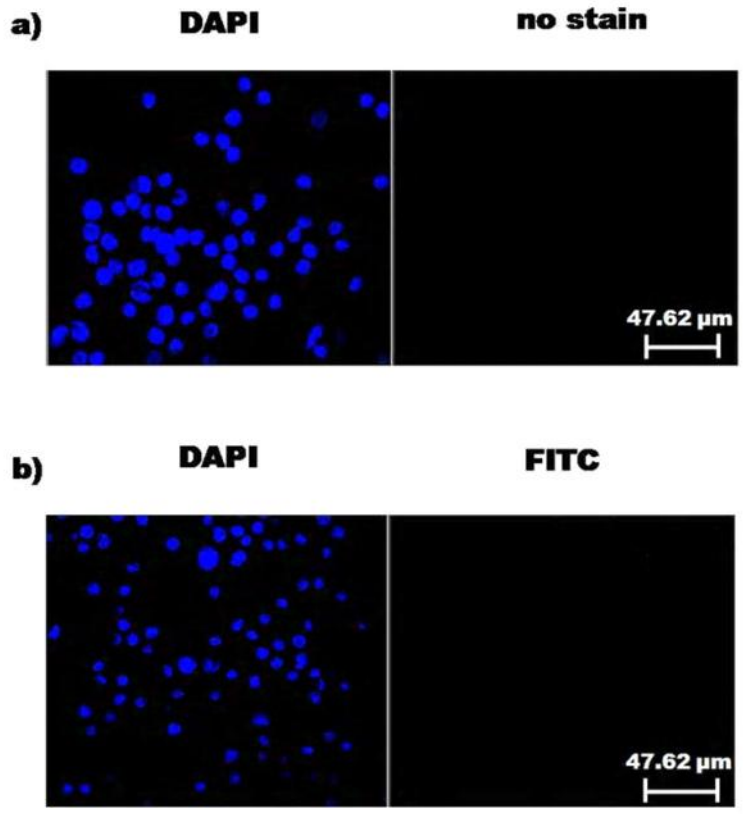

Figure 2.18 Settings for the confocal laser scanning microscope and the standard fluorescence microscope

THP-1 monocytes were processed for fluorescence microscopy and analysed on a confocal laser scanning microscope. Sensitivity settings of the confocal laser scanning microscope were adjusted to a background level of FITC fluorescence of cells stained with the secondary antibody only (b). Cells without a FITC stain were included as an additional control for autofluorescence (a).

2.7 Immunoprecipitation and Western blot

2.7.1 Lysis of cells

4 x 10^6 THP-1 monocytes were collected and centrifuged for 5 min at 2000 rpm, 20 °C. The cell pellet was resuspended in 100 µl (500 µl for immunoprecipitation, IP, see 2.7.2) cell lysis buffer (0.05 M HEPES, w/v; 0.1 M NaCl, w/v, BDH Laboratory supplies; 0.5 % Nonidet-P40, v/v; 0.01 M EDTA, w/v) and incubated on ice for 15 min. The cell lysate was centrifuged for 10 min at 12000 g, 4 °C. 20 µl of the supernatant were retained for total protein analysis as described in 2.7.3. 80 µl were diluted 1:1 with sample loading buffer (0.09 M Tris, pH 6.8, w/v; 20 % Glycerol, v/v, BDH Laboratory supplies; 2 % SDS, w/v; 0.02 % Bromophenol Blue, w/v; 0.1 M DTT, w/v) and frozen at -20 °C for future analysis.

2.7.2 Immunoprecipitation

The Sigma Protein G Immunoprecipitation kit was used for immunoprecipitation of PU.1, following the manufacturer's instructions. Cells were collected and lysates prepared as described above (2.7.1). 500 µl of cell lysate was centrifuged for 15 min, at 13000 rpm, 4 °C. Supernatants were transferred to mini spin columns as described by the manufacturer, 2 µl of rabbit-anti human PU.1 (PU.1 (H-135) rabbit polyclonal, Santa Cruz, Biotechnology) were added and columns were incubated over night head over tail on a rotating wheel (Dynal Sample mixer, Invitrogen) at 4 °C. The next day, 30 µl of Protein G sepharose beads were washed 3 times with 1 ml of ice cold PBS for 30 s, at 12000 g, 4 °C. The beads were resuspended in 50 µl ice cold PBS and transferred to each IP reaction column. Columns were incubated for 1 h head over tail on a rotating wheel at 4 °C. The bottoms of the columns were opened, the columns placed in an

eppendorf tube and centrifuged for 1 min at 13000 rpm, 4 °C. The flow through was discarded and the beads in each of the columns were washed 5 times with 600 µl cell lysis buffer for 1min, at 13000 rpm, 4 °C. The bottoms of the columns were closed and beads were resuspended in 40 µl sample loading buffer. The columns were placed in eppendorf tubes and heated for 5 min, at 95 °C on a dry heat block (Techne Dri-Block, DB.2A, Sigma-Aldrich). The bottoms of the columns were opened again and the columns centrifuged in an eppendorf tube for 1 min at 13000 rpm, 4 °C. The immunoprecipitate was processed immediately for Western blot.

2.7.3 Western blot

Protein concentration and phosphorylation state of total cell lysates and immunoprecipitates were analysed with Western blot. A 12 % SDS-Polyacrylamide gel (resolving: 4.8 ml H2O; 6 ml 30 % Bis-Acrylamide, w/v; 3.9 ml 1.5 M Tris, pH 8.8, w/v; 150 µl 10 % SDS, w/v; 150 µl 10 % Ammoniumpersulfate, w/v; 6 µl TEMED; stack: 5.6 ml H2O; 1.3 ml 30 % Bis-Acrylamide, w/v; 1 ml 1 M Tris, pH 6.8, w/v; 80 µl 10 % SDS, w/v; 80 µl 10 % Ammoniumpersulfate, w/v; 8 µl TEMED) was prepared with the Atto system (AE-6450, Genetic research Instr. Ltd., Essex, UK).

Samples were heated for 5 min, at 95 °C on a dry heat block and 20 µl of sample were loaded on each lane of the SDS-Polyacrylamide gel in running buffer (0.2 M Glycine, 0.025 M Tris, 0.1 % SDS, w/v). A broad range prestained protein marker (New England Biolabs, Hitchin, UK) was equally loaded on the same gel for reference of protein sizes. The electrophoresis was run for 1 ½ h at 120 V, 180 mAmp (Consort, Flowgen Instruments Ltd., Kent, UK).

Proteins were transferred to a Hybond P PVDF Membrane (GE Healthcare, Amersham, Buckinghamshire, UK) with the wet transfer system from Bio-Rad (Mini Trans Blot, Bio-Rad Laboratories, Hertfordshire, UK) in transfer buffer (0.2 M Glycine, 0.025 M Tris, w/v), on ice. Prior to transfer, the membrane was pre-wetted with methanol and equilibrated in transfer buffer according to the manufacturer's instructions. The transfer was run for 1 h at 100 V, 180 mAmp (Consort, Flowgen Instruments Ltd.). To confirm transfer of proteins from gel to membrane, membrane was covered with Ponceau red (Ponceau S solution) for 2 min to detect protein lanes. The membrane was washed with TBS-T (8.5 g NaCl, BDH Laboratory supplies; 6.5 g Tris; 1 ml Tween 20 in 1 l H_2O, pH 7.5) until clear. The membrane was blocked for 1 h at room temperature with blocking solution (45 ml TBS-T, 2.5 ml Horse Serum, 2.5 g Marvel non-fat dried milk, Premier International Foods, Lincs, UK), followed by overnight incubation with the primary antibody mouse anti-Phosphoserine (clone PSR-45, 2.5 µg/ml, in blocking solution) or rabbit-anti human PU.1 (PU.1 (H-135) rabbit polyclonal, 1:500, in blocking solution) at 4 °C. Next day, the membrane was washed 5 times with TBS-T and incubated with the secondary HRP-linked polyclonal antibody (sheep-anti mouse 1:10000, or donkey-anti rabbit 1:20000, in blocking solution, GE Healthcare, Amersham) for 1 h at room temperature. The membrane was washed 2 times for 5 min in TBS-T and further 3 times in TBS (TBS-T without Tween). The membrane was developed with the ECLPlus Western Blotting Detection System (GE Healthcare, Amersham) according to manufacturer's instructions and the chemiluminescence was detected on an X-ray film (Lumi-Film Chemiluminescent Detection Film, Roche Diagnostics Ltd., Welwyn Garden City, UK) with an X-ray developer (Konica SRX-101A).

2.8 Subjects

The cross-sectional and longitudinal investigation of serum and GCF adipokine concentrations in T2DM patients and non-diabetic controls with and without periodontal disease was conducted with the help of a clinical team. The clinical team (Prof. Philip Preshaw, Rebecca Wassall, Susan Bissett, Hannah Fraser, Kerry Stone, School of Dental Sciences, Newcastle University) was involved in the recruitment, diagnosis and treatment of subjects, the obtaining of blood and GCF samples from subjects and in the overall conducting of the clinical study.

2.8.1 Recruitment of subjects

T2DM patients were recruited from GP practices and secondary care diabetes clinics, non-diabetic controls from consultant clinics at the Newcastle Dental Hospital or from staff of the Dental School, Newcastle University. Subjects were matched for age and smoking status. Table 2.8 gives an overview of the general characteristics of the study group. No significant differences between female T2DM patients and non-diabetic controls in subject numbers, age, smoking status or ethnicity were detected. There were significantly more male T2DM patients than non-diabetic controls ($p < 0.05$) and significantly more male T2DM ex-smokers than non-diabetic controls ($p < 0.05$). No significant differences between male T2DM patients and non-diabetic controls in subject age, ethnicity or the other smoking status were detected.

		T2DM male/female	Non-diabetic control male/female
n		66*/34	44/32
age (*years*)		49 ± 6/48 ± 7	48 ± 7/48 ± 8
years since T2DM diagnosis		7 ± 5/7 ± 5	–/–
ethnicity (*n*)	**caucasian**	63/32	44/32
	black	–/1	–/–
	asian	3/1	–/–
smoking status (*n*)	**current**	3/2	5/3
	ex	17*/7	14/9
	non	46/25	25/20

Table 2.8 General characteristics of the study population

The table shows characteristics of the study groups according to diabetic status and gender. Age and years since T2DM diagnosis are given as mean ± SD. Statistics: X^2-tests and Student's *t*-tests *$p < 0.05$ compared to non-diabetic controls within the corresponding gender group.

2.8.2 Screening and treatment of subjects

Subjects were classified according to periodontal status as periodontally healthy, or having gingivitis or chronic periodontitis following consideration of diagnostic criteria that were proposed by the 2005 European Workshop on Periodontology and the 2007 Centres for Disease Control and Prevention-American Academy of Periodontology collaboration (Tonetti and Claffey, 2005; Page and Eke, 2007). Alveolar bone loss was confirmed by radiography. After screening, periodontal non-surgical treatment was provided for patients with periodontal disease using oral hygiene instruction and a full mouth instrumentation approach (Quirynen *et al.*, 2000). The subjects were followed up after three and six months and further periodontal treatment was provided as necessary. Subjects with gingivitis were given oral hygiene instruction and a full mouth prophylaxis at the time of screening but were not followed up any further. The study was approved by the Sunderland Research Ethics Committee (ref 06/Q0904/8).

2.8.3 Obtaining and processing of blood and GCF samples

Blood and GCF samples were taken by the clinical team pre-treatment from all subjects at the screening appointment and at month 3 and month 6 after periodontal treatment for subjects with periodontitis (Figure 2.19). Blood samples were obtained by venepuncture and were non-fasting. One part of the blood sample was sent to a clinical laboratory (Haematology and Clinical Biochemistry labs of the Royal Victoria Infirmary, Newcastle upon Tyne, UK) for analysis of HbA1c and hsCRP. The other part of the blood sample was retained for analysis of serum adipokine concentrations and was kept on ice until further processing. Within 1 h, blood samples were centrifuged for 15 min at 4 °C and 1500 g in a Sigma 3K10 centrifuge (Sigma) and serum was collected and frozen at -80 °C until further analysis with ELISAs. For GCF samples, PerioPaper strips

(ProFlow, New York, USA) were inserted for 30 s into the gingival crevice and subsequently placed in a sterile cryovial containing 150 µl PBS. Four GCF samples were taken from four different teeth in each subject. At the screening appointment, GCF samples were obtained from the first molars, choosing the visibly most inflamed sites with likely the deepest pocket depths. After conduction of a full mouth probing, the chosen sites were confirmed to be of ≥ 5 mm pocket depths. GCF was resampled at the treatment appointment in the event of the sites having a probing depth of < 5 mm. GCF samples were frozen at -80 °C within 1 h. For elution of GCF from the PerioPaper strips, GCF samples were thawed and 50 µl of 1% BSA in PBS (w/v) was added. Samples were then centrifuged (Sigma 3K10 centrifuge) for 60 min at 300 rpm, 4 °C, followed by a centrifugation step at 12000 rpm, 2 min, 4 °C. The PerioPaper strips were removed with tweezers. Samples were frozen again at -80 °C until further analysis with ELISAs.

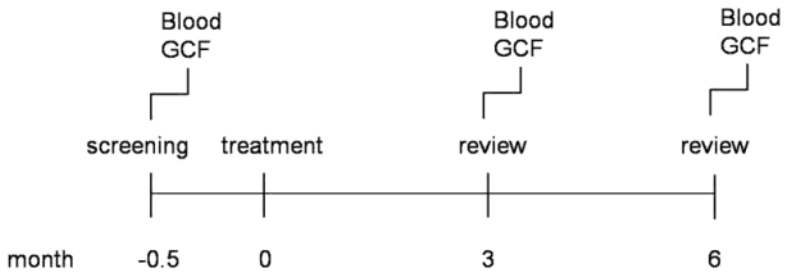

Figure 2.19 Overview of the study timecourse

Blood and GCF samples were taken from all study subjects at the screening appointment. Treatment for patients with periodontal disease was commenced within 2 weeks from initial screening. Subjects with periodontal disease were followed up 3 and 6 months after treatment and blood and GCF samples were obtained.

2.9 Statistical analysis

Statistical analysis was perfomed in SPSS 15.0. All graphs except for box plots were created in Sigmaplot 11.0. Box plots were created in SPSS 15.0.

2.9.1 Analysis of cell culture data

Parametric data were analysed with ANOVA. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. Standard transformations such as *square root*, *common log* or *inverse* were used to achieve normal distribution and equality of variance. Non-parametric data were analysed with Kruskal-Wallis. Student's *t*-test or Mann-Whitney *U* test were applied for *post hoc* analyses of parametric or non-parametric data, respectively. *P*-values were corrected for multiple comparisons with Bonferroni-Holm. A p-value of < 0.05 was considered significant.

Flow cytometry and Realtime RT-PCR data

The percentage data obtained from flow cytometry were transformed with *arcsine* prior to further analysis. Statistical analyses of Realtime RT-PCR data were performed on δCt values as described by Yuan and colleagues (Yuan *et al.*, 2006).

2.9.2 Analysis of clinical data

X^2-test was used to analyse discrete variables. Crossectional parametric data were analysed with ANOVA. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. Data were transformed with *common log* to achieve normal distribution when necessary. Non-parametric data were analysed with Kruskal-Wallis. Student's *t*-test or Mann-Whitney *U* test were

applied for *post hoc* analysis of parametric or non-parametric data, respectively. Longitudinal parametric data were analysed with repeated measures ANOVA and paired samples *t*-test. Shapiro-Wilk testing for normal distribution and Mauchly's test of sphericity was performed prior to analysis. Data were transformed with *common log* to achieve normal distribution when necessary. Non-parametric data were analysed with Friedman test and Wilcoxon Mann-Whitney test. A p-value of < 0.05 was considered significant. *P*-values were corrected for multiple comparisons with Bonferroni-Holm. Spearman correlations were used to analyse relationships between two parameters. Linear regression analysis was used to determine the ability of BMI to predict adipokine concentrations. Multinomial logistic regression analysis was applied to test the value of BMI and adipokines as predictors of periodontal status categorized as periodontal health, gingivitis and periodontitis.

Chapter 3 The effect of leptin on TNF-α expression in monocytes

Chapter 3 The effect of leptin on TNF-α expression in monocytes

3.1 Introduction

The adipose tissue is not only a direct source for a number of pro-inflammatory cytokines and chemokines itself, through the production of other adipokines such as leptin, visfatin or resistin, it is also a source for proteins which themselves have potent pro-inflammatory immune regulatory properties (Kershaw and Flier, 2004). Whereas knowledge on the role of visfatin and resistin in immunity is still relatively limited, several studies have investigated responses of both leukocytes and other immune cell types to the adipokine leptin in humans. For example, in lymphocytes and NK cells, leptin was shown to promote cellular immune responses and to support a T_h1 cytokine expression pattern by enhancing IFN-γ secretion and suppressing IL-4 (Lord *et al.*, 1998; Martin-Romero *et al.*, 2000; Zhao *et al.*, 2003). Furthermore, in non-immune cells such as fibroblasts and endothelial cells, leptin induces the expression of pro-inflammatory chemokines (Bouloumie *et al.*, 1999; Aleffi *et al.*, 2005; Tong *et al.*, 2008). In addition, some studies demonstrate that leptin can induce pro-inflammatory cytokines such as IL-6 and TNF-α in PBMCs and in monocytes (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001; Zarkesh-Esfahani *et al.*, 2004). Thus, Santos-Alvarez *et al.* (1999) and Zarkesh-Esfahani *et al.* (2001) found increased intracellular accumulation of pro-inflammatory cytokines in $CD14^+$ cells after leptin stimulation. Furthermore, Zarkesh-Esfahani *et al.* (2001) detect increased IL-6 and TNF-α concentrations in supernatants and enhanced IL-6 and TNF-α mRNA expression in leptin stimulated PBMC cultures. In combined cultures of monocytes and neutrophils these authors also demonstrate leptin-dependent activation of neutrophils through

increased TNF-α production by monocytes (Zarkesh-Esfahani *et al.*, 2004). Additionally, increased pro-inflammatory cytokine concentrations were detected in supernatants of leptin stimulated cultures of DCs (Mattioli *et al.*, 2005). In contrast, reports on cultures of isolated murine peritoneal macrophages or Kupffer cells show that leptin alone had no capacity to induce TNF-α or IL-6 secretion in these cells (Loffreda *et al.*, 1998; Shen *et al.*, 2005).

Although it is possible that species differences or the different cell types studied play a role in the different findings between the studies, it appears more likely that in isolated cell cultures in some cases leptin alone is an insufficient signal for the release of pro-inflammatory cytokines. For example, TNF-α is produced as a membrane bound pro-form and the active form is only released after enzymatic processing which can be induced by stimuli such as LPS or PMA (Mullberg *et al.*, 2000). Strikingly, although Loffreda *et al.*(1998) and Shen *et al.* (2005) find that leptin alone had no effect, they do show that pre- or simultaneous incubation of cells with leptin enhanced LPS-induced TNF-α and IL-6 secretion. This LPS-enhancing effect of leptin on pro-inflammatory cytokine production was also demonstrated in humans in the studies of Santos-Alvarez *et al.* (1999) in PBMCs and Mattioli *et al.* (2005) in monocyte-derived DCs. However, no studies yet have investigated the effect of leptin or leptin and LPS stimulation in single human monocyte cultures and thus it is unknown if this adipokine on its own or in combination with LPS induces a pro-inflammatory immune response in human monocytes or if this effect is exclusive to more differentiated myeloid cells such as DCs (Mattioli *et al.*, 2005).

As one of the first immune cells in contact with pathogens and potent producers of TNF-α (see 1.2.1), any changes of monocyte-immune responses to pathogens could

have an effect on recruitment or responses of other immune cells and eventually alter monocyte differentiation into macrophages and DCs under inflammatory conditions. Clearly, the LPS-enhancing effect of leptin is of biological relevance. Leptin or leptin receptor deficient animals show decreased IL-6 and TNF-α serum concentrations after LPS stimulation (Loffreda *et al.*, 1998) and have a higher susceptibility to endotoxaemia (Yang *et al.*, 1997). However, while the LPS-enhancing effect of leptin may be beneficial in short term acute situations to promote a potent inflammatory immune response against pathogens, an exaggerated immune response could prove detrimental in situations of chronic exposure to inflammatory stimuli. Thus, although *ob*/*ob* mice are more susceptible to endotoxaemia (Yang *et al.*, 1997), at the same time they are protected against toxic effects of TNF-α (Takahashi *et al.*, 1999) and in several models of inflammation such as autoimmune encephalomyelitis, collagen induced arthritis or colitis (Matarese *et al.*, 2001; Busso *et al.*, 2002; Siegmund *et al.*, 2002). Hence, it is conceivable that chronically elevated leptin concentrations in obesity and diabetes potentially lead to exaggerated immune responses.

Diabetes is a risk factor for severe periodontal disease (Khader *et al.*, 2006), an inflammatory condition of the gingival and periodontal tissues induced by an overreaction of the immune system towards bacterial plaque (Dixon *et al.*, 2004). Although other factors such as AGEs (King, 2008) and physiological changes in gingival tissues (Brennan, 1989; Tepper *et al.*, 2002; Bhatwadekar *et al.*, 2008) likely contribute to the exaggerated immune response, potentially the LPS-enhancing effect of leptin is a major factor as well. However, all studies so far have investigated this leptin effect in combination with LPS from the enterogenic pathogen *E. coli* whereas actually plaque is composed of numerous different bacterial species, including key periodontal

pathogens such as *P. gingivalis* (Lamont and Jenkinson, 1998; Socransky *et al.*, 1998). Furthermore, in contrast to the TLR4 ligand *E. coli* LPS, LPS from *P. gingivalis* is a known TLR2 ligand and induces differential cytokine gene and secretion patterns (Zhou *et al.*, 2005; Barksby *et al.*, 2009). Thus, it is not known if leptin would also have an LPS-enhancing effect for *P. gingivalis*, which could eventually alter pro-inflammatory cytokine expression and hence periodontal disease pathogenesis. Therefore, the aims of the present study were first to investigate the effect of leptin or *E. coli* and *P. gingivalis* LPS alone on TNF-α secretion and mRNA expression in human monocytes and second to investigate the effect of a combined challenge with leptin and *E. coli* or *P. gingivalis* LPS on TNF-α secretion and mRNA expression in human monocytes.

3.2 Results

3.2.1 Investigation of the effect of leptin and LPS stimulation on TNF-α protein secretion in THP-1 and primary human monocytes

THP-1 monocytes (0.5×10^6) were stimulated with 500 ng/ml leptin for 5 h. LPS from *E. coli* or *P. gingivalis* (100 ng/ml) was then added for 2 or 24 h. Unstimulated cells and cells stimulated with leptin or LPS alone served as controls. Supernatants were collected and analysed for TNF-α with ELISA as described in 2.3.

Single LPS stimulations induced TNF-α secretion in THP-1 monocytes at both timepoints. At 2 h, a concentration of 1535 ± 358 and 2089 ± 281 pg/ml TNF-α was detected in cell culture supernatants for *E. coli* (Figure 3.1) or *P. gingivalis* (Figure 3.2) LPS, respectively. At 24 h, TNF-α concentrations were lower and reached a concentration of 484 ± 223 and 724 ± 195 pg/ml for *E. coli* (Figure 3.3) or *P. gingivalis* (Figure 3.4) LPS, respectively. At both timepoints, neither cells stimulated with leptin alone nor unstimulated controls produced detectable levels of TNF-α in cell culture

supernatants (Figure 3.1-Figure 3.4). At 2 h, TNF-α concentrations in cell culture supernatants were significantly higher in cells stimulated with both leptin and LPS than in cells stimulated with LPS alone. Compared to single LPS stimulations, leptin enhanced LPS-induced TNF-α secretion by 29 % ($p < 0.05$) for both *E. coli* and *P. gingivalis* LPS (Figure 3.1, Figure 3.2). At 24 h, the stimulatory effect of leptin on LPS-induced TNF-α secretion did not reach statistical significance (Figure 3.3, Figure 3.4).

At 2 h, TNF-α concentrations in cell culture supernatants of monocytes stimulated with *P. gingivalis* LPS were 36 % higher ($p < 0.05$) than in cell culture supernatants stimulated with *E. coli* LPS (Figure 3.1, Figure 3.2). No significant difference was detected between cells stimulated with *P. gingivalis* or *E. coli* LPS at 24 h (Figure 3.3, Figure 3.4).

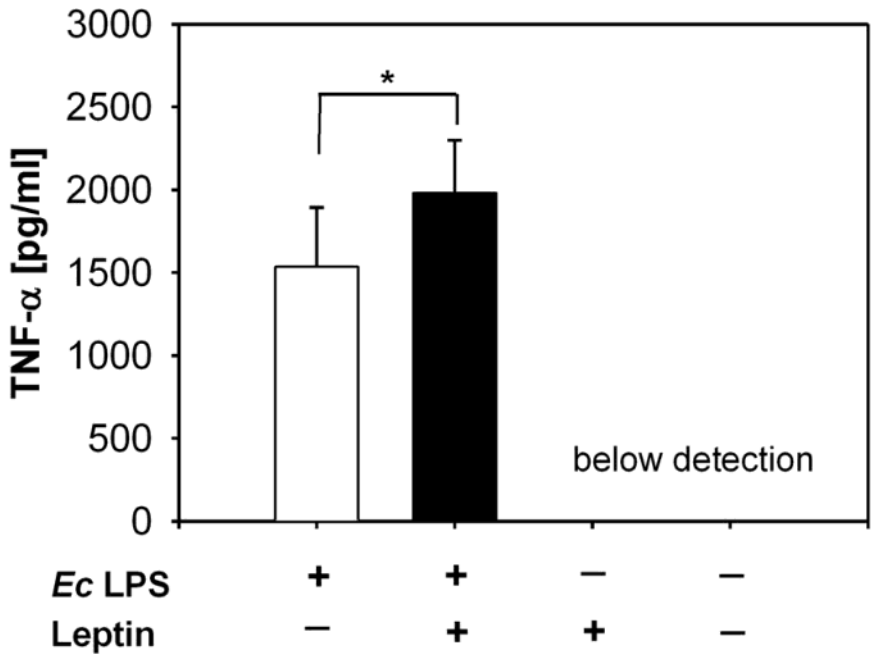

Figure 3.1 The effect of leptin on TNF-α secretion in THP-1 monocytes stimulated with *E. coli* LPS for 2 h

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. LPS from *E. coli* (100 ng/ml) was then added for 2 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents mean + SD from three independent cell culture experiments. Statistics: ANOVA, Student's *t*-test $*p < 0.05$. *Ec*: *E. coli*.

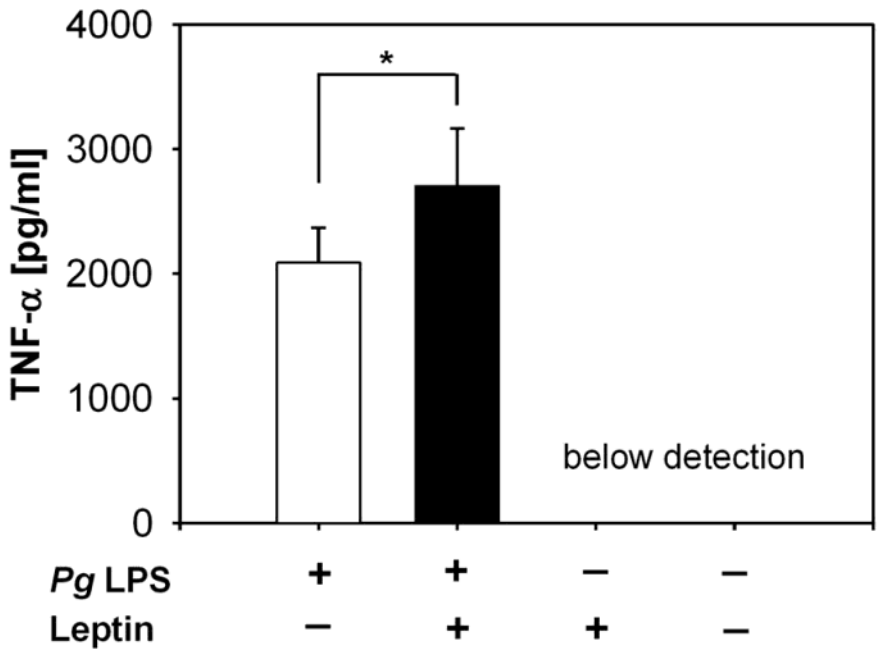

Figure 3.2 The effect of leptin on TNF-α secretion in THP-1 monocytes stimulated with *P. gingivalis* LPS for 2 h

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. LPS from *P. gingivalis* (100 ng/ml) was then added for 2 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents mean + SD from three independent cell culture experiments. Statistics: ANOVA, Student's *t*-test $*p < 0.05$. *Pg*: *P. gingivalis*.

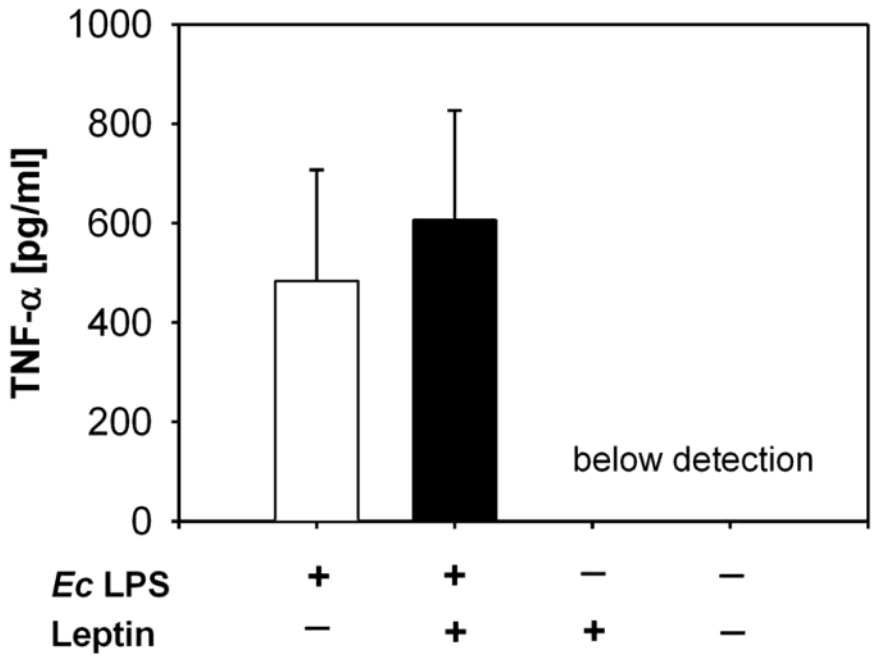

Figure 3.3 The effect of leptin on TNF-α secretion in THP-1 monocytes stimulated with *E. coli* LPS for 24 h

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. LPS from *E. coli* (100 ng/ml) was then added for 24 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents mean + SD from three independent cell culture experiments. Statistics: ANOVA n.s. *Ec*: *E. coli.*

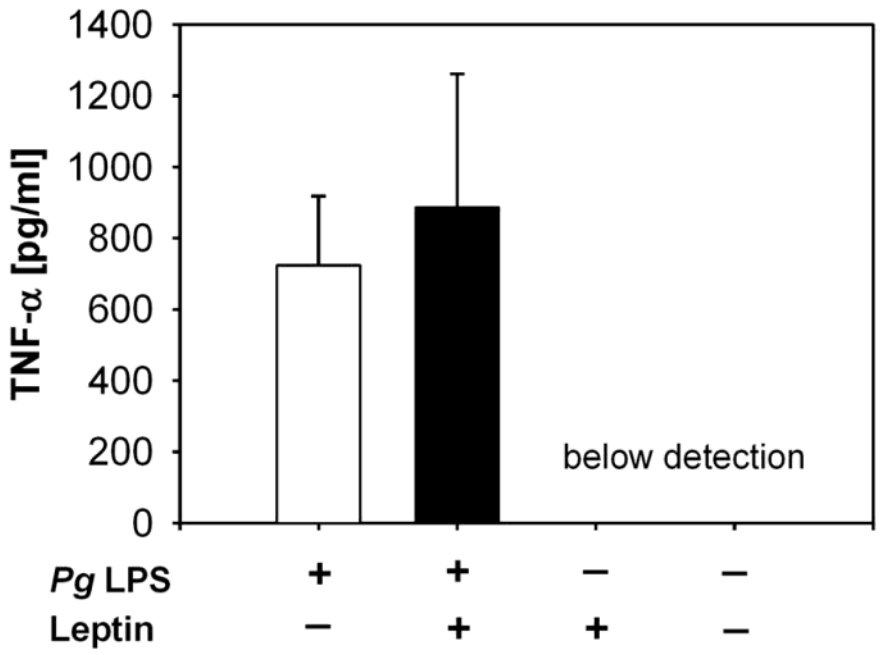

Figure 3.4 The effect of leptin on TNF-α secretion in THP-1 monocytes stimulated with *P. gingivalis* LPS for 24 h

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. LPS from *P. gingivalis* (100 ng/ml) was then added for 24 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents mean + SD from three independent cell culture experiments. Statistics: ANOVA n.s. *Pg*: *P. gingivalis*.

TNF-α is one of the first cytokines induced in response to pro-inflammatory stimuli and is downregulated during the continuing course of the immune response (Schindler *et al.*, 1990b). Since no TNF-α was detected in cell culture supernatants after leptin stimulation in experiments with longer incubation times (e.g. 7 and 29 h, Figure 3.1, Figure 3.2 and Figure 3.3, Figure 3.4, respectively), the effect of leptin stimulation on TNF-α secretion was tested at earlier timepoints. THP-1 monocytes (0.5×10^6) were stimulated with 500 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 0.5, 3 or 5 h. Unstimulated cells served as control. Supernatants were collected and analysed for TNF-α with ELISA as described in 2.3.

TNF-α secretion in response to *E. coli* LPS increased with time from a concentration of 315 pg/ml at 0.5 h up to 3513 pg/ml at 5 h. At all timepoints, neither cells stimulated with leptin alone nor unstimulated controls produced detectable levels of TNF-α in cell culture supernatants of THP-1 monocytes.

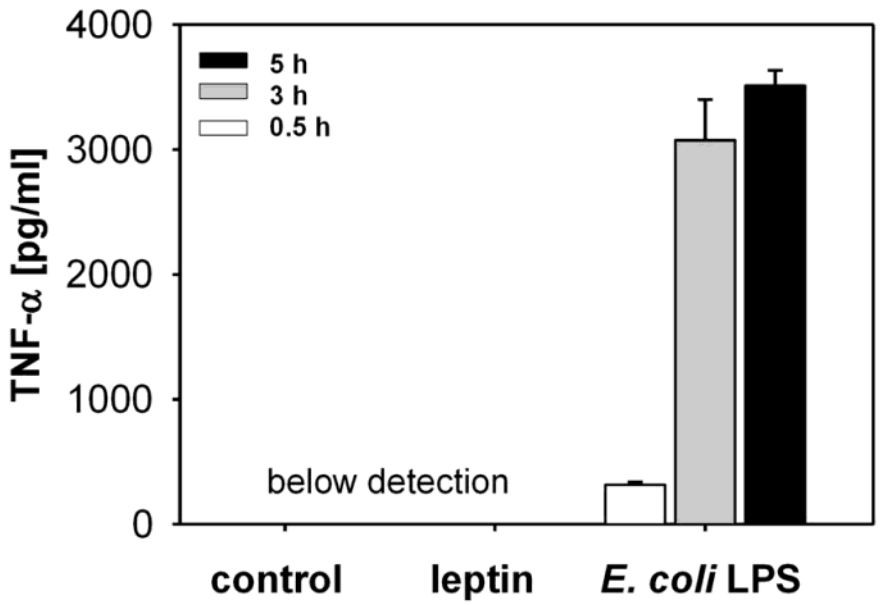

Figure 3.5 The effect of leptin on TNF-α secretion in THP-1 monocytes at early timepoints

THP-1 monocytes were stimulated with 500 ng/ml leptin or 100 ng/ml *E. coli* LPS for 0.5, 3 or 5 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents mean + SD from one cell culture experiment.

To further evaluate the potential of leptin alone on TNF-α protein secretion, a stimulation experiment was repeated in primary human monocytes. Primary human monocytes were isolated from a buffy coat via CD14 expression using the magnetic beads method as described in 2.1.3.2. After purification, 4×10^6 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 24 h. Unstimulated cells served as control. Supernatants were collected and analysed for TNF-α with ELISA as described in 2.3.

TNF-α protein concentrations in cell culture supernatants of primary human monocytes increased after stimulation with *E. coli* LPS (Figure 3.6). Compared to control, LPS stimulation induced a significant 2.7-fold upregulation ($p < 0.05$) in TNF-α protein concentrations. A baseline TNF-α concentration of 417 and 383 pg/ml was detected for control and leptin stimulated cells, respectively (Figure 3.6). Compared to control, leptin did not alter TNF-α secretion in primary human monocytes ($p = 0.49$).

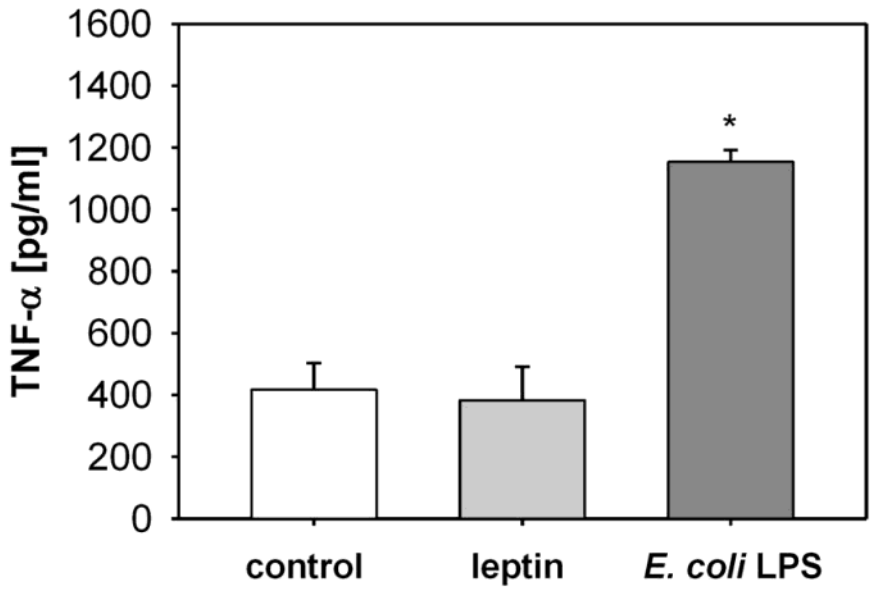

Figure 3.6 The effect of leptin on TNF-α expression in primary human monocytes

Primary human monocytes were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 24 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents median + IQR from two independent cell culture experiments. Statistics: Kruskal-Wallis, Mann-Whitney *U* test $*p < 0.05$, compared to control.

3.2.2 Investigation of the effect of leptin on TNF-α mRNA expression in THP-1 monocytes

To evaluate the relationship of protein secretion to mRNA expression, the effect of leptin and LPS on TNF-α mRNA expression was investigated. THP-1 monocytes (4 x 10^6) were stimulated with 500 ng/ml leptin for 5 h. LPS from *E. coli* or *P. gingivalis* (100 ng/ml) was then added for 2 h. Unstimulated cells and cells stimulated with leptin or LPS alone served as controls. Cells were collected and analysed for TNF-α mRNA expression with Realtime RT-PCR as described in 2.5.3.

Single LPS stimulations of THP-1 monocytes induced a significant upregulation of TNF-α mRNA expression compared to unstimulated cells (Figure 3.7). An 79-fold ($p < 0.001$) and 120-fold ($p < 0.001$) increase of TNF-α mRNA expression in THP-1 monocytes was detected for *E. coli* or *P. gingivalis* LPS, respectively. In this experimental setup, leptin alone did not significantly up regulate TNF-α mRNA expression compared to control ($p = 0.12$), however leptin significantly enhanced LPS-induced TNF-α mRNA expression (Figure 3.7). TNF-α mRNA expression was 58 % higher ($p < 0.001$) in cells stimulated with both leptin and *E. coli* LPS than in cells stimulated with *E. coli* LPS alone (Figure 3.7). The leptin effect on *P. gingivalis* LPS-stimulated TNF-α secretion did not reach statistical significance ($p = 0.22$, Figure 3.7).

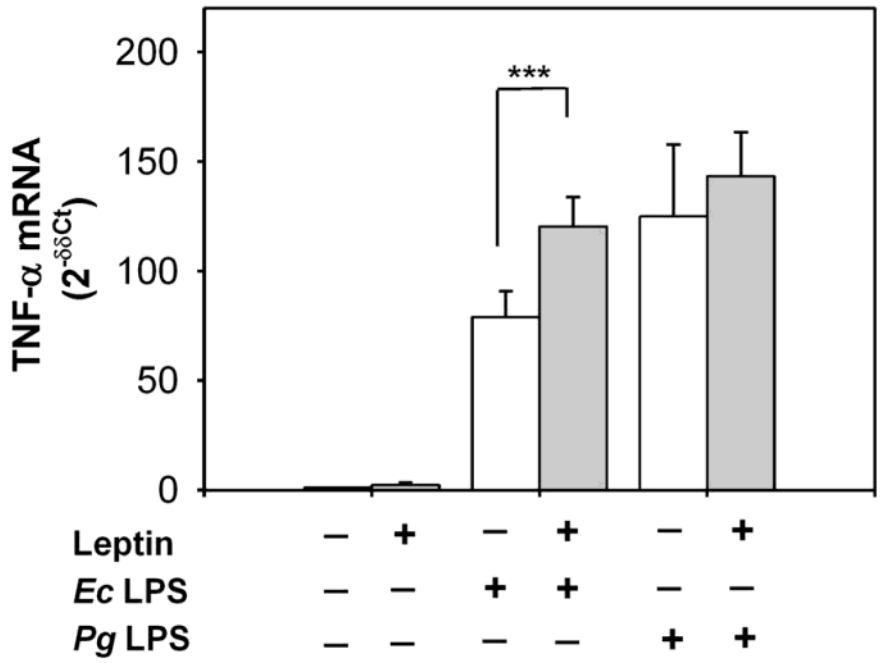

Figure 3.7 The effect of leptin on TNF-α mRNA expression in THP-1 monocytes stimulated with *E. coli* or *P. gingivalis* LPS for 2 h

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. LPS from *E. coli* or *P. gingivalis* (100 ng/ml) was then added for 2 h. Cells were collected and analysed for TNF-α mRNA expression with Realtime RT-PCR. Graph represents mean + SD from two independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: ANOVA, Student's *t*-test ***$p < 0.001$. *Ec*: *E. coli*, *Pg*: *P. gingivalis*.

In contrast to TNF-α protein concentrations, Realtime RT-PCR produced detectable results for stimulations with leptin alone. Therefore, analysis of TNF-α mRNA expression with single leptin stimulations was repeated with a higher leptin concentration and at different timepoints. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Unstimulated cells served as controls. Cells were collected and analysed for TNF-α mRNA expression with Realtime RT-PCR as described in 2.5.3.

Compared to control, both leptin and *E. coli* LPS stimulation induced a significant upregulation at 4 and 16 h in TNF-α mRNA expression (Figure 3.8). At 4 h, a 25-fold ($p < 0.001$) and 77-fold ($p < 0.001$) increase in TNF-α mRNA expression was detected for leptin or *E. coli* LPS stimulated cells, respectively. TNF-α mRNA expression decreased over time and reached a 3-fold ($p < 0.05$) and 5-fold ($p < 0.001$) upregulation for leptin or *E. coli* LPS stimulated cells, respectively, at 16 h (Figure 3.8).

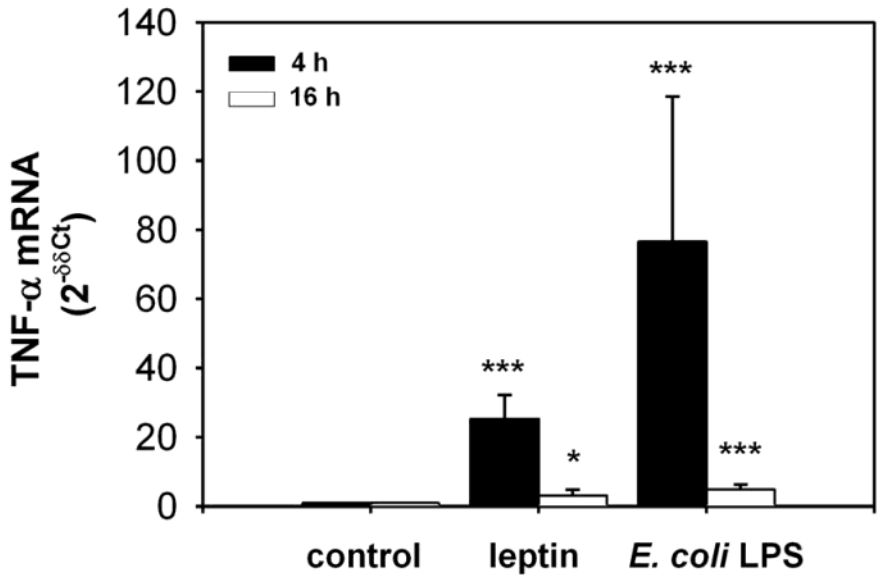

Figure 3.8 The effect of leptin alone on TNF-α mRNA expression in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Cells were collected and analysed for TNF-α mRNA expression with Realtime RT-PCR. Graph represents mean + SD from three independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: ANOVA, Student's *t*-test $*p < 0.05$, $***p < 0.001$, compared to control.

3.2.3 The effect of blocking leptin signalling on LPS and leptin-induced TNF-α secretion in THP-1 monocytes

To further validate the role of leptin in enhancing LPS-induced TNF-α secretion and to identify a possible signalling mechanism, preliminary experiments for blocking leptin receptor signalling and pathways were conducted. THP-1 monocytes (0.5×10^6) were stimulated with 500 ng/ml leptin for 5 h. A soluble leptin receptor (Recombinant human leptin R Fc chimera, R&D systems) was added at the same time at different concentrations as indicated. The range of concentration was chosen as recommended by the manufacturer. LPS from *E. coli* (100 ng/ml) was then added for 2 h. Unstimulated cells and cells stimulated with LPS alone served as controls. The experiment was set up in single stimulations. Supernatants were collected and analysed for TNF-α with ELISA as described in 2.3.

Combined leptin and LPS-stimulation yielded a higher TNF-α concentration in cell culture supernatants of THP-1 monocytes than LPS-stimulation alone (6740 versus 6232 pg/ml) (Figure 3.9). Unexpectedly, addition of a soluble leptin receptor to block leptin signalling increased TNF-α secretion in a dose-dependent manner. TNF-α concentrations as high as 12790 pg/ml were detected in cells stimulated with leptin and LPS in addition with the soluble receptor. At the lowest dose of soluble leptin receptor TNF-α concentrations were still higher than in cells stimulated with LPS and leptin only (8136 versus 6740 pg/ml). No TNF-α expression was detected in unstimulated cells. The results are derived from single stimulations and therefore no statistical analysis was performed.

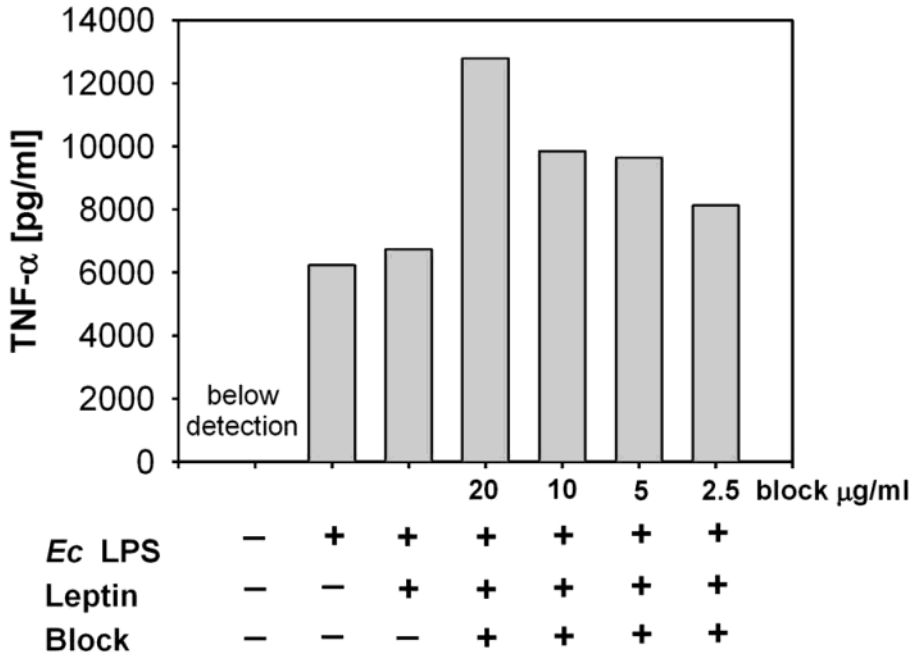

Figure 3.9 The effect of a soluble leptin receptor on leptin-enhancement of LPS-induced TNF-α secretion in THP-1 monocytes

THP-1 monocytes were stimulated with 500 ng/ml leptin for 5 h. A soluble leptin receptor was added at the same time at different concentrations as indicated. LPS from *E. coli* (100 ng/ml) was then added for 2 h. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents single stimulations from one cell culture experiment. *Ec*: *E. coli*; block: soluble leptin receptor.

Due to the unexpected results, it was investigated if the soluble leptin receptor potentially interacts with LPS. THP-1 monocytes (0.5×10^6) were either stimulated with 500 ng/ml leptin for 5 h and then 2 h with LPS from *E. coli* (100 ng/ml), stimulated for 2 h with LPS *E. coli* (100 ng/ml) alone or the soluble leptin receptor (5 µg/ml) was added for 5 h, followed by 2 h of LPS *E. coli* (100 ng/ml) stimulation. Unstimulated cells served as control. The experiment was set up in single stimulations. Supernatants were collected and analysed for TNF-α with ELISA as described in 2.3.

Combined leptin and LPS-stimulation yielded a higher TNF-α concentration in cell culture supernatants of THP-1 monocytes than LPS-stimulation alone (6182 versus 5352 pg/ml) (Figure 3.10). Likewise, combined soluble leptin receptor and LPS-stimulation resulted in a higher TNF-α concentration in cell culture supernatants of THP-1 monocytes than LPS-stimulation alone (6787 versus 5352 pg/ml), reaching an even higher level than the leptin-LPS combination (6787 versus 6182 pg/ml). No TNF-α expression was detected in unstimulated cells. The results are derived from single stimulations and therefore no statistical analysis was performed.

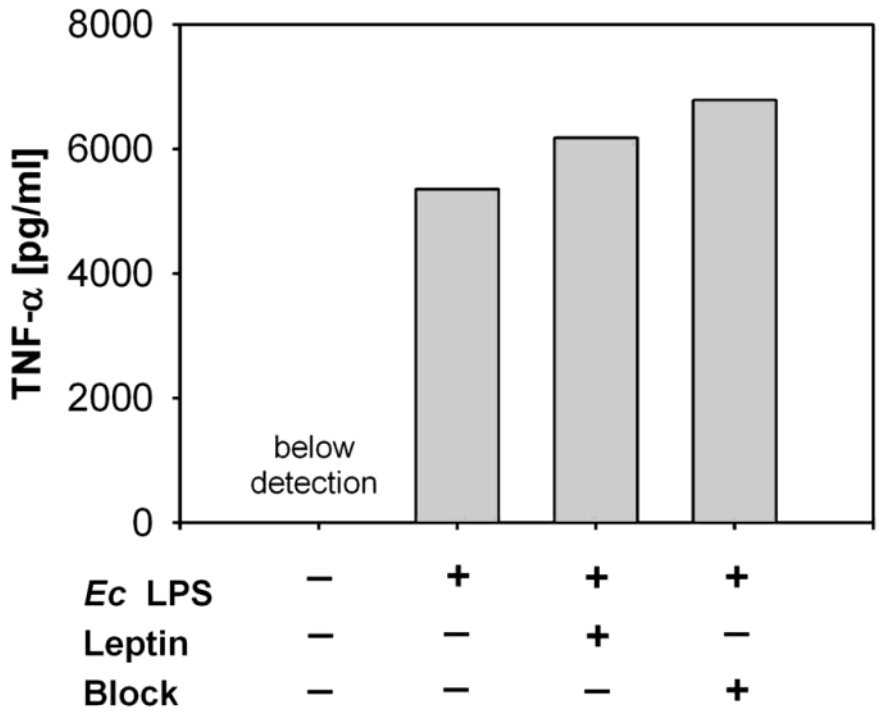

Figure 3.10 The effect of a soluble leptin receptor on LPS-induced TNF-α secretion in THP-1 monocytes

THP-1 monocytes were either stimulated with 500 ng/ml leptin for 5 h and then 2 h with LPS from *E. coli* (100 ng/ml), stimulated for 2 h with LPS *E. coli* (100 ng/ml) alone or the soluble leptin receptor (5 µg/ml) was added for 5 h, followed by 2 h of LPS *E. coli* (100 ng/ml) stimulation. Supernatants were collected and analysed for TNF-α with ELISA. Graph represents single stimulations from one cell culture experiment. *Ec*: *E. coli*; block: soluble leptin receptor.

Results of both experiments with the soluble leptin receptor indicate that this blocking reagent is not suitable to further validate the role of leptin in enhancing LPS-induced TNF-α secretion. To identify a possible pathway of leptin receptor signalling in enhancing LPS-induced TNF-α secretion, THP-1 monocytes (4×10^6) were incubated overnight or for 30 min, 2 h and 3 h with 50 µM of JAK2 inhibitor II (Merck Chemicals) to inhibit the JAK2 signalling pathway of the leptin receptor. The JAK2 inhibitor II is specific for JAK2 only and the concentration was chosen as recommended by the manufacturer and as described in Sandberg *et al.* (2005). However, the inhibitor proved toxic to the cells as observed by losing adherence from tissue culture plates and cell shrinkage and experiments were not continued.

3.3 Discussion

The periodontal pathogen *P. gingivalis* is one of the key bacteria associated with periodontal disease (Lamont and Jenkinson, 1998; Socransky *et al.*, 1998). In contrast to the Lipid A from *E. coli* LPS, Lipid A from *P. gingivalis* LPS is highly variable, capable of signalling through TLR2 and disrupting the normal host response to other bacteria (Zhou *et al.*, 2005; Coats *et al.*, 2007). The antagonistic actions of *P. gingivalis* on TLR4 signalling from other pathogens and the TLR2-induced differential cytokine and chemokine expression pattern is thought to play a role in the strategy of this periodontal pathogen to evade immune responses and thus contribute to disease pathogenesis (Yoshimura *et al.*, 2002; Bostanci *et al.*, 2007). A number of studies have shown an enhancing effect for the adipokine leptin in pro-inflammatory immune responses towards *E. coli* LPS (Loffreda *et al.*, 1998; Santos-Alvarez *et al.*, 1999; Mattioli *et al.*, 2005; Shen *et al.*, 2005). However, due to the unique properties and signalling of *P. gingivalis* LPS no definite conclusions can be drawn as to the effect of

leptin on pro-inflammatory immune responses to this LPS. The present study confirms results from Loffreda *et al.* (1998) and Shen *et al.* (2005) in murine peritoneal macrophages and Kupffer cells and shows the enhancing effect of leptin on *E. coli* LPS-induced TNF-α secretion for the first time in human monocytes. In addition, it was demonstrated that leptin enhances *P. gingivalis* LPS-induced TNF-α secretion in human monocytes with a comparable magnitude as its effect in combination with *E. coli* LPS. TNF-α is a strong chemotactic factor and activator of neutrophils (Vassalli, 1992) and is one of the key inflammatory cytokines involved in tissue destruction and bone breakdown in periodontal disease (Graves and Cochran, 2003; Graves, 2008).

Therefore, the first observation of an effect of leptin to enhance *P. gingivalis* LPS-induced immune responses in the present study is an important finding and may prove relevant in a link between diabetes and periodontal disease. Leptin concentrations are strongly correlated with BMI and generally elevated in T2DM (Considine *et al.*, 1996; Sinha *et al.*, 1996), a condition with an increased risk for severe periodontal disease (Khader *et al.*, 2006). Thus, increased leptin concentrations in diabetes could potentiate the expression of pro-inflammatory mediators such as TNF-α in response to *P. gingivalis* LPS and contribute to the exaggerated host immune response in periodontal disease. The enhancing effect of leptin for LPS-induced TNF-α secretion was diminished after 24 h. However, the production of TNF-α is downregulated during continuing exposure to LPS and other pro-inflammatory cytokines such as IL-6 become more prominent (Schindler *et al.*, 1990b). The effect of leptin on LPS-induced secretion of other pro-inflammatory cytokines than TNF-α was not determined in the present study, yet enhanced IL-6 production was observed after 24 h of LPS and leptin stimulation in cultures of murine peritoneal macrophages and human monocyte-derived

DCs (Loffreda *et al.*, 1998; Mattioli *et al.*, 2005). Thus, leptin likely has a prolonged and not only an acute effect on the alteration of myeloid cell immune responses to LPS, which further implicates a role for this adipokine in chronic inflammatory conditions such as periodontal disease.

In addition, the present study complements findings on direct pro-inflammatory immune responses to leptin alone in myeloid cells. In accordance with results from Loffreda *et al.* (1998) and Shen *et al.* (2005) on murine peritoneal macrophages and Kupffer cells, leptin stimulation did not induce secretion of TNF-α in THP-1 and primary human monocytes. Yet, two other studies on human myeloid cells show the opposite (Zarkesh-Esfahani *et al.*, 2004; Mattioli *et al.*, 2005). In these studies, leptin enhanced neutrophilic CD11b expression indirectly through inducing TNF-α secretion in monocytes (Zarkesh-Esfahani *et al.*, 2004) and cultures of monocyte-derived dendritic cells directly responded to leptin with increased TNF-α secretion (Mattioli *et al.*, 2005). There are a number of possible explanations for these discrepancies. Thus, although Zarkesh-Esfahani *et al.* (2004) reveal monocyte-derived TNF-α as a mediator for the leptin-induced effect on neutrophils, they do so only in a combined culture of monocytes with neutrophils. However, several studies demonstrate a complex cross-talk between neutrophils and monocytes (Maus *et al.*, 2002; Sabroe *et al.*, 2002; Sabroe *et al.*, 2003), which likely interferes with the process of TNF-α secretion (Mullberg *et al.*, 2000). This is further supported by findings of leptin-induced increase in intracellular TNF-α concentration in $CD14^+$ cells in human PBMCs (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001), although TNF-α concentrations in monocyte cell culture supernatants were not determined in these studies and therefore no direct conclusion regarding actual leptin-induced induction of monocyte TNF-α secretion can be drawn.

TNF-α is a potent toxic pro-inflammatory cytokine (Vassalli, 1992) and thus it would seem plausible that its release is tightly regulated and dependent on other stimuli such as LPS. Nonetheless, single cultures of monocyte-derived human DCs show increased TNF-α secretion after leptin stimulation (Mattioli *et al.*, 2005). However, these cells already display a baseline TNF-α secretion which simply appears to be further enhanced by leptin (Mattioli *et al.*, 2005). In contrast, in the present study, basal TNF-α secretion was observed in primary human monocytes and was not enhanced after leptin stimulation. In this case, the differentiation stage of myeloid cells potentially plays a role. Thus, *in vivo* dendritic cells differentiate from monocytes under inflammatory conditions (Auffray *et al.*, 2009), which is replicated *in vitro* through addition of IL-4 and GM-CSF to the growth medium of primary monocyte cultures (Mattioli *et al.*, 2005). Hence, the monocyte-derived DCs used in the study of Mattioli *et al.* (2005) already are in a more inflammatory state than primary monocytes and therefore likely react different to leptin stimulation.

Although leptin alone does not appear to be sufficient to induce TNF-α secretion in human monocytes, the present study demonstrates leptin-induced upregulation of TNF-α mRNA expression. Possibly, leptin-induced upregulation of TNF-α mRNA expression is dose-dependent, as this effect was only significant at a higher leptin concentration. However, a lower leptin dose was sufficient to enhance *E. coli* LPS-induced TNF-α mRNA expression and since TNF-α mRNA expression declined over time it is likely that the chosen experimental setup and time to study combined effects of leptin and LPS masked the effect of leptin alone on TNF-α mRNA expression. In future studies, it would be interesting to further evaluate time and dose-dependent effects of leptin on TNF-α mRNA expression in human monocytes to reach a better understanding of leptin

kinetics. Similar differences between leptin-induced TNF-α mRNA expression and protein secretion were also observed after leptin stimulation in PBMCs (Zarkesh-Esfahani *et al.*, 2001). In addition, the apparent uncoupling of TNF-α transcription and translation/secretion was recognized in other settings as well. Thus, Shook *et al.* (1994) demonstrate that both IL-2 and IFN-γ induce TNF-α mRNA expression in bone-marrow derived macrophages, however TNF-α secretion was only induced after LPS stimulation. Likewise, a complement factor (C5a) induced TNF-α mRNA expression in PBMCs, yet LPS was necessary to induce translation (Schindler *et al.*, 1990a). Studies on the effect of LPS on TNF-α transcription and translation revealed that LPS strongly enhances both transcription and translation efficiency (Han *et al.*, 1990; Raabe *et al.*, 1998). Thus, although leptin induces TNF-α mRNA, the level of expression might not reach a specific threshold for efficient translation and LPS is needed as a second signal for TNF-α translation and release.

Interestingly, although the LPS-enhancing effect of leptin on TNF-α protein secretion in monocytes was observed for both *E. coli* and *P. gingivalis* LPS, at the mRNA level the effect only was significant for *E. coli* LPS. However, *P. gingivalis* LPS appeared to be a more potent inducer of TNF-α mRNA expression than *E. coli* LPS and possibly the combined challenge of leptin and *P. gingivalis* LPS reached the limit of capability of the monocytes to increase TNF-α mRNA expression. It would be interesting to further evaluate the combined effect of leptin and different TLR ligands on TNF-α mRNA expression with different LPS concentrations in future studies, to clarify this finding. In addition, differential LPS signalling for *E. coli* or *P. gingivalis* may play a role as well. For example, Hajishengallis *et al.* (2002) reveal induction of LPS-tolerance for *E. coli*, but not for *P. gingivalis* LPS and show prolonged TNF-α production in THP-1

monocytes after a secondary stimulation with LPS from the periodontal pathogen. Furthermore, they observe antagonistic actions of *P. gingivalis* LPS on *E. coli* LPS induced TNF-α secretion (Hajishengallis *et al.*, 2002).

Mutual leptin and LPS intracellular signalling mechanisms likely are involved in the LPS-enhancing effect of leptin. Thus, Lam *et al.* (2007) demonstrate that both leptin and LPS individually induce JAK2/STAT and PI3K/Akt pathway phosphorylation events in bone marrow-derived DCs, however the phosphorylation status of these signalling pathways is markedly increased in the combination of LPS and leptin. Two of the main leptin signalling pathways, the p38 and ERK MAPK pathways, were shown to be involved in LPS-induced transcription of TNF-α in murine macrophages (Zhu *et al.*, 2000). Furthermore, Zhu *et al.* (2000) demonstrate that although each pathway individually leads to TNF-α transcription, combined activation of several pathways results in increased transcriptional activity compared to each pathway alone. It is therefore possible that both leptin and LPS signalling independently activate these pathways and that in a combination of both stimuli TNF-α transcription is increased compared to each stimuli alone. Additionally, leptin signalling could impact on the post-transcriptional regulation of TNF-α mRNA, effecting factors for translation initiation, mRNA stability or polyadenylation. These processes are known to play a key role in TNF-α expression (Anderson, 2000) and are under the influence of a number of signalling pathways such as the PI3K/Akt, ERK and p38 MAPK pathways (Gingras *et al.*, 1999), which all are potentially induced by leptin.

To further investigate the mechanisms of combined leptin and LPS stimulation on TNF-α secretion, experiments involving blocking of leptin signalling were conducted. However, these experiments were not reliable and returned some unexpected results. In

a first experiment, a soluble leptin receptor was used to block leptin signalling. This approach was successfully applied in the study of Lam *et al.* (2007) to investigate the effects of leptin on dendritic cell CD40 expression. According to the manufacturer, the soluble leptin receptor has a higher affinity for leptin than ObRb and therefore is a potent leptin antagonist. However, in the present study, when used in combination with leptin and *E. coli* LPS, this protein was very potent in enhancing TNF-α secretion to levels even higher than the combined effect of leptin and LPS. Additionally, a combination of the soluble receptor and *E. coli* LPS stimulation induced increased TNF-α secretion as well in comparison to LPS alone or the combination of leptin and LPS. The soluble leptin receptor was produced in a mouse cell line as a chimeric protein consisting of human leptin ObRb and IgG and therefore any endotoxin contamination was unlikely. However, it was thought that the observed unexpected increase in TNF-α secretion was possibly due to some type of Fc receptor signalling (Loegering and Lennartz, 2004), although this was not further investigated. In a second experiment, a specific chemical JAK2 inhibitor was used to block leptin signalling. JAK2 phosphorylation is the first step in several key leptin signalling events (O'Rourke *et al.*, 2001; Sanchez-Margalet and Martin-Romero, 2001; Cui *et al.*, 2006; Cao *et al.*, 2007; Tong *et al.*, 2008) and therefore potentially involved in the effect of leptin on TNF-α expression. Although concentrations for the JAK2 inhibitor were chosen as recommended by the manufacturer and Sandberg *et al.* (2005) detect no cytotoxic effects for higher concentrations and long incubation times in an epithelial cell line (BSC-40), the inhibitor proved toxic to the THP-1 monocytes. Unable to use TNF-α secretion in cell culture supernatants as a direct and easy accessible output measurement for leptin stimulation alone further complicated experiments to determine the optimum time and concentration needed for the JAK2 inhibitor to be non-toxic while still

inhibiting leptin signalling through JAK2. Studying leptin signalling events was not the main aim of the present study and therefore inhibitor experiments were not continued. However, in the meantime it was noted in our laboratory that leptin directly induces IL-18 secretion in THP-1 monocytes (Jitprasertwong, unpublished) and this output measurement might be a useful tool in future studies to investigate leptin intracellular signalling events with inhibitors.

In summary, the present study shows for the first time an LPS-enhancing effect of leptin on TNF-α production in response to *P. gingivalis* LPS. In addition, it was demonstrated that human monocytes do not respond to leptin with TNF-α secretion. The leptin-induced enhanced TNF-α secretion in response to LPS is potentially mediated through increased leptin and LPS-induced TNF-α mRNA expression, although other mechanisms likely contribute to this effect as well. The leptin-induced altered inflammatory response of monocytes to *P. gingivalis* LPS may contribute to an increased risk for severe periodontal disease in conditions with increased leptin concentrations such as T2DM.

Chapter 4 The effect of leptin on expression of cell surface markers in monocytes

Chapter 4 The effect of leptin on expression of cell surface markers in monocytes

4.1 Introduction

Leptin not only induces expression of pro-inflammatory cytokines (Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001; Zarkesh-Esfahani *et al.*, 2004; Mattioli *et al.*, 2005), a number of studies also report an effect of the adipokine on the expression of cell surface markers in several immune cells. Thus, leptin enhanced expression of late (CD11b, CD11c, CD25, CD38, CD71, HLA-DR) and early (CD69) activation markers in human primary monocytes, T cells and neutrophils (Santos-Alvarez *et al.*, 1999; Martin-Romero *et al.*, 2000; Zarkesh-Esfahani *et al.*, 2004). These activation markers play a role in antigen presentation, phagocytosis and cell proliferation (Meuer *et al.*, 1984; Hofman *et al.*, 2000) and are generally upregulated by LPS (Santos-Alvarez *et al.*, 1999). In addition, Lam *et al.* (2007) demonstrated that leptin could both induce CD40 and enhance LPS-stimulated cell surface expression of CD40 in murine dendritic cells. A LPS-enhancing effect of leptin was also observed for CD69 expression in human primary monocytes (Santos-Alvarez *et al.*, 1999). Thus, leptin could potentiate the effects of LPS for expression of cell surface activation markers in a similar way as observed for pro-inflammatory cytokines such as TNF-α or IL-6 in the present and in other studies (Loffreda *et al.*, 1998; Shen *et al.*, 2005). The effect of leptin on the expression of cell surface activation markers potentially is of importance in conditions with increased leptin concentrations such as obesity and diabetes (Considine *et al.*, 1996; Sinha *et al.*, 1996). For example, increased expression of the cell surface activation markers CD25 and HLA-DR is associated with angina (Neri Serneri *et al.*, 1997; van der Wal *et al.*, 1998), a coronary syndrome which is regarded as a major risk

factor in obesity and diabetes (Opie *et al.*, 2006). Thus, increased leptin concentrations potentially alter expression of cell surface activation markers and increase the risk for obesity and diabetes associated complications, however this remains to be elucidated.

Interestingly, in a number of inflammatory conditions such as human sepsis, tuberculosis and diabetes expression of TLR2, TLR4 and the accessory molecule CD14, is upregulated (Patino *et al.*, 2000; Creely *et al.*, 2007; Devaraj *et al.*, 2008; Schaaf *et al.*, 2009). Compared to health, the TLR and CD14 expression pattern in oral tissues also appears to differ in periodontal disease (Asai *et al.*, 2001; Wang *et al.*, 2001a; Mori *et al.*, 2003; Ren *et al.*, 2005; Uehara *et al.*, 2007) and increased TLR2 and TLR4 expression in gingival tissues is associated with advanced stages of periodontal disease (Mori *et al.*, 2003). In addition, the percentage of $CD14^{low}CD16^{+}$ monocytes, a monocyte subset with enhanced TLR2 expression (Belge *et al.*, 2002), is increased in periodontal disease (Nagasawa *et al.*, 2004). Using vector-induced overexpression of TLR4 in macrophages or vitamin D_3 dose-dependent increase of CD14 expression in THP-1 monocytes, *in vitro* studies directly correlate increased TLR4 and CD14 expression with enhanced production of pro-inflammatory cytokines in response to LPS stimulation (Martin *et al.*, 1994; Du *et al.*, 1999). Thus, differential TLR expression patterns likely impact on pro-inflammatory immune responses to LPS and potentially modulate pathogenesis of inflammatory conditions such as periodontal disease.

A number of studies investigated LPS-induced changes of TLR2 and TLR4 expression in human monocytes and macrophages *in vivo* and *in vitro* and results vary considerably between different TLRs, ligands and experimental conditions. While *P. gingivalis* LPS generally appears to induce an upregulation of TLR2 and TLR4 expression at both mRNA and protein level (Hajishengallis *et al.*, 2002; Muthukuru *et al.*, 2005; Foster *et*

al., 2007), *E. coli* LPS only consistently up regulates TLR2 mRNA and protein expression (Flo *et al.*, 2001; Marsik *et al.*, 2003; Armstrong *et al.*, 2004; Muthukuru *et al.*, 2005; Maris *et al.*, 2006; Foster *et al.*, 2007). The effect of *E. coli* LPS on TLR4 mRNA and protein expression is not always uniform and differs between studies, inducing both TLR4 up- or downregulation (Bosisio *et al.*, 2002; Muthukuru *et al.*, 2005; Maris *et al.*, 2006). It is possible, that different experimental conditions such as different LPS stimulation times provide an explanation for these inconsistent findings (Nomura *et al.*, 2000; Armstrong *et al.*, 2004; Foster *et al.*, 2007). In addition, basal TLR4 protein expression is very low and highly variable between individuals, which may impact on efficient analysis of cell surface protein expression (Visintin *et al.*, 2001).

TLR expression is not exclusively altered by LPS alone and a variety of cytokines such as IL-4, IL-10, GM-CSF, IFN-γ or TNF-α also induce a down or upregulation of TLR expression (Flo *et al.*, 2001; Kurt-Jones *et al.*, 2002). In addition, it was demonstrated that in adipocytes, leptin appears to have some regulatory function on several TLR mRNA expression patterns (Batra *et al.*, 2007). Thus, in *ob/ob* and *db/db* mice, Batra *et al.* (2007) observed increased TLR mRNA expression in comparison to the wild type controls. However, although altered TLR expression is observed in both diabetes and periodontal disease (Mori *et al.*, 2003; Creely *et al.*, 2007; Devaraj *et al.*, 2008) and diabetics generally exhibit increased leptin concentrations (Sinha *et al.*, 1996), no studies to date have investigated an effect of this adipokine on TLR mRNA and protein expression in monocytes or any other immune cells as a possible link between diabetes and an increased risk for severe periodontal disease (Khader *et al.*, 2006). Therefore, the aims of the present study were first to investigate the effect of leptin or *E. coli* and *P.*

gingivalis LPS on TLR2 and TLR4 cell surface expression and second to relate findings at the protein level to leptin or LPS-induced alterations of TLR2 and TLR4 expression at the mRNA level in human monocytes.

4.2 Results

4.2.1 Analysis of the effect of leptin and LPS on expression of cell surface markers in THP-1 monocytes

To investigate the effect of leptin and LPS stimulation on expression of the main receptor for LPS, TLR4 expression was analysed on the cell surface of THP-1 monocytes. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Unstimulated cells served as controls. Cells were collected, stained with a TLR4 antibody and 10,000 events were acquired on a flow cytometer as described in 2.4. Acquired data were analysed on dot plots of TLR4 and FSC settings using the region tool of the flow cytometry analysis programme. FSC (indicating cell size) was simply chosen for the requirement of two parameters for dot plot analysis and was not further evaluated. The two regions were drawn corresponding to specific custom settings using TLR4 as the main parameter. Region 1 was set at the upper endpoint of the isotype control (see Figure 2.15) and at the lower endpoint of the control. Region 2 was set in line with the upper endpoint of region 1 and drawn wide enough to include the highest observed TLR4 fluorescence intensity of each experimental sample set. Region 2 was then analysed for percentage changes in acquired events for TLR4. For further information refer to 2.4.

In the R2 analysis setting, 21 % of THP-1 monocytes were positive for TLR4. The TLR4 cell surface expression was not significantly altered by leptin or *E. coli* LPS

stimulation (Figure 4.1, Figure 4.2). Compared to unstimulated cells, *P. gingivalis* LPS induced a 7 % increase ($p < 0.05$) in TLR4 cell surface expression (Figure 4.1, Figure 4.2).

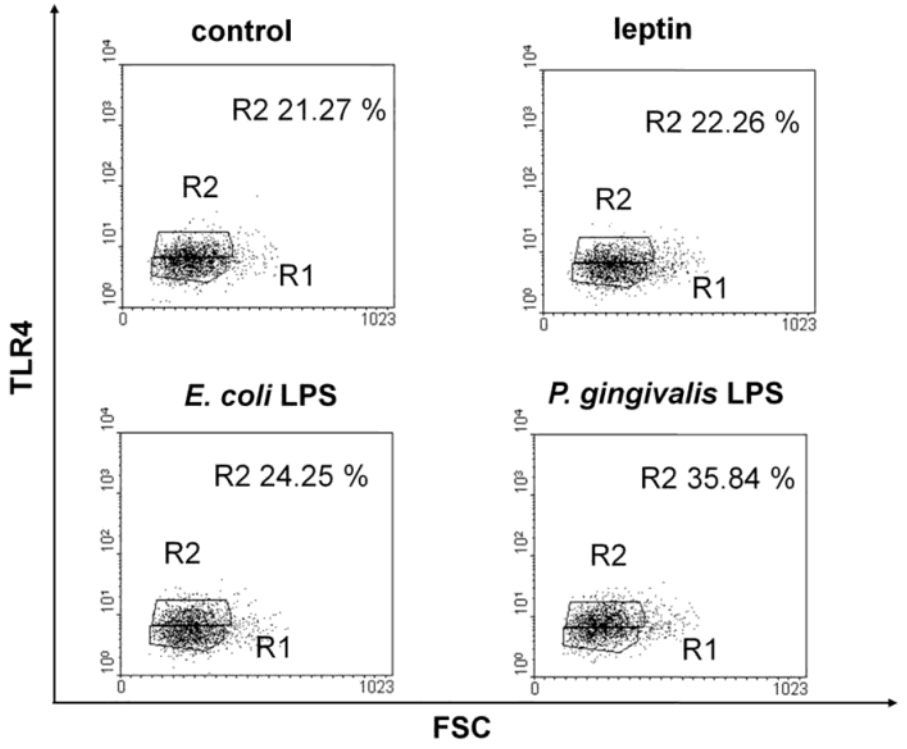

Figure 4.1 The effect of leptin or LPS on TLR4 cell surface expression in THP-1 monocytes (graphical representation)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR4 expression with flow cytometry. Graphs are representative for one of five independent experiments. R1: Region 1, R2: Region 2, FSC: Forward Scatter.

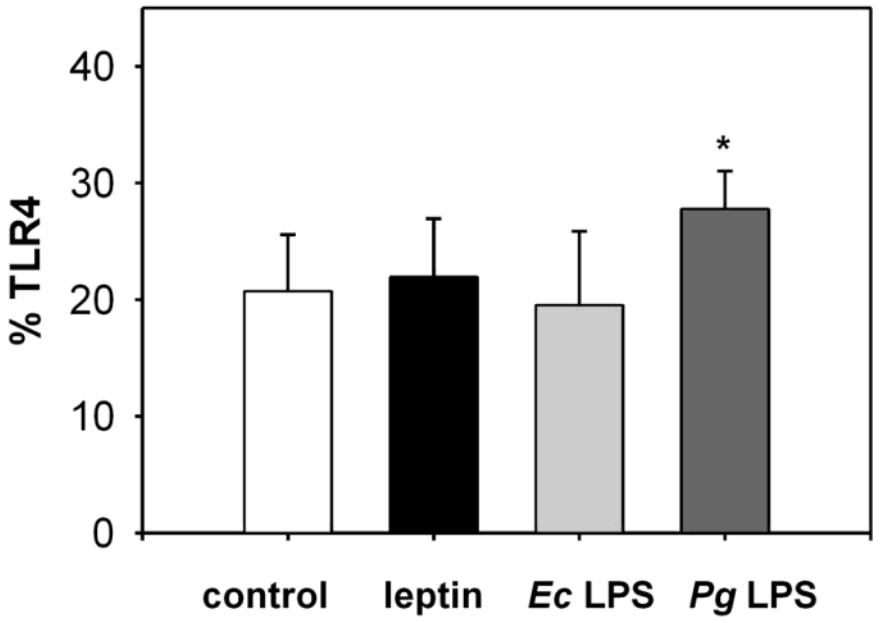

Figure 4.2 The effect of leptin or LPS on TLR4 cell surface expression in THP-1 monocytes (analysis of R2)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR4 expression with flow cytometry. Graph represents mean + SD from five independent cell culture experiments on events in Region 2. Statistics: ANOVA, Student's *t*-test $*p < 0.05$, compared to control. *Ec*: *E. coli, Pg: P. gingivalis.*

To investigate the effect of leptin and LPS stimulation on expression of the receptor for *P. gingivalis* LPS, TLR2 expression was analysed on the cell surface of THP-1 monocytes. Additionally, CD14 cell surface expression was analysed in the same experiment. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Unstimulated cells served as controls. Cells were collected, stained with a TLR2 and CD14 antibody and 10,000 events were acquired on a flow cytometer as described in 2.4.

For TLR2, acquired data were analysed on dot plots of TLR2 and CD14 settings using the quadrant tool of the flow cytometry analysis programme, which divides each dot plot in 4 quadrants. The lines of the quadrant were set at the endpoints of TLR2 and CD14 fluorescence intensity for the isotype control in a way so that the isotype control composed the lower left quadrant (see Figure 2.16). The upper right quadrant of each dot plot was then used to analyse percentage changes in acquired events for TLR2. For further information refer to 2.4. The difference between isotype control and CD14 staining was too large for dot plot analysis and therefore acquired data for cell surface CD14 expression were analysed on histograms using CD14 median fluorescence intensity as described in 2.4, Figure 2.17. A representative example is shown in Figure 4.5.

Compared to an isotype control, 19 % of THP-1 monocytes were positive for TLR2 (Figure 4.3, Figure 4.4). Leptin stimulation and both *E. coli* and *P. gingivalis* LPS stimulation induced an upregulation of cell surface TLR2 expression. In comparison to unstimulated cells, leptin induced an increase of 13 % ($p < 0.05$) in cell surface TLR2 expression, *E. coli* and *P. gingivalis* LPS stimulation increased cell surface TLR2 expression by 21 % ($p < 0.01$) and 29 % ($p < 0.001$), respectively (Figure 4.3, Figure

4.4). A similar pattern of upregulation is observed for cell surface CD14 expression. Compared to control, leptin increased cell surface CD14 expression by 28 % ($p < 0.05$, Figure 4.5, Figure 4.6). *E. coli* and *P. gingivalis* LPS induced a 1.9 ($p < 0.001$) and 2.1-fold increase ($p < 0.001$) in cell surface CD14 expression (Figure 4.5, Figure 4.6), respectively.

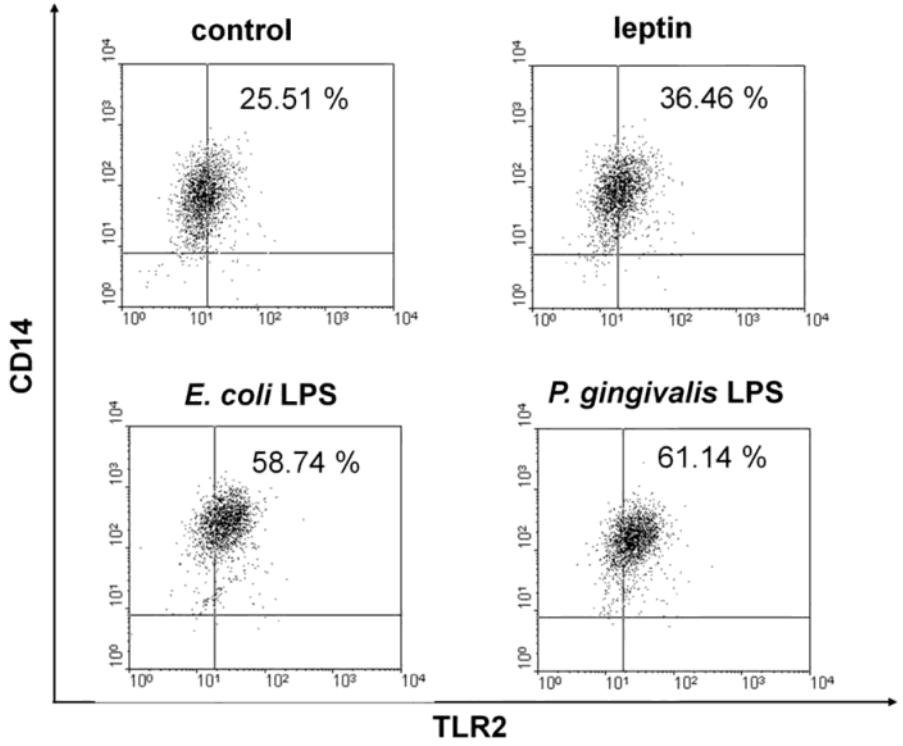

Figure 4.3 The effect of leptin or LPS on TLR2 cell surface expression in THP-1 monocytes (graphical representation for TLR2 analysis)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graphs are representative for one of three independent experiments. Numbers present the percentage of events in the upper right quadrant.

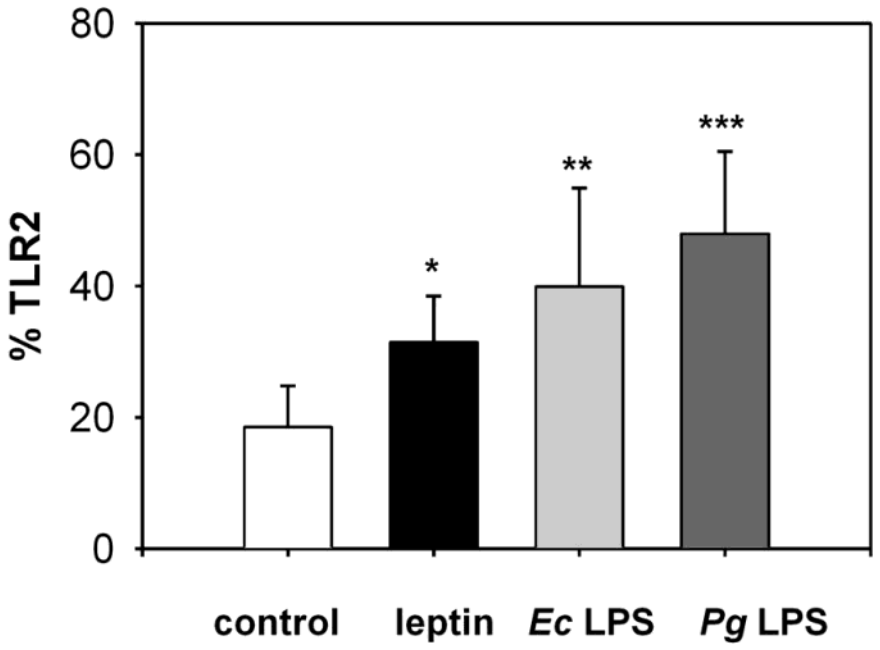

Figure 4.4 The effect of leptin or LPS on TLR2 cell surface expression in THP-1 monocytes (analysis of upper right quadrant)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graph represents mean + SD from three independent cell culture experiments on events in the upper right quadrant ($CD14^+TLR2^+$). Statistics: ANOVA, Student's *t*-test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to control. *Ec*: *E. coli, Pg: P. gingivalis.*

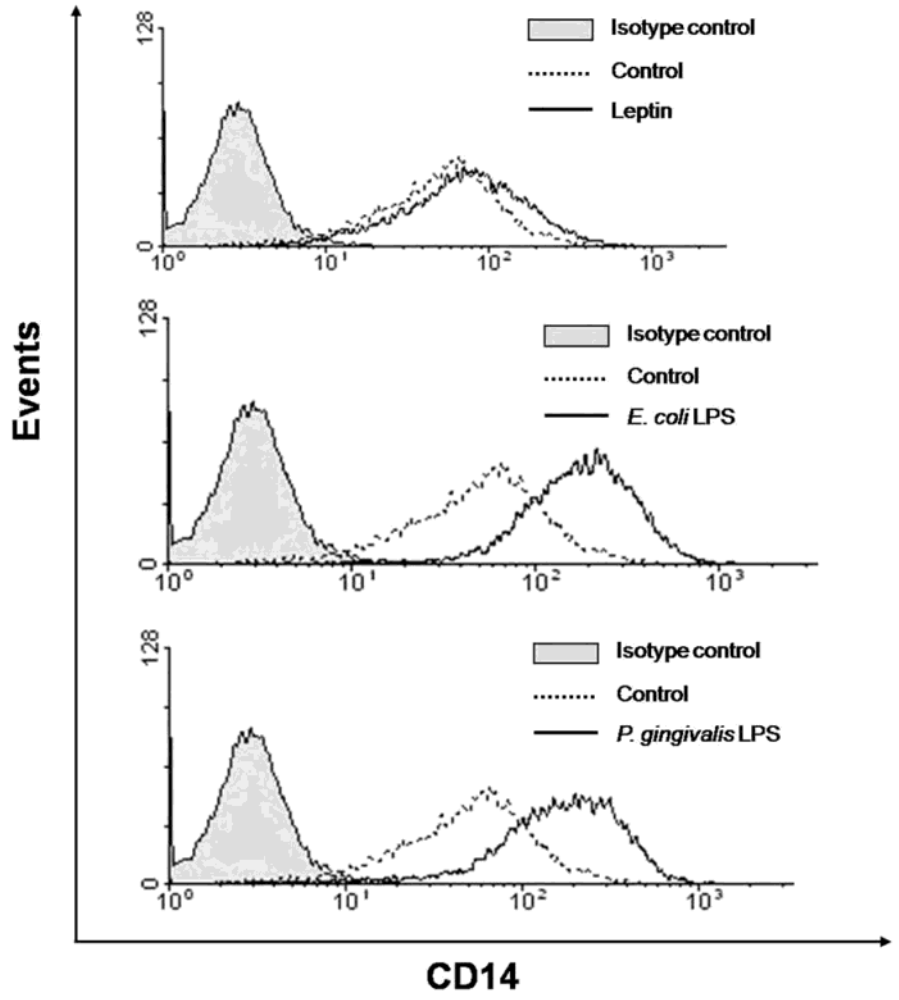

Figure 4.5 The effect of leptin or LPS on CD14 cell surface expression in THP-1 monocytes (graphical representation for CD14 analysis)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graphs show events for CD14 staining compared to an isotype control and are representative for one of three independent experiments.

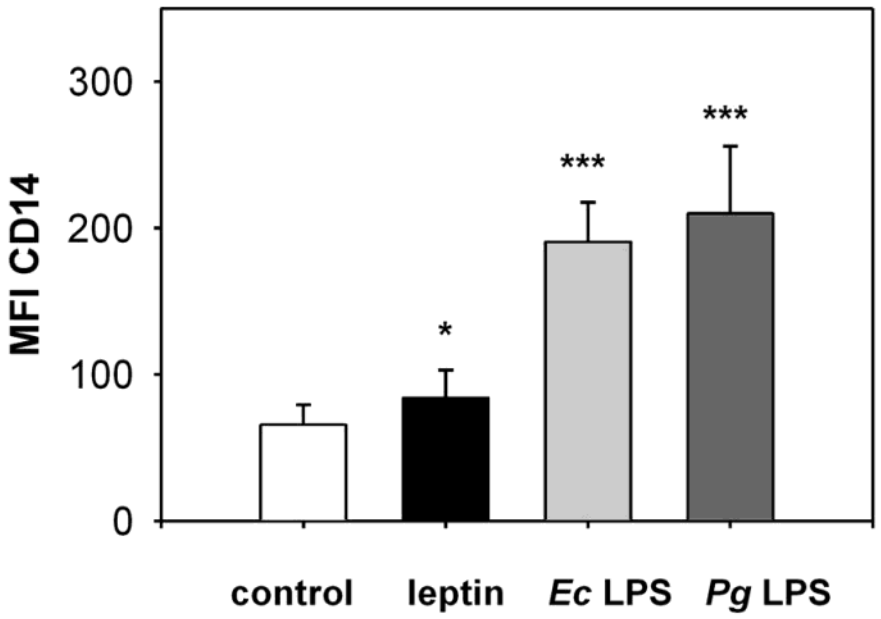

Figure 4.6 The effect of leptin or LPS on CD14 cell surface expression in THP-1 monocytes (analysis of CD14 MFI)

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graph represents mean + SD from three independent cell culture experiments on CD14 MFI. Statistics: ANOVA, Student's *t*-test $*p < 0.05$, $***p < 0.001$, compared to control. *Ec*: *E. coli, Pg: P. gingivalis.* MFI: median fluorescence intensity.

To investigate if leptin has an LPS-enhancing effect on TLR2 expression, THP-1 monocytes (4 x 10^6) were stimulated with 1000 ng/ml leptin and LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h and TLR2 cell surface expression was analysed. Unstimulated cells and single stimulations served as controls. Cells were collected, stained with a TLR2 and CD14 antibody and 10,000 events were acquired on a flow cytometer as described in 2.4. For TLR2, acquired data were analysed on dot plots of TLR2 and CD14 settings in comparison to an isotype control using the quadrant tool of the flow cytometry analysis programme applying the same settings as described above and in 2.4. Analysis of a preliminary experiment (n = 2) revealed no apparent differences in cell surface TLR2 expression between LPS and LPS plus leptin stimulated cells (Figure 4.7). Compared to an isotype control, 30 % of THP-1 monocytes were positive for TLR2 after *E. coli* LPS or *E. coli* LPS plus leptin stimulation. After *P. gingivalis* LPS or *P. gingivalis* LPS plus leptin stimulation, 37 % or 35 % of cells were positive for TLR2 cell surface expression, respectively. The results are derived from one experiment with two stimulations and therefore no statistical analysis was performed.

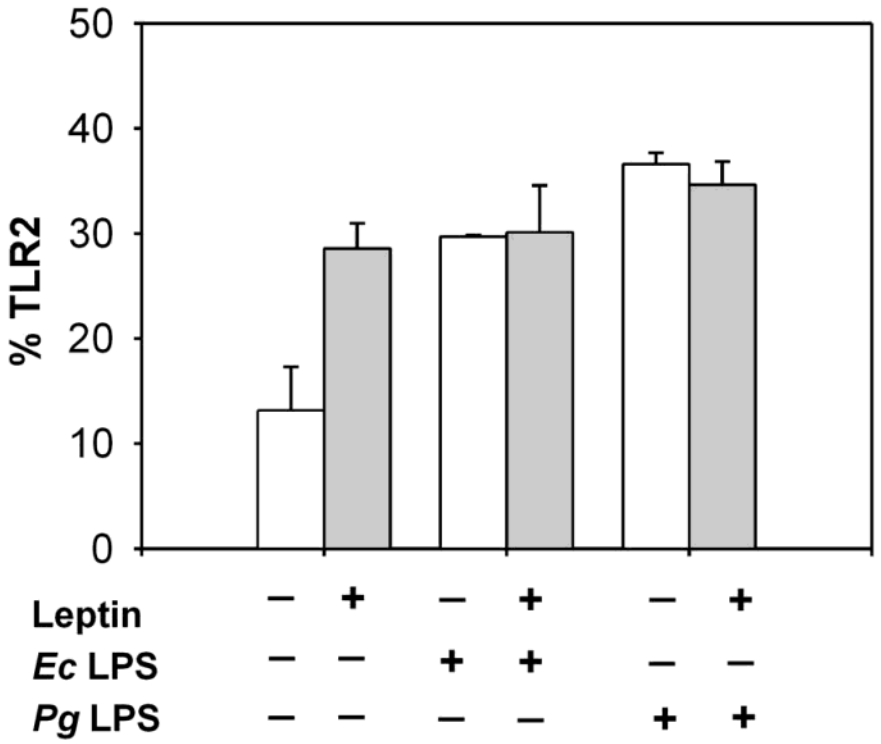

Figure 4.7 The effect of combined leptin and LPS stimulation on TLR2 cell surface expression in THP-1 monocytes (analysis of upper right quadrant)

THP-1 monocytes were stimulated with 1000 ng/ml leptin and LPS from *E. coli* or *P. gingivalis* (100 ng/ml) for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graph represents mean + SD from one cell culture experiments on events in the upper right quadrant ($CD14^+TLR2^+$). *Ec*: *E. coli*, *Pg: P. gingivalis.*

4.2.2 Analysis of the effect of leptin and LPS on expression of cell surface markers in primary human monocytes

The effect of leptin or LPS on TLR2 and CD14 expression was analysed in primary human monocytes. Primary human monocytes were isolated from a buffy coat via CD14 expression using the magnetic beads method as described in 2.1.3.2. After purification, 4 x 10^6 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 24 h. Unstimulated cells served as control. Cells were collected, stained with a TLR2 and CD14 antibody and 10,000 events were acquired on a flow cytometer as described in 2.4. For TLR2, acquired data were analysed on dot plots of TLR2 and CD14 settings in comparison to an isotype control using the quadrant tool of the flow cytometry analysis programme applying the same settings as described above and in 2.4. The difference between isotype control and CD14 staining was too large for dot plot analysis and therefore acquired data for cell surface CD14 expression were analysed on histograms using CD14 median fluorescence intensity as described in 2.4.

Compared to an isotype control, 41 % of primary human monocytes were positive for TLR2 (Figure 4.8). Significant differences were observed between the unstimulated cells and cells stimulated with leptin or *E. coli* LPS (Kruskal-Wallis $p < 0.05$) and a strong trend ($p = 0.057$) for increased cell surface TLR2 expression was found for both leptin and *E. coli* LPS stimulated cells. In comparison to an isotype control, 46 % of leptin-stimulated monocytes and 50 % of *E. coli* LPS-stimulated cells were positive for TLR2 (Figure 4.8). No significant differences in cell surface CD14 expression were observed for either leptin or *E. coli* LPS stimulated monocytes (Figure 4.9).

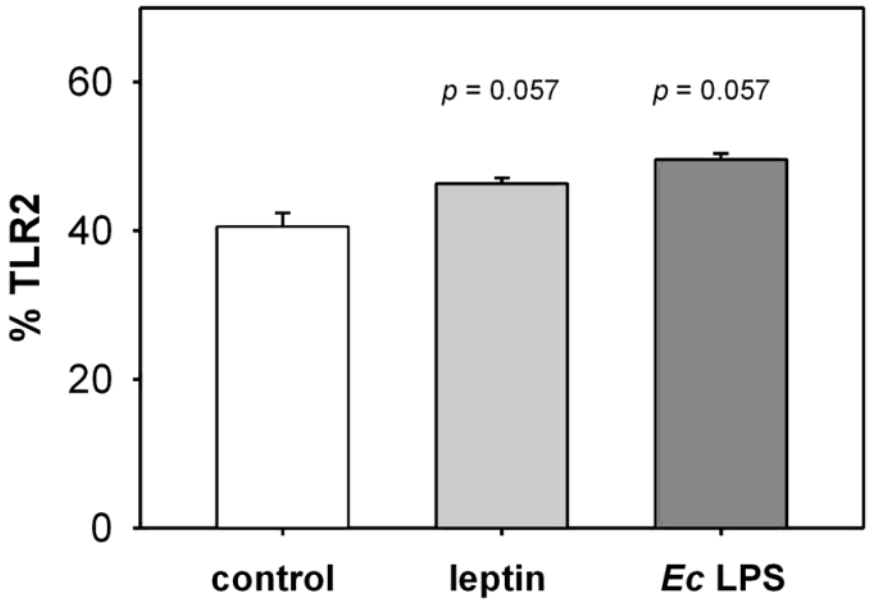

Figure 4.8 The effect of leptin or LPS on TLR2 cell surface expression in primary human monocytes (analysis of upper right quadrant)

Primary human monocytes were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graph represents median + IQR from two independent cell culture experiments on events in the upper right quadrant ($CD14^+TLR2^+$). Statistics: Kruskal-Wallis, Mann-Whitney *U* test $p = 0.057$, compared to control. *Ec*: *E. coli.*

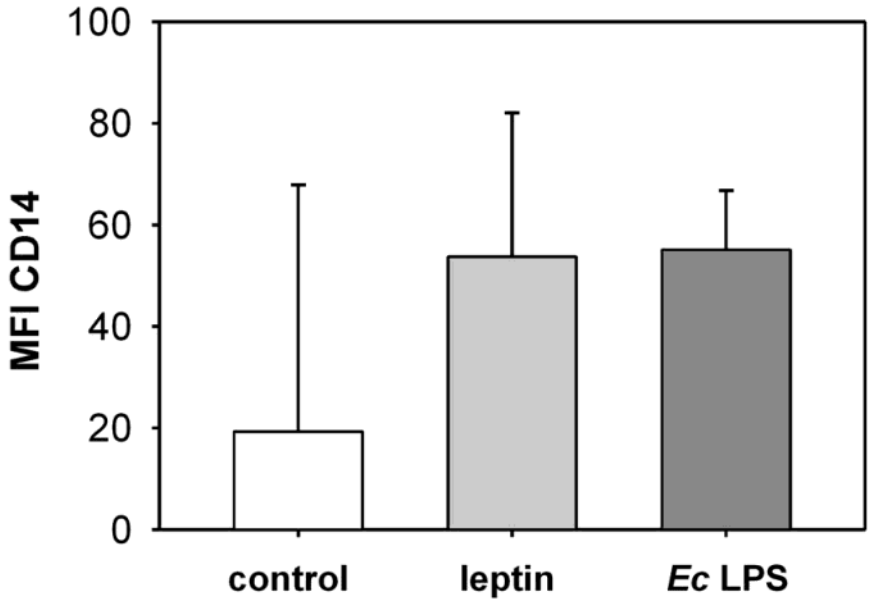

Figure 4.9 The effect of leptin or LPS on CD14 cell surface expression in primary human monocytes (analysis of CD14 MFI)

Primary human monocytes were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 24 h. Cells were collected and analysed for cell surface TLR2 and CD14 expression with flow cytometry. Graph represents median + IQR from two independent cell culture experiments on CD14 MFI. Statistics: Kruskal-Wallis n.s. *Ec*: *E. coli*. MFI: median fluorescence intensity.

4.2.3 Analysis of the effect of leptin and LPS on TLR mRNA expression in THP-1 monocytes

To evaluate a possible mechanism for leptin-induced alteration of TLR expression, mRNA expression for TLR2 and TLR4 was analysed. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Unstimulated cells served as controls. Cells were collected and analysed for TLR2 and TLR4 mRNA expression with Realtime RT-PCR as described in 2.5.3.

Compared to control, both leptin and *E. coli* LPS stimulation induced a significant upregulation at 4 and 16 h in TLR2 mRNA expression (Figure 4.10). At 4 h, TLR2 mRNA expression was doubled ($p < 0.05$) for leptin and increased by 80 % ($p < 0.05$) for *E. coli* LPS stimulated cells. At 16 h, the effect of leptin on TLR2 mRNA expression was less prominent and reached an upregulation of 50 % ($p < 0.05$), whereas *E. coli* LPS further enhanced TLR2 mRNA expression, inducing a 3.7-fold increase ($p < 0.01$, Figure 4.10). TLR4 mRNA expression was not altered by leptin at the analysed timepoints (Figure 4.11). In contrast, *E. coli* LPS induced a downregulation of TLR4 mRNA expression over time, with a significant reduction of 84 % ($p < 0.05$) at 16 h (Figure 4.11).

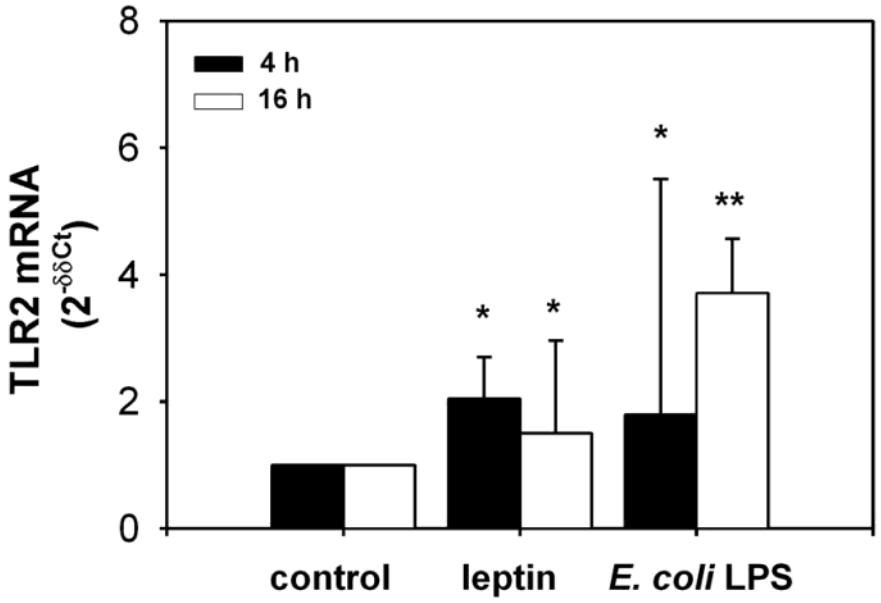

Figure 4.10 The effect of leptin or LPS on TLR2 mRNA expression in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Cells were collected and analysed for TLR2 mRNA expression with Realtime RT-PCR. Graph represents median + IQR from three independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: Kruskal-Wallis, Mann-Whitney *U* test $*p < 0.05$, $**p < 0.01$, compared to control.

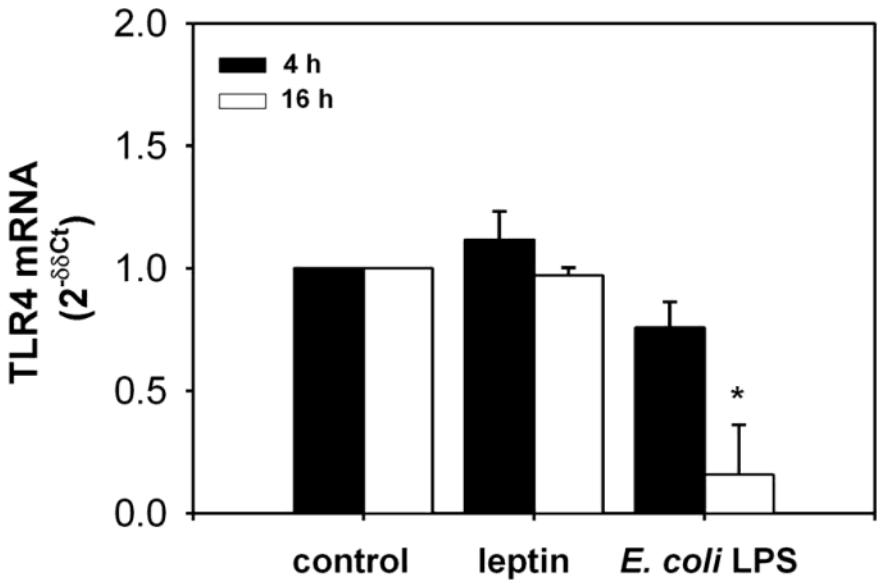

Figure 4.11 The effect of leptin or LPS on TLR4 mRNA expression in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Cells were collected and analysed for TLR4 mRNA expression with Realtime RT-PCR . Graph represents median + IQR from three independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: Kruskal-Wallis, Mann-Whitney *U* test $*p < 0.05$, compared to control.

4.3 Discussion

An increase in TLR or CD14 expression can be directly correlated to an increased production of pro-inflammatory cytokines such as IL-1β, TNF-α or IL-6 in response to LPS (Martin *et al.*, 1994; Du *et al.*, 1999). Therefore, it seems plausible that altered TLR and accessory protein expression patterns in inflammatory conditions such as human sepsis (Marsik *et al.*, 2003; Armstrong *et al.*, 2004; Schaaf *et al.*, 2009), tuberculosis (Prabha *et al.*, 2008) or periodontal disease (Asai *et al.*, 2001; Wang *et al.*, 2001a; Mori *et al.*, 2003; Ren *et al.*, 2005; Uehara *et al.*, 2007; Shin *et al.*, 2009) likely contribute to disease pathogenesis. A number of studies also describe altered TLR and CD14 expression patterns in diabetes (Patino *et al.*, 2000; Fogelstrand *et al.*, 2004; Creely *et al.*, 2007; Devaraj *et al.*, 2008). Although diabetes pathogenesis is not as clearly associated with inflammatory responses to PAMPs as, for example sepsis or tuberculosis, changes in TLR expression are nonetheless relevant, at least in obesity-induced diabetes. Thus, dietary fatty acids can induce both TLR4 and TLR2 signalling and trigger the expression of pro-inflammatory cytokines (Lee *et al.*, 2004; Shi *et al.*, 2006; Milanski *et al.*, 2009). In addition, any alteration of TLR expression patterns in diabetes potentially alters immune responses to periodontal pathogens and hence may contribute to an increased risk for periodontal disease (Khader *et al.*, 2006).

The present study and other studies in monocytes or macrophages demonstrate LPS-induced alterations in TLR2 and TLR4 expression patterns for both *E. coli* and *P. gingivalis* LPS. Thus, the present study shows an upregulation of TLR2 and TLR4 cell surface expression on THP-1 monocytes by *P. gingivalis* LPS. This finding is in agreement with results from other studies in THP-1 or primary human monocytes (Hajishengallis *et al.*, 2002; Muthukuru *et al.*, 2005; Foster *et al.*, 2007). In contrast, *E.*

coli LPS induced an upregulation of TLR2 cell surface expression only, no effect was observed for cell surface TLR4 expression in THP-1 monocytes. The effect of *E. coli* LPS on TLR2 expression was also seen in primary human monocytes, where a strong trend for *E. coli* LPS-induced increased TLR2 cell surface expression was observed. Furthermore, these *in vitro* findings confirm results from *in vivo* studies on monocytes in human sepsis and experimental endotoxaemia (Marsik *et al.*, 2003; Armstrong *et al.*, 2004). However, other studies report a different effect of *E. coli* LPS on TLR2 and TLR4 cell surface expression in monocytes. Thus, in THP-1 and primary human monocytes Foster *et al.* (2007) and Muthukuru *et al.* (2005) show an upregulation of both TLR2 and TLR4 cell surface expression after *E. coli* LPS stimulation while another study reports an *E. coli* LPS-induced downregulation of TLR4 cell surface expression in primary human monocytes (Bosisio *et al.*, 2002).

The studies with conflicting findings to the present report have all been conducted in human THP-1 or primary monocytes, with an *E. coli* LPS stimulation of 24 h. Therefore, experimental conditions were very similar between these studies and in comparison to the present study. However, it should be noted that basal TLR4 protein expression is very low in unstimulated monocytes and highly variable between individuals (Visintin *et al.*, 2001), making an efficient analysis of cell surface expression with flow cytometry technically challenging. Although great care was taken in the present study to ensure correct and consistent settings for acquisition and analysis of flow cytometry data, due to the low cell surface expression a technical error in the TLR4 data cannot be completely excluded. TLR4 expression in the present study was only detected after changing from FITC to the brighter PE dye. However, in a combined stain with CD14-FITC and TLR4-PE, a strong interference between the two dyes was

observed, which could not be compensated and thus made analysis of TLR4 expression in primary human monocytes impossible. Therefore, in future studies novel dyes such as the Alexa dyes with a higher fluorescence intensity and a different wave length spectrum (Panchuk-Voloshina *et al.*, 1999) may provide an alternative to the classic PE/FITC dyes for analysis of TLR4 cell surface expression.

The present study also confirms findings of *E. coli* LPS-induced upregulation of CD14 cell surface expression in monocytes (Otterlei *et al.*, 1995; Landmann *et al.*, 1996; Flo *et al.*, 2001) and is, to the best of my knowledge the first study to quantitatively describe this effect for *P. gingivalis* LPS as well. The effect of LPS on CD14 expression was only observed in THP-1 monocytes and was not significant in primary human monocytes. However, a large variation in basal cell surface CD14 expression was observed in primary cells and likely the sample size was too small to overcome individual differences. Nonetheless, the observed upregulation of CD14 expression by *P. gingivalis* LPS may be relevant as CD14 is an essential accessory protein for both TLR4 and TLR2 signalling (Wright *et al.*, 1990; Manukyan *et al.*, 2005) and thus an increased CD14 expression potentially also increases production of pro-inflammatory cytokines (Martin *et al.*, 1994).

Studies in diabetes show an upregulation of TLR2, TLR4 and CD14 cell surface expression in primary human monocytes (Patino *et al.*, 2000; Fogelstrand *et al.*, 2004; Devaraj *et al.*, 2008) and skeletal muscle (Reyna *et al.*, 2008). No clear explanations were provided for these findings and it could be speculated that alterations in free fatty acids, AGEs or cholesterol play a role. Recently, two reports described increased circulating endotoxin concentrations in diabetics (Creely *et al.*, 2007; Al-Attas *et al.*, 2009) which may explain the upregulated TLR and CD14 expression. However, the

findings of the present study may provide an additional explanation. A number of studies report an effect of the adipokine leptin on the expression of cell surface activation markers such as CD11b, CD40 or CD69 in several immune cells (Santos-Alvarez *et al.*, 1999; Martin-Romero *et al.*, 2000; Lam *et al.*, 2006; Konstantinidis *et al.*, 2009). In the present study, leptin induces an upregulation of TLR2 and CD14 cell surface expression on human THP-1 monocytes. In addition, a strong trend for leptin-induced upregulation of TLR2 cell surface expression was observed in primary human monocytes. Leptin did not induce an upregulation of CD14 expression in primary human monocytes, but, as in a comparable way as for *E. coli* LPS, the large variation in basal cell surface CD14 expression and the small sample size likely impair any conclusions. Due to the above mentioned technical difficulties, it was not possible to study an effect of leptin on TLR4 expression on the cell surface of primary human monocytes. However, no effect of leptin was observed on TLR4 cell surface expression in THP-1 monocytes. In fact, only *P. gingivalis* (and not *E. coli* LPS) had any effects on TLR4 cell surface expression. Thus, it appears that TLR2 and TLR4 expression is regulated differentially and that *P. gingivalis* LPS and *E. coli* LPS or leptin likely induce different mechanisms of TLR expression.

This is also supported by the results obtained in the present study for leptin or *E. coli* LPS-induced alterations in TLR mRNA expression. While both leptin and *E. coli* LPS increased TLR2 mRNA expression in THP-1 monocytes, leptin had no effect on TLR4 mRNA expression. In contrast, *E. coli* LPS induced a downregulation of TLR4 mRNA expression over time. The findings for *E. coli* LPS are in line with results of an *in vivo* study on human macrophages in experimental endotoxaemia where equally an *E. coli* LPS-induced upregulation of TLR2 mRNA but a downregulation of TLR4 mRNA was

observed (Maris *et al.*, 2006). However, as seen for TLR protein expression, reports on the effect of LPS on TLR mRNA expression reveal conflicting findings as well. Thus, Muthukuru *et al.* (2005) find an upregulation of both TLR2 and TLR4 mRNA expression by *E. coli* or *P. gingivalis* LPS, while Muzio *et al.* (2000) and Armstrong *et al.* (2004) show no change in TLR2 mRNA but an upregulation of TLR4 mRNA by *E. coli* LPS.

While the consistent *E. coli* or *P. gingivalis* LPS-induced upregulation of TLR2 expression at both mRNA and protein level presumably points to a regulation at the transcriptional level, observations for *E. coli* but not *P. gingivalis* LPS-induced TLR4 mRNA upregulation yet decreased protein expression (Bosisio *et al.*, 2002) may be a mechanism of LPS tolerance towards *E. coli* LPS. This indicates an activation of different signalling pathways for TLR2 and TLR4 ligands (Martin *et al.*, 2001; Hajishengallis *et al.*, 2002), especially as no such effect of LPS tolerance was reported for *P. gingivalis* LPS (Martin *et al.*, 2001). It is interesting to note that except for strict MyD88 dependency, the TLR2 intra-cellular signalling pathway is similar to TLR4 signalling (O'Neill and Bowie, 2007). Nonetheless, *E. coli* or *P. gingivalis* LPS signalling can induce differential immune responses which is potentially biologically relevant in inflammatory conditions such as periodontal disease. For example, in contrast to *E. coli* LPS, *P. gingivalis* LPS was found to be only a weak inducer of the pro-inflammatory cytokines and chemokines IL-1β, IFN-γ, MCP-5 and CXCL5 in monocytes and macrophages (Hirschfeld *et al.*, 2001; Barksby *et al.*, 2009). It was also demonstrated that *P. gingivalis* LPS primes dendritic cells for inducing a T_h-2 response, whereas *E. coli* LPS favors a T_h-1 response (Jotwani *et al.*, 2003). Additionally, a study in human monocyte-derived DCs showed that only *P. gingivalis* but not *E. coli* LPS

could up regulate CD14 and CD16 expression, thereby creating a unique DC subset with distinct stimulatory properties (Kanaya *et al.*, 2004). It is thought that in combination with its antagonistic actions on TLR4 signalling, the differential TLR signalling by *P. gingivalis* LPS is part of a strategy of this periodontal pathogen to evade immune responses and thus the differential regulation of TLR expression by *E. coli* or *P. gingivalis* LPS potentially is a relevant element in periodontal disease pathogenesis (Yoshimura *et al.*, 2002; Bostanci *et al.*, 2007).

The regulatory mechanisms of TLR expression are complex and still incompletely understood. At the cell surface and in cytoplasm, they involve translocation (Nagai *et al.*, 2002) and lateral diffusion (Triantafilou *et al.*, 2004) of TLR proteins through glycosylation processes (Nagai *et al.*, 2002; Ohnishi *et al.*, 2003) and the ESCRT machinery (Williams and Urbe, 2007). At the transcriptional level, TLR expression is regulated through the transcription factors PU.1 and Sp1. A consensus-binding site for the transcription factor PU.1 can be detected in both TLR2 and TLR4 promoters (Rehli *et al.*, 2000; Haehnel *et al.*, 2002; Lin and Rikihisa, 2004), while only the TLR2 but not the TLR4 promoter contains a consensus-binding site for Sp1 (Rehli *et al.*, 2000; Wang *et al.*, 2001b; Haehnel *et al.*, 2002). Possibly both *E. coli* and *P. gingivalis* LPS, as well as leptin, induce an upregulation or change in activity of TLR2 transcription factors which then leads to increased TLR2 mRNA and protein expression. In contrast, TLR4 mRNA and cell surface expression may be regulated more independently and changes at the mRNA level do not necessarily affect the expression of TLR4 protein. As part of the present study, experiments were conducted to evaluate the effect of leptin on the expression levels and activity of TLR transcription factors to determine a possible mechanism for the leptin-induced changes in TLR expression and these will be

discussed in more detail in the next chapter. Meanwhile, it is important to note that compared to the strong effect of LPS on TNF-α mRNA expression, observed in the previous chapter with over a 100-fold change, only small changes (up to 3-fold) in mRNA expression could be detected for TLR2 and TLR4. However, the small changes in TLR mRNA expression are consistent with other studies using the same methodology for analysis (Sha *et al.*, 2004; Franchini *et al.*, 2006). A possible explanation could be that TLR mRNA is more stable, can be transcribed more efficiently than TNF-α mRNA, or simply that TLR proteins have a longer half-life than TNF-α and therefore less mRNA is needed. Alternatively, one could assume that only one TLR is needed to respond to LPS and induce a whole array of different intra-cellular signalling pathways and cytokine responses, while a threshold of a certain TNF-α protein concentration must be reached to evoke a response.

Lam *et al.* (2007) demonstrate that leptin enhances *E. coli* LPS-induced upregulation of CD40 expression in murine bone marrow-derived DCs through simultaneous activation of JAK2/STAT and PI3K/Akt signalling events. The present study does not support an LPS-enhancing effect of leptin on TLR2 cell surface expression for either *E. coli* or *P. gingivalis* LPS. However, to date, the mechanism for how leptin may affect TLR expression is largely unknown and it could be that at the chosen LPS concentration, the TLR2 receptor was already maximally stimulated and there was no capacity for any further upregulation of TLR2 cell surface expression. In future experiments, it would be interesting to study the effect of leptin on TLR2 cell surface expression in a combination with a variety of different LPS concentrations. In addition, it should be considered that TLR2 associates with TLR1 or TLR6 (Ozinsky *et al.*, 2000) and while leptin may not have an LPS-enhancing effect on TLR2 expression, it possibly does with

TLR1 or TLR6. Nonetheless, the enhancing effect of leptin alone on TLR2 expression potentially is an important link between diabetes and an increased risk for severe periodontal disease (Khader *et al.*, 2006). Leptin concentrations are increased in diabetes (Sinha *et al.*, 1996) and thus by inducing increased TLR2 expression, leptin may pre-condition monocytes for an exaggerated immune response towards periodontal pathogens such as *P. gingivalis* LPS which are known to induce TLR2 signalling. Furthermore, leptin-induced upregulation of TLR2 expression may also impact on monocyte differentiation and might enhance development of the $CD14^{low}CD16^{+}$ monocyte subset, which displays increased TLR2 expression (Iwahashi *et al.*, 2004) and is increased in periodontal disease (Nagasawa *et al.*, 2004).

In summary, the present study contributes to the literature on LPS-induced alterations in TLR expression and supports findings for consistent TLR2 upregulation at both the mRNA and protein level by *P. gingivalis* or *E. coli* LPS and more diverse regulations for LPS-induced alterations in TLR4 expression, with a downregulation at the mRNA level yet no change in TLR4 protein expression. In addition, the present study shows for the first time that leptin can induce an upregulation of TLR2 cell surface and TLR2 mRNA expression in human monocytes. Although leptin had no direct effect on TLR4 expression, the leptin-induced upregulation of CD14 is likely relevant for both TLR2 and TLR4 signalling. Together, these findings suggest that leptin alters monocyte TLR expression patterns and this may be relevant in a link between conditions with increased leptin concentrations such as T2DM and the increased risk for periodontal disease.

Chapter 5 The effect of leptin on monocyte PU.1 and GM-CSF expression

Chapter 5 The effect of leptin on monocyte PU.1 and GM-CSF expression

5.1 Introduction

Changes in TLR expression patterns in immune cells and various tissues are observed in several inflammatory diseases such as sepsis (Armstrong *et al.*, 2004; Schaaf *et al.*, 2009), tuberculosis (Prabha *et al.*, 2008), periodontal disease (Mori *et al.*, 2003) and diabetes (Creely *et al.*, 2007; Devaraj *et al.*, 2008) but the mechanisms which underpin these changes are still incompletely understood. PU.1 is recognized as a key transcription factor in TLR mRNA expression and both TLR2 and TLR4 promoter contain a consensus-binding site for PU.1 (Rehli *et al.*, 2000; Haehnel *et al.*, 2002; Lin and Rikihisa, 2004). LPS stimulation of murine macrophages can induce the association of PU.1 with the TLR4 promoter (Pedchenko *et al.*, 2005). Furthermore, siRNA-induced inhibition of PU.1 results in reduced TLR4 mRNA expression (Joo *et al.*, 2008). Another relevant TLR transcription factor is Sp1. The TLR2 promoter has a consensus-binding site for Sp1 but the TLR4 promoter does not (Rehli *et al.*, 2000), suggesting that their expression is regulated differentially. Although putative consensus-binding sites for other transcription factors such as STAT or AP-1 were identified by Rehli (2002), these have not been experimentally validated and thus PU.1 and Sp1 remain the only confirmed TLR transcription factors to date.

Some evidence suggests that PU.1 auto regulates its own expression through an upstream regulatory promoter element (Chen *et al.*, 1995; Okuno *et al.*, 2005). Hence, increased PU.1 activity after LPS or PMA stimulation (Shackelford *et al.*, 1995; Lodie *et al.*, 1997; Ito *et al.*, 2005) should result in increased PU.1 protein expression. However, results of the few studies that have investigated PU.1 protein expression

under inflammatory conditions do not always support this concept. While Ito *et al.* (2005) find increased PU.1 protein expression after LPS or PMA stimulation in mouse mast cells, LPS-stimulation of mouse macrophages has no effect on PU.1 protein expression in the studies of Lodie *et al.* (1997) or Buras *et al.* (1995). Comparable findings to the protein expression were obtained at the mRNA level for PU.1 after LPS or PMA stimulation (Carey *et al.*, 1996; Ishii *et al.*, 2008; Suzuki *et al.*, 2009). Clearly, more research is needed to evaluate the effect of inflammatory conditions on PU.1 protein and mRNA expression in animals and humans. During granulocyte and monocyte differentiation, GM-CSF was shown to be an important regulator of PU.1 mRNA expression (Voso *et al.*, 1994) and GM-CSF deficiency results in reduced PU.1 expression in both murine knock-out models and human patients (Shibata *et al.*, 2001; Bonfield *et al.*, 2003). Importantly, the murine GM-CSF knock-out model also exhibited reduced TLR2 expression, which could be rescued after restoring either PU.1 or GM-CSF expression (Shibata *et al.*, 2001). No further data are available on GM-CSF and its effect on PU.1 and TLR expression. Moreover, Shibata *et al.* (2001) are the only group to evaluate the effect of other stimuli than LPS or PMA on PU.1 and TLR expression. Several studies report the expression of various pro-inflammatory cytokines such as IL-2, IL-6, IFN-γ or TNF-α (Santos-Alvarez *et al.*, 1999; Martin-Romero *et al.*, 2000; Zarkesh-Esfahani *et al.*, 2001; Mattioli *et al.*, 2005) after leptin stimulation in myeloid cells and T cells. Gainsford *et al.* (1996) show that GM-CSF secretion was not affected by leptin in murine peritoneal macrophages. However, to date, no studies have investigated an effect of the adipokine on GM-CSF expression in humans and it is unknown if leptin potentially could effect PU.1 expression through GM-CSF.

A number of studies have revealed changes in PU.1 activity as an important factor for its efficiency in the transcription of several genes. PU.1 has at least 5 phosphorylation sites (Lodie *et al.*, 1997) and depending on the cell type and function studied, phosphorylation of different sites is essential for increased PU.1 activity. For example, Pongubala *et al.* (1993) demonstrated that PU.1 phosphorylation at Ser 148 is needed for recruitment of a B-cell transcription factor whereas Celada *et al.* (1996) revealed a critical role for PU.1 phosphorylation at Ser 41 and Ser 45 in macrophage proliferation. No data are available to date on PU.1 phosphorylation status and TLR expression. Increased DNA-binding of PU.1 was reported after LPS or PMA stimulation as another indicator for increased PU.1 activity (Shackelford *et al.*, 1995; Carey *et al.*, 1996). However, Pongubala *et al.* (1993) demonstrated in mouse B cells that both phosphorylated and dephosphorylated PU.1 bind DNA yet only the phosphorylated form was capable of interaction with the B cell restricted transcription factor NF-EM5. This result indicates that enhanced DNA-binding capacity is not always associated with increased transcriptional activity. Thus, PU.1 phosphorylation status is likely one of the main determinants for changes in TLR expression at the transcriptional level.

PU.1 phosphorylation was shown to be induced via the p38 and the PI3K/Akt pathway (Rieske and Pongubala, 2001; Wang *et al.*, 2003) and is mainly mediated through casein kinase II (CKII) (Meisner and Czech, 1991; Pongubala *et al.*, 1993; Lodie *et al.*, 1997; Joo *et al.*, 2009). Interestingly, the p38 and PI3K/Akt pathways are two of the main pathways activated by leptin signalling in myeloid cells (van den Brink *et al.*, 2000; Ktori *et al.*, 2003; Gruen *et al.*, 2007). In addition, it was demonstrated recently that leptin can induce CKII activation (Ning *et al.*, 2006; Ning *et al.*, 2009). Therefore, leptin can potentially alter PU.1 activity and thereby expression of TLR2 and TLR4.

Furthermore, Sp1 activity is mediated through CKII (Armstrong *et al.*, 1997; Dunzendorfer *et al.*, 2004) and a study in murine hepatic stellate cells demonstrates direct leptin-induced phosphorylation of Sp1 (Lin *et al.*, 2006), supporting a potential role for this adipokine in the regulation of TLR expression. Increased leptin concentrations have been observed in diabetics (Sinha *et al.*, 1996) and TLR expression is upregulated (Creely *et al.*, 2007; Devaraj *et al.*, 2008). Thus, although increased endotoxin concentrations in diabetics (Creely *et al.*, 2007; Al-Attas *et al.*, 2009) may impact on TLR transcription factors, leptin potentially affects PU.1 and Sp1 activity as well. However, except for the studies by Lin *et al.* (2006) and Gainsford *et al.* (1996), an effect of leptin on TLR transcription factors activity or expression has not been investigated and a role for this adipokine further needs to be evaluated.

PU.1 is not only involved in TLR expression, it is a member of the Ets family, a group of transcription factors which are involved in cell maturation, growth and differentiation (Oikawa and Yamada, 2003). Hence, PU.1 is essential in the development of granulocytes, lymphocytes and monocytes and PU.1 knock-out mice do not produce lymphoid or myeloid progenitors (Scott *et al.*, 1994; Iwasaki *et al.*, 2005). Furthermore, PU.1 expression is upregulated during macrophage differentiation (Shibata *et al.*, 2001; Bonfield *et al.*, 2003) and important for the expression of typical macrophage cell surface receptors such as CD11b (Pahl *et al.*, 1993) or the GM-CSF (Hohaus *et al.*, 1995) and M-CSF (Zhang *et al.*, 1994) receptors which are associated with phagocytosis and cell growth (Baek *et al.*, 2009). Thus, stimulation of the relatively immature monocytic cell line THP-1 with PMA induces PU.1 upregulation and differentiation into cells with macrophage like morphology and altered cell surface receptor expression patterns (Tsuchiya *et al.*, 1982; Baqui *et al.*, 1999; Suzuki *et al.*,

2009). Interestingly, leptin was revealed as an important regulator of haematopoiesis and myeloid cell differentiation. Thus, Claycombe *et al.* (2008) report 70% less absolute numbers of B cells, 40 % less absolute numbers of granulocytes and 25 % absolute numbers of monocytes in *ob/ob* mice compared to wild-type controls. In addition, they also observed a near normalisation of cell numbers after treatment of the *ob/ob* mice with leptin (Claycombe *et al.*, 2008). The mechanisms of regulatory functions of leptin in haematopiesis and differentiation are not identified yet, however possibly its anti-apoptotic effects play a role in sustaining lymphoid and myeloid progenitor cell populations (Lam *et al.*, 2006; Claycombe *et al.*, 2008). Alternatively, leptin potentially influences cell differentiation via regulation of PU.1, however this remains to be investigated.

Knowledge on any manipulations of monocyte differentiation or maturation through leptin is potentially important in conditions with increased leptin concentrations such as diabetes as this may change immune responses to PAMPs and thus impact on the susceptibility to periodontal disease. The aims of the present study were therefore to investigate an effect of leptin on expression and activity of the transcription factor PU.1 in THP-1 and primary human monocytes. In addition, an effect of leptin on GM-CSF expression was studied. Finally, the potential for leptin to induce differentiation in THP-1 monocytes was evaluated.

5.2 Results

5.2.1 Investigation of the effect of leptin on expression and intracellular location of PU.1 in THP-1 and primary human monocytes

Immunofluorescence staining was performed to evaluate if leptin has an effect on monocytic PU.1 expression at the protein level and to determine an effect of leptin on PU.1 nuclear translocation. 0.5 x 10^6 THP-1 or primary human monocytes (isolated with the adherent cell method as described in 2.1.3.1) were cultured on glass cover slips and stimulated with 1000 ng/ml leptin or 50 ng/ml PMA for 24 h. Unstimulated cells served as controls. Cells were processed for fluorescence microscopy and incubated with the primary antibody rabbit-anti-human PU.1 followed by incubation with the secondary antibody sheep-anti-rabbit IgG-FITC (see 2.6). Fluorescence of THP-1 monocytes was analysed on a confocal laser scanning microscope, fluorescence analysis of primary human monocytes was performed on a standard fluorescence microscope as described in 2.6. Microscope settings were based on the negative control (secondary antibody stain only) and were kept the same throughout each individual experiment. The fluorescent cells were analysed for the FITC (PU.1) stain alone, for DAPI staining (nucleus) and for an overlay of FITC and DAPI. Also, a light image overlay with the FITC stain was conducted, however this function was not available on the standard fluorescence microscope. Additionally, for THP-1 monocytes FITC pixel intensities of the 5 brightest cells per slide were evaluated with the histogram function of the image processing and analysis software ImageJ.

Figure 5.1a and Figure 5.2a show that PU.1 (green stain) is constitutively expressed at the protein level in THP-1 and primary human monocytes, respectively. The protein is

evenly distributed throughout the cells and no apparent concentration to a particular cell region is visible. The overlay image of nucleus (blue stain) and PU.1 in THP-1 monocytes confirms that the protein can be detected in both nucleus (open arrow) and cytoplasm (filled arrow) (Figure 5.1b) in unstimulated cells. Figure 5.2b shows that PU.1 can be detected in cytoplasm (filled arrow) of primary human monocytes. The deep blue nuclear stain in unstimulated primary human monocytes interferes with the green stain of PU.1 (Figure 5.2b), however the green stain in the nuclear region can be seen in the corresponding single stain image of the overlay (Figure 5.2a).

After leptin stimulation, fluorescence for PU.1 staining is more intense in both THP-1 and primary human monocytes, indicating increased PU.1 protein expression (Figure 5.1d, Figure 5.2c). In contrast to the even distribution in unstimulated THP-1 monocytes, several THP-1 monocytes show a more intense stain for PU.1 in the nuclear region after leptin stimulation (open arrows, Figure 5.1d). This nuclear staining pattern is further confirmed in the corresponding PU.1-nuclear stain overlay (Figure 5.1e). PU.1 is not exclusively detected in the nucleus and staining can also be observed in the cytoplasm (filled arrows, Figure 5.1d, e), although the stain intensity is somewhat weaker than in the nucleus, which might suggest a leptin-induced nuclear translocation event of PU.1. However, compared to control leptin up regulates PU.1 throughout the cell but the fluorescence staining is too intense to allow conclusions about sub-cellular localisation to be made. After leptin stimulation in primary human monocytes, the staining intensity between nucleus (open arrows) and cytoplasm (filled arrows) does not differ as noticeably as in the cell line (Figure 5.2c, d) and in comparison to unstimulated cells the overall increase in staining intensity is more apparent.

To further investigate possible nuclear translocation events of PU.1, without interference of the blue nuclear stain, a light image overlay with the PU.1 stain was taken for THP-1 monocytes (Figure 5.1c, f). Detection of the cell membrane (filled arrow) and nuclear membrane (open arrow) is possible, yet the monocytic nucleus almost completely fills up the whole cell and the two membranes are in close proximity to each other, leaving only a small cytoplasmatic region. In combination with the overall increased staining intensity after leptin stimulation, the proximity of the two membranes again does not allow for conclusions about sub-cellular localisation of PU.1 to be made.

Foster *et al.* (2007) used PMA stimulation of THP-1 monocytes as a positive control for nuclear translocation of PU.1. However, results of the present study to analyse PMA-induced nuclear translocation of PU.1 in THP-1 monocytes are inconclusive. Although overlays of nuclear and PU.1 stain reveal that after PMA stimulation, PU.1 is almost exclusively detected in the nucleus (open arrow, Figure 5.3e), an increased PU.1 staining intensity in comparison to control (Figure 5.3b) was also observed. The increased staining intensity and the small cytoplasmatic region again do not allow for a clear differentiation between nuclear translocation and increased PU.1 protein expression. Furthermore, although the same experimental setup was used, results were not always consistent and in some experiments staining appeared more intense in control than in PMA stimulated cells (Figure 5.3c, f), indicating downregulation of PU.1 expression.

To quantify the stain for PU.1 before and after leptin stimulation, PU.1 FITC pixel intensity in THP-1 monocytes was analysed. Figure 5.4 shows that compared to control, FITC pixel intensity doubles after leptin stimulation ($p < 0.001$), which suggests an

increase in PU.1 protein expression. Data from primary human monocytes are derived from one experiment only and therefore FITC pixel intensity was not evaluated.

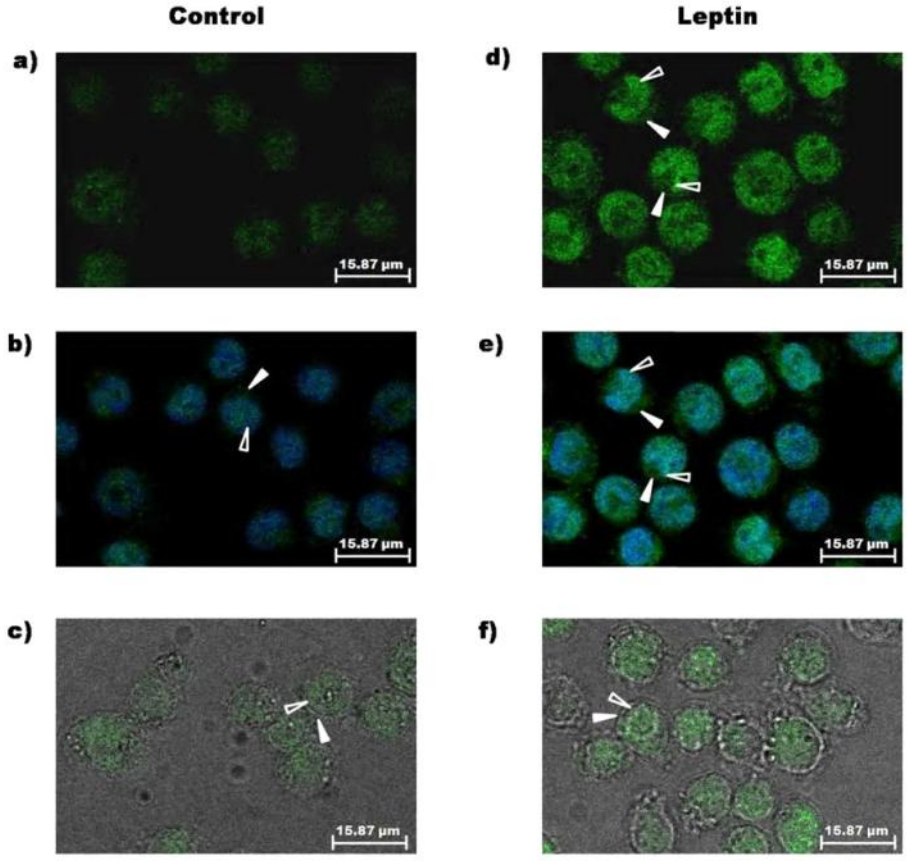

Figure 5.1 The effect of leptin on PU.1 protein expression and localization in THP-1 monocytes (confocal microscopy pictures)

THP-1 monocytes were stimulated with 1000 ng/ml leptin for 24 h. Cells were processed for fluorescence microscopy and analysed on a confocal laser scanning microscope. Pictures are representative examples from three independent cell culture experiments. a, d) PU.1 FITC; b, e) PU.1 FITC and DAPI (nuclear stain, blue) overlay; c, f) PU.1 FITC and light image overlay. Open arrow: nucleus; filled arrow: cytoplasm.

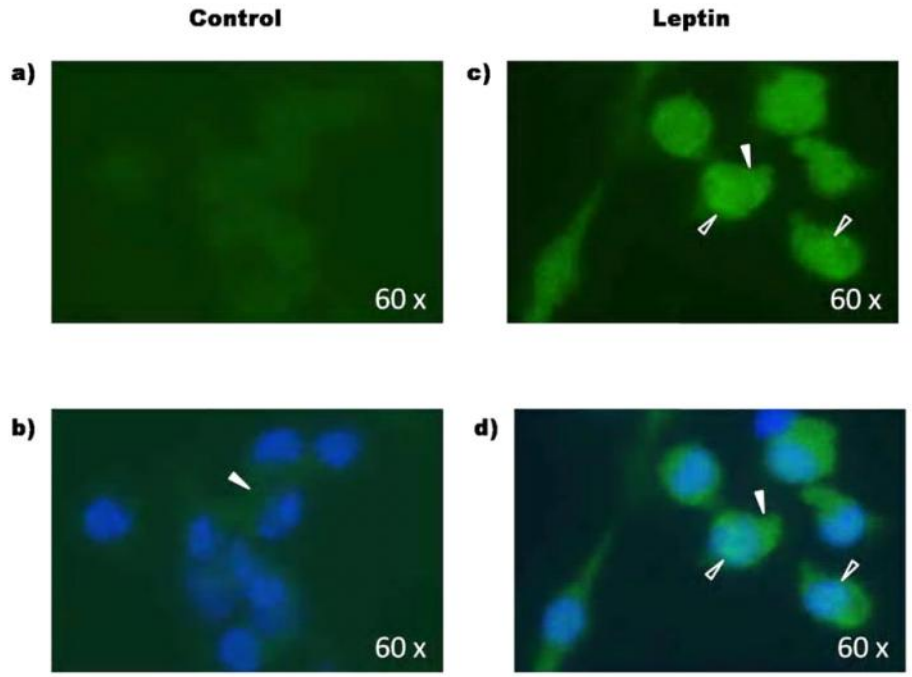

Figure 5.2 The effect of leptin on PU.1 protein expression and localization in primary human monocytes (fluorescence microscopy pictures)

Primary human monocytes were stimulated with 1000 ng/ml leptin for 24 h. Cells were processed for fluorescence microscopy and analysed on a fluorescence microscope using a 60 x magnification. Pictures are a representative view derived from one cell culture experiment. a, c) PU.1 FITC; b, d) PU.1 FITC and DAPI (nuclear stain, blue) overlay. Open arrow: nucleus; filled arrow: cytoplasm.

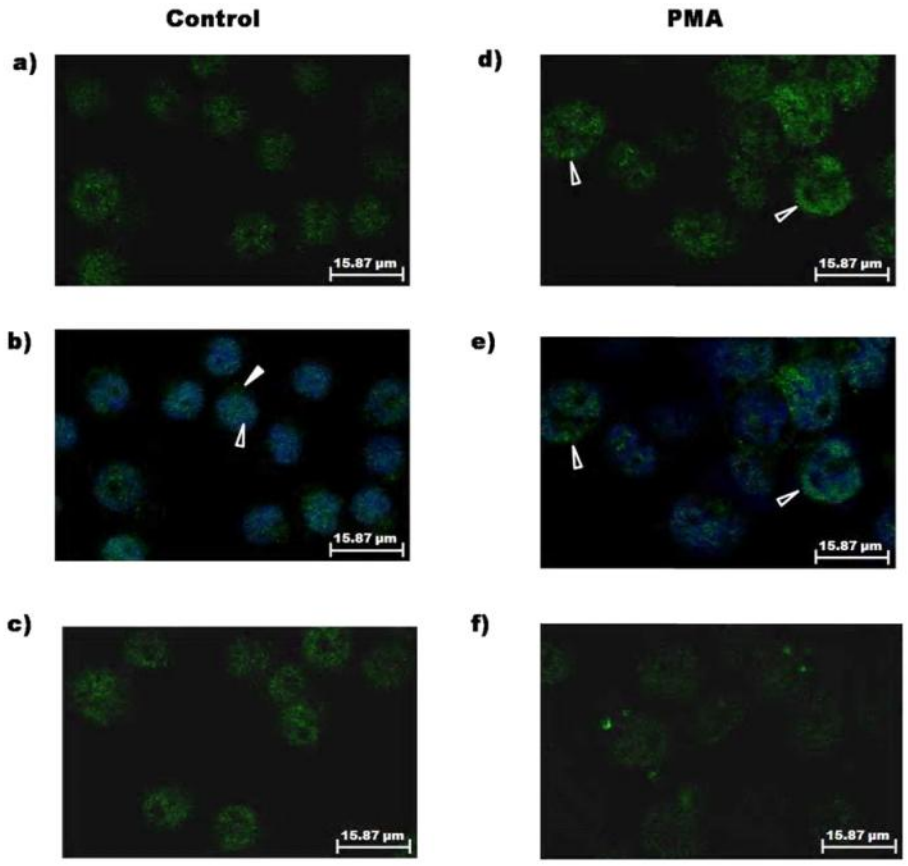

Figure 5.3 The effect of PMA on PU.1 protein expression and localization in THP-1 monocytes (confocal microscopy pictures)

THP-1 monocytes were stimulated with 50 ng/ml PMA for 24 h. Cells were processed for fluorescence microscopy and analysed on a confocal laser scanning microscope. Pictures are representative examples from two independent cell culture experiments. a, c d, f) PU.1 FITC; b, e) PU.1 FITC and DAPI (nuclear stain, blue) overlay. Open arrow: nucleus; filled arrow: cytoplasm.

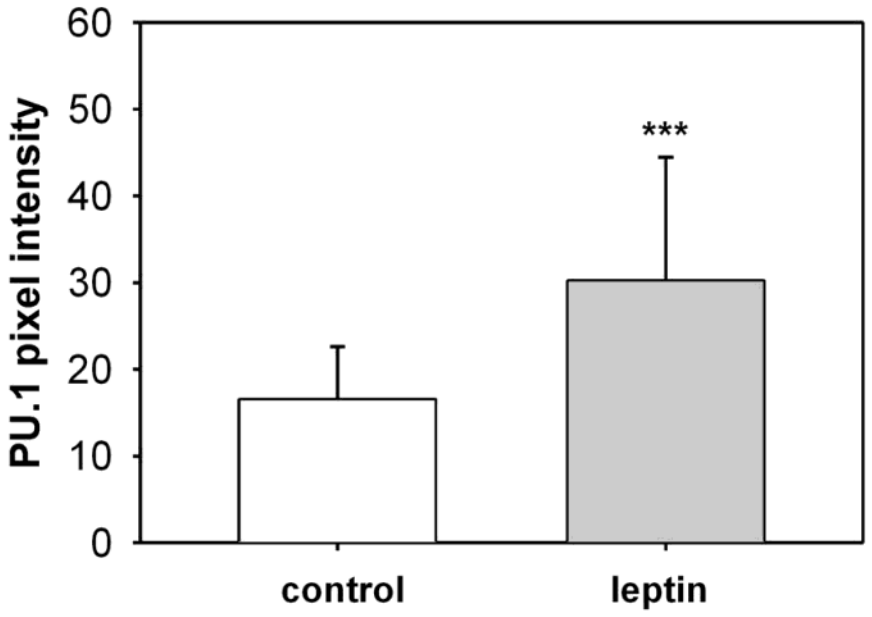

Figure 5.4 The effect of leptin on PU.1 protein expression in THP-1 monocytes (analysis of pixel intensity)

THP-1 monocytes were stimulated with 1000 ng/ml leptin for 24 h. Cells were processed for fluorescence microscopy and analysed on a confocal laser scanning microscope. Graph represents median + IQR from three independent cell culture experiments. Statistics: Mann-Whitney *U* test ***$p < 0.001$, compared to control.

5.2.1.1 Investigation of the effect of leptin on PU.1 mRNA expression in THP-1 monocytes

To further evaluate an effect of leptin on PU.1 expression, mRNA for PU.1 was analysed. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or PMA (50 ng/ml) for 24 h. Unstimulated cells served as controls. Cells were collected and analysed for PU.1 mRNA expression with Realtime RT-PCR as described in 2.5.3.

PU.1 mRNA is constitutively expressed in THP-1 monocytes however compared to unstimulated THP-1 monocytes, leptin had no effect on PU.1 mRNA expression ($p = 0.94$) (Figure 5.5). PMA stimulation induced a 172 % ($p < 0.01$) upregulation in PU.1 mRNA expression in comparison with control. PMA rather than LPS was used as a positive control for studying PU.1 mRNA expression as recent studies showed that PMA but not LPS upregulated PU.1 mRNA expression in myeloid cells (Ishii *et al.*, 2008; Suzuki *et al.*, 2009). The different effects of leptin and PMA on PU.1 mRNA expression possibly suggest additional regulation of PU.1 at the post-transcriptional level.

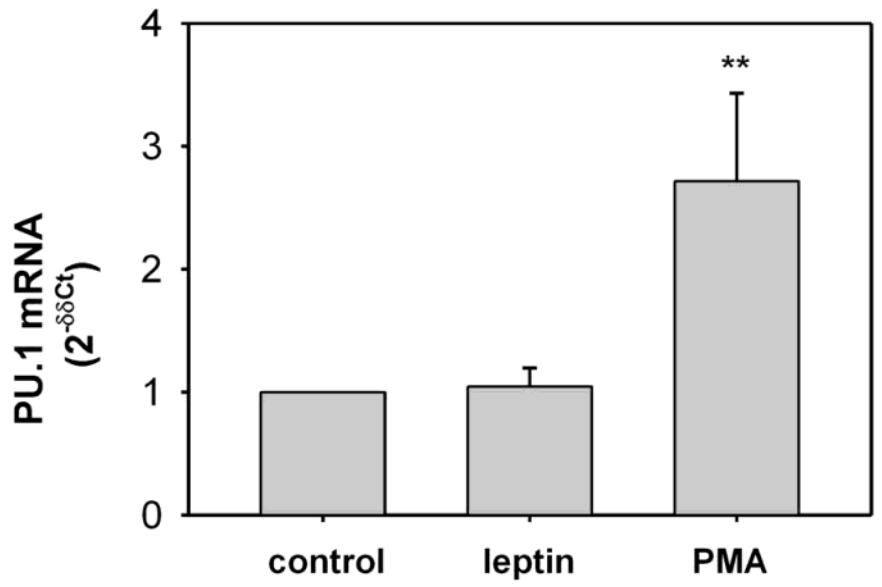

Figure 5.5 The effect of leptin or PMA on PU.1 mRNA expression in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin or PMA (50 ng/ml) for 24 h. Cells were collected and analysed for PU.1 mRNA expression with Realtime RT-PCR . Graph represents median + IQR from three independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: Kruskal-Wallis, Mann-Whitney *U* test $^{**}p < 0.01$, compared to control.

5.2.2 Investigation of the effect of leptin on PU.1 activity in THP-1 monocytes

To evaluate PU.1 activity, changes in the serine phosphorylation status of the protein were analysed after leptin and LPS stimulation of THP-1 monocytes. No phosphorylation antibodies are commercially available for PU.1 and therefore PU.1 was first immunoprecipitated and subsequently analysed using a generic phosphorylated serine antibody for Western blot.

Immunoprecipitation of PU.1

An immunoprecipitation protocol for PU.1 was established and tested for its efficiency. THP-1 monocytes (4×10^6) were lysed and PU.1 was immunoprecipitated with a PU.1 antibody as described in 2.7.2. Cell lysates without immunoprecipitation served as control. Cell lysates were analysed for PU.1 with Western blotting using the same PU.1 antibody as for immunoprecipitation. Methodological details are described in 2.7.3.

Figure 5.6 shows a representative example of the Western blot for PU.1 after immunoprecipitation. In a first IP, only faint bands were detected at the correct size for PU.1 (~ 40 kDa) (lanes 3 and 4). However, after adjusting salt concentrations in IP buffers and increasing the antibody incubation time, strong bands were detected at the correct size of PU.1 (lanes 5 and 6). In order to show that the IP was efficient, immunoprecipitates were compared to 5 % of total cell lysates from unprecipitated samples (lanes 1 and 2), as described by Harlow and Lane (1999). Bands for 5 % of unprecipitated samples were fainter than bands of the improved IP method (lanes 5 and 6), confirming that the established immunoprecipitation protocol was efficient. Additionally, a strong band of ~ 50 kDa was detected in immunoprecipitates which

corresponds to the heavy IgG chain of the PU.1 anti-human rabbit antibody. The heavy IgG chain is detected as a result of using an antibody with the same IgG source (rabbit) for both IP and Western blotting. The secondary antibody used in the Western blot is directed at rabbit IgG and will therefore detect any rabbit IgG that was eluted together with PU.1 in the IP. The light rabbit IgG chain (~ 25 kDa) was not detectable.

Western blot for PU.1 serine phosphorylation

After establishing the efficiency of PU.1 immunoprecipitation, changes in the serine phosphorylation status of PU.1 were analysed. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 1 h or 24 h. Unstimulated cells served as controls. Cells were lysed and PU.1 was immunoprecipitated with a PU.1 antibody as described in 2.7.2. An IP without cell lysate was included as a negative control. Immunoprecipitates were analysed for PU.1 serine phosphorylation with Western blot as described in 2.7.3. Cell lysate from an unprecipitated LPS-stimulated sample was included as a positive control for serine phosphorylation.

Results from a Western blot for serine phosphorylation of PU.1 which was developed under different conditions are shown in Figure 5.7 a) and b). The IP without cell lysate in lane 1 reveals strong bands of nonspecific binding of the serine phosphorylation antibody to some unknown component within the IP procedure. These same bands can be observed in all other IPs as well (lanes 3-8). LPS is a known inducer of phosphorylation (Lodie *et al.*, 1997). Therefore, while establishing the method for the serine phosphorylation Western blot, unprecipitated cell lysates of LPS-stimulated THP-1 monocytes were used as a positive control for the antibody. A first antibody did

detect little or no serine phosphorylation (blots not shown), despite switching to a highly sensitive developing reagent (ECL advanced instead of ECL plus) and addition of phosphatase inhibitors to the IP. After changing to a serine phosphorylation antibody from a different company, detection of several bands of various sizes in unprecipitated cell lysates of LPS stimulated THP-1 monocytes was possible (Figure 5.7, lane 2). Lanes 3 to 8 of the blots in Figure 5.7 a) and b) show cell lysates immunoprecipitated for PU.1 of THP-1 monocytes stimulated for 1 or 24 h with LPS (lane 3 and 6), leptin (lane 4 and 7) or left unstimulated as control (lane 5 and 8). The blot of Figure 5.7 a) was blocked in milk and horse serum. Although in some lanes very faint bands at the correct size for PU.1 (~ 40 kDa) can be seen, the signal is too weak to allow for any conclusions to be made on differences in the serine phosphorylation status of PU.1 before and after leptin or LPS stimulation. However, phosphorylation antibodies may interact with phosphorylated sites in milk proteins (Kit *et al.*, 1996), possibly reducing interaction with the actual phosphorylated sites of interest in PU.1. Therefore, the blot was equilibrated again and blocked in 3 % BSA instead of milk, as shown in Figure 5.7 b). However, while the bands at the correct size for PU.1 were stronger than in the first blot, the background stain increased drastically, likely due to now insufficient blocking. The poor signal to noise ratio again does not allow for any conclusions to be made on differences in the serine phosphorylation status of PU.1 before and after leptin or LPS stimulation.

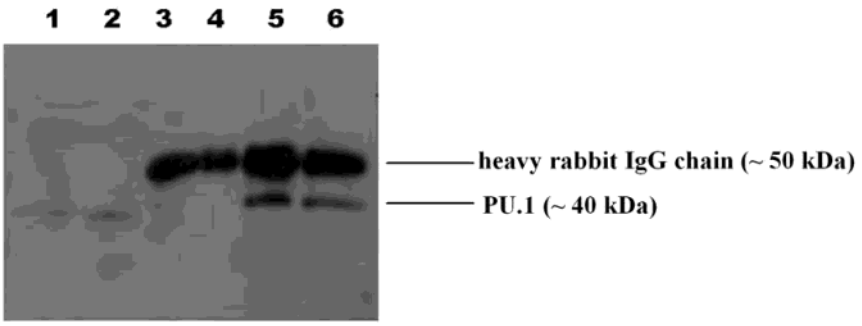

Figure 5.6 Immunoprecipitation for PU.1

Unstimulated THP-1 monocytes were immunoprecipitated for PU.1. Immunoprecepitates were analysed for PU.1 with Western blotting. The shown blot is representative for two independent cell culture experiments. All samples were run in duplicates. Lane 1 and 2: 5 % of total cells; lane 3 and 4: IP first method; lane 5 and 6: IP improved method.

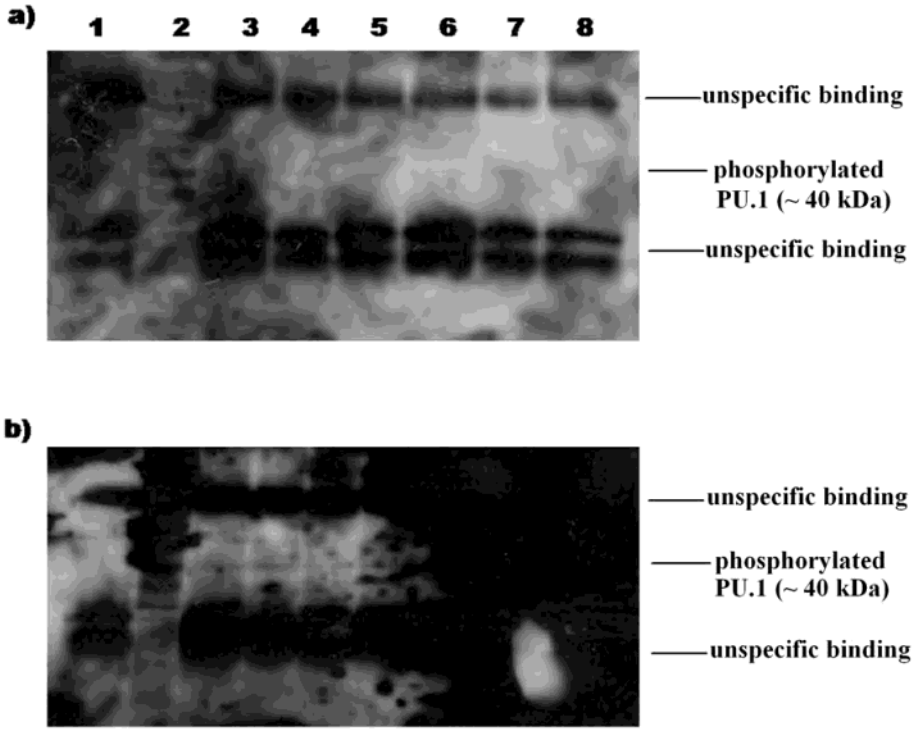

Figure 5.7 Western blot for PU.1 serine phosphorylation

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 1 h or 24 h. Cell lysates were immunoprecipitated for PU.1 and analysed for serine phosphorylation with Western blot. The same Western blot was developed under two different conditions and is derived from one experiment. Blot a) was blocked using milk and horse serum, blot b) was blocked using 3 % BSA instead of milk and horse serum. Lane 1: IP without cell lysate (negative control), lane 2: unprecipitated THP-1 monocytes stimulated for 1 h with LPS (positive control), lane 3: IP of 1 h LPS stimulation, lane 4: IP of 1 h leptin stimulation, lane 5: IP of 1 h control, lane 6: IP of 24 h LPS stimulation, lane 7: IP of 24 h leptin stimulation, lane 8: IP of 24 h control.

5.2.3 The role of GM-CSF in leptin regulated TLR expression in human monocytes

5.2.3.1 The role of GM-CSF in leptin regulated TLR expression in THP-1 monocytes

To investigate if leptin possibly alters TLR expression indirectly via inducing GM-CSF, the effect of leptin on GM-CSF secretion was determined in cell culture supernatants of THP-1 monocytes. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin, LPS from *E. coli* or *P. gingivalis* (100 ng/ml), or PMA (50 ng/ml) for 24 h. Unstimulated cells served as controls. Supernatants were collected and analysed for GM-CSF with ELISA as described in 2.3.

No detectable levels of GM-CSF were observed in cell culture supernatants of unstimulated THP-1 monocytes (Figure 5.8). Neither leptin, *E. coli* LPS nor *P. gingivalis* LPS induced GM-CSF secretion. Stimulation of THP-1 monocytes with PMA induced GM-CSF secretion and a concentration of 667 pg/ml was detected in cell culture supernatants. GM-CSF was shown to be an important regulator of PU.1 mRNA expression during monocyte differentiation (Voso *et al.*, 1994) and is associated with regulating TLR2 expression (Shibata *et al.*, 2001). However, the finding of the present study does not support a role for leptin or LPS in GM-CSF secretion.

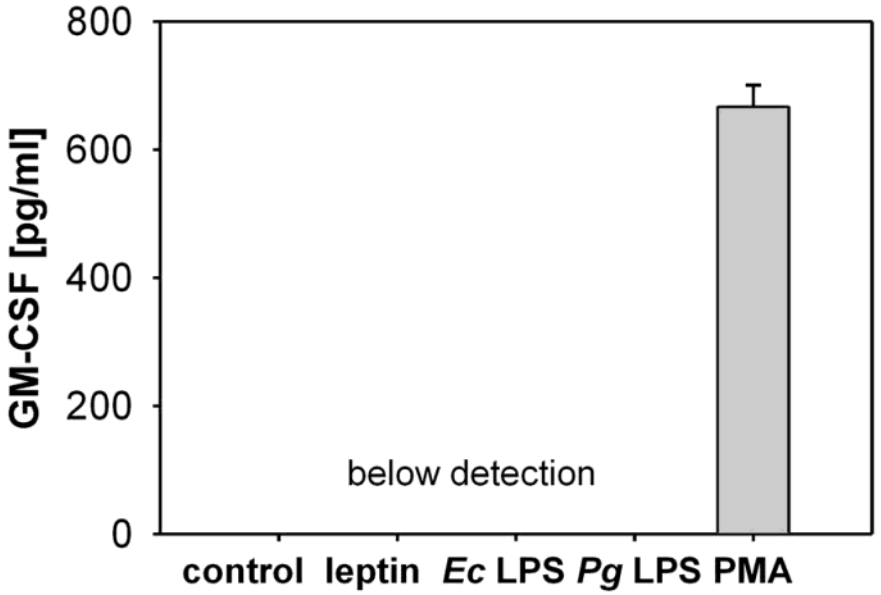

Figure 5.8 The effect of leptin on GM-CSF secretion in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin, LPS from *E. coli* or *P. gingivalis* (100 ng/ml) or PMA (50 ng/ml) for 24 h. Supernatants were collected and analysed for GM-CSF with ELISA. Graph represents mean + SD from three independent cell culture experiments. *Ec*: *E. coli, Pg: P. gingivalis*.

5.2.3.2 The role of GM-CSF in leptin regulated TLR expression in primary human monocytes

To further evaluate the effect of leptin on GM-CSF secretion, a stimulation experiment was repeated in primary human monocytes. Primary human monocytes were isolated from a buffy coat via CD14 expression using the magnetic beads method as described in 2.1.3.2. After purification, 4 x 10^6 monocytes were stimulated with 1000 ng/ml leptin, LPS from *E. coli* (100 ng/ml) or PMA (50 ng/ml) for 24 h. Unstimulated cells served as control. Supernatants were collected and analysed for GM-CSF with ELISA as described in 2.3.

No detectable levels of GM-CSF were observed in cell culture supernatants of unstimulated primary human monocytes (Figure 5.9). Neither leptin nor *E. coli* LPS induced GM-CSF secretion. Stimulation of primary human monocytes with PMA induced GM-CSF secretion and a concentration of 357 pg/ml was detected in cell culture supernatants.

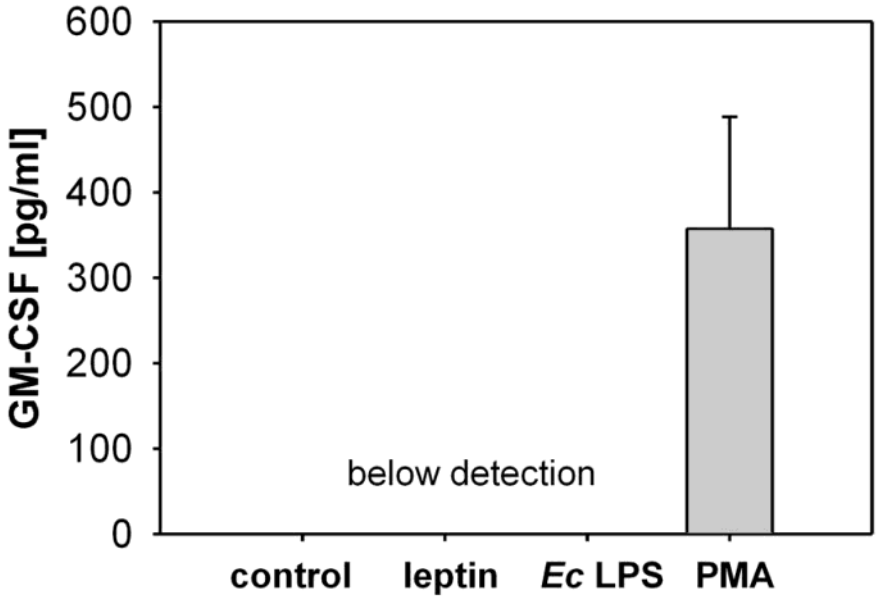

Figure 5.9 The effect of leptin on GM-CSF secretion in primary human monocytes

Primary human monocytes were stimulated with 1000 ng/ml leptin, LPS from *E. coli* (100 ng/ml) or PMA (50 ng/ml) for 24 h. Supernatants were collected and analysed for GM-CSF with ELISA. Graph represents mean + SD from three independent cell culture experiments. *Ec*: *E. coli.*

5.2.4 Investigation of the effect of leptin on GM-CSF mRNA expression in THP-1 monocytes

To study an effect of leptin on GM-CSF at the transcriptional level, mRNA expression for GM-CSF was analysed. THP-1 monocytes (4×10^6) were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Unstimulated cells served as controls. Cells were collected and analysed for GM-CSF mRNA expression with Realtime RT-PCR as described in 2.5.3.

Leptin and *E. coli* LPS stimulation both induced a significant upregulation at 4 h in GM-CSF mRNA expression (Figure 5.10). Compared to control, GM-CSF mRNA expression was 5.2-fold higher ($p < 0.01$) after leptin and 141-fold higher ($p < 0.001$) after *E. coli* LPS stimulation. After 16 h, only a trend of increased GM-CSF mRNA expression after leptin stimulation was observed ($p = 0.07$), whereas *E. coli* LPS further enhanced GM-CSF mRNA expression, inducing a 511-fold increase ($p < 0.001$, Figure 5.10). The leptin and LPS-induced upregulation of GM-CSF at the mRNA but not at the protein level possibly suggests a mechanism for regulating GM-CSF at the level of protein secretion.

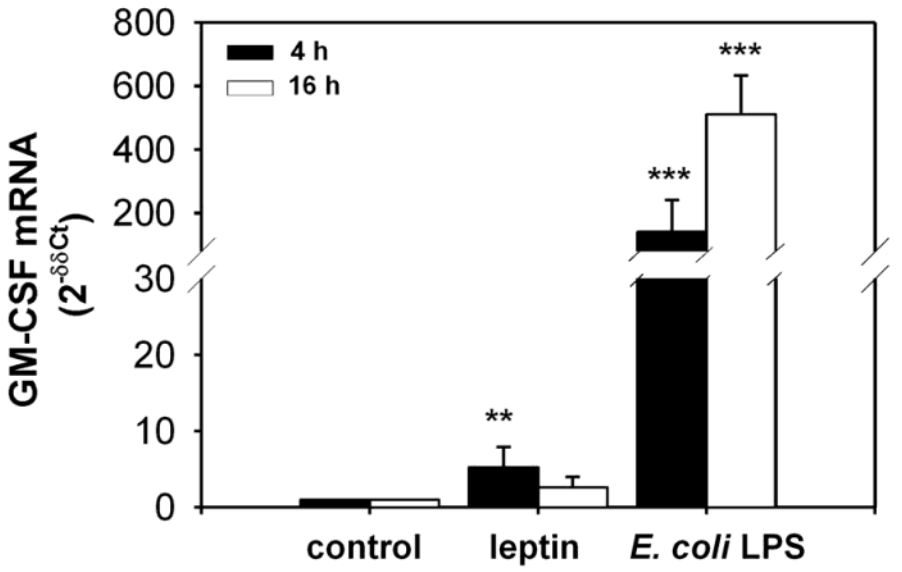

Figure 5.10 The effect of leptin or LPS on GM-CSF mRNA expression in THP-1 monocytes

THP-1 monocytes were stimulated with 1000 ng/ml leptin or LPS from *E. coli* (100 ng/ml) for 4 or 16 h. Cells were collected and analysed for GM-CSF mRNA expression with Realtime RT-PCR. Graph represents mean + SD from three independent cell culture experiments. mRNA expression was normalized to RNA polymerase II and is expressed as $2^{-\delta\delta Ct}$ values. Statistics: ANOVA, Student's *t*-test $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

5.2.5 The effect of leptin on monocyte morphology

During routine observations of THP-1 monocyte cell cultures after leptin stimulation, slight changes in cell morphology were noted. For documentation, photographs of cells after stimulation with a variety of agents were taken. THP-1 monocytes (4×10^6) were pre-treated with $VitD_3$ for 48 h as under normal experimental conditions (see 2.1.1.4). Cells were then stimulated with 1000 ng/ml leptin, LPS from *E. coli* (100 ng/ml) or PMA (50 ng/ml) for 24 h. THP-1 pro-monocytes and unstimulated cells ($VitD_3$ treatment only) served as controls. Photographs of cell cultures were taken under a light microscope and cell morphology was evaluated.

Photographs in Figure 5.11-Figure 5.15 show the original picture of a 40 x magnification as obtained from the microscope and an enlarged view generated with Microsoft Power Point. The different stimulations had distinguished effects on THP-1 cell morphology and adherence to cell culture plates. THP-1 pro-monocytes show a round, even morphology, most cells being of the same size (Figure 5.11). In addition, these cells are non-adherent. After $VitD_3$ treatment, cells start to adhere and increase in size, yet mostly cells retained the round, even morphology (Figure 5.12). Leptin stimulation increases cell adherence and some cells greatly increase in cell size, which may indicate monocyte maturation (Figure 5.13, **M**). Other cells display a more stretched out morphology, possibly starting a differentiation process towards macrophages (Figure 5.13, **S**). The greatly increased cell size was observed in leptin-stimulated cells alone. LPS or PMA stimulation further increased cell adherence to cell culture plates. In addition, after LPS stimulation some cells stretch out in several directions, forming podosomes (Figure 5.14, **P**). These podosomes are specialised structures containing actin filaments and function as adherence contacts to the extra-

cellular matrix (Linder and Kopp, 2005). Additionally, they may be indicative for a differentiation process towards dendritic cells (Burns *et al.*, 2001). Furthermore, some cells appeared to show increased granularity after LPS stimulation (Figure 5.14, **R**), which might either suggest apoptotic events or differentiation into neutrophil-like cells. Dendritic cell shaped morphology or granularity were not observed in any of the other stimulations.

PMA stimulation induced a differentiation of several monocytes into macrophage-like cells, displaying characteristic macrophage morphology with a long stretched, amoeboid shape (Figure 5.15, **G**). Finally, in all stimulations it was noted that not every cell showed a changed morphology under a specific treatment but several cells retained the same features as before stimulation.

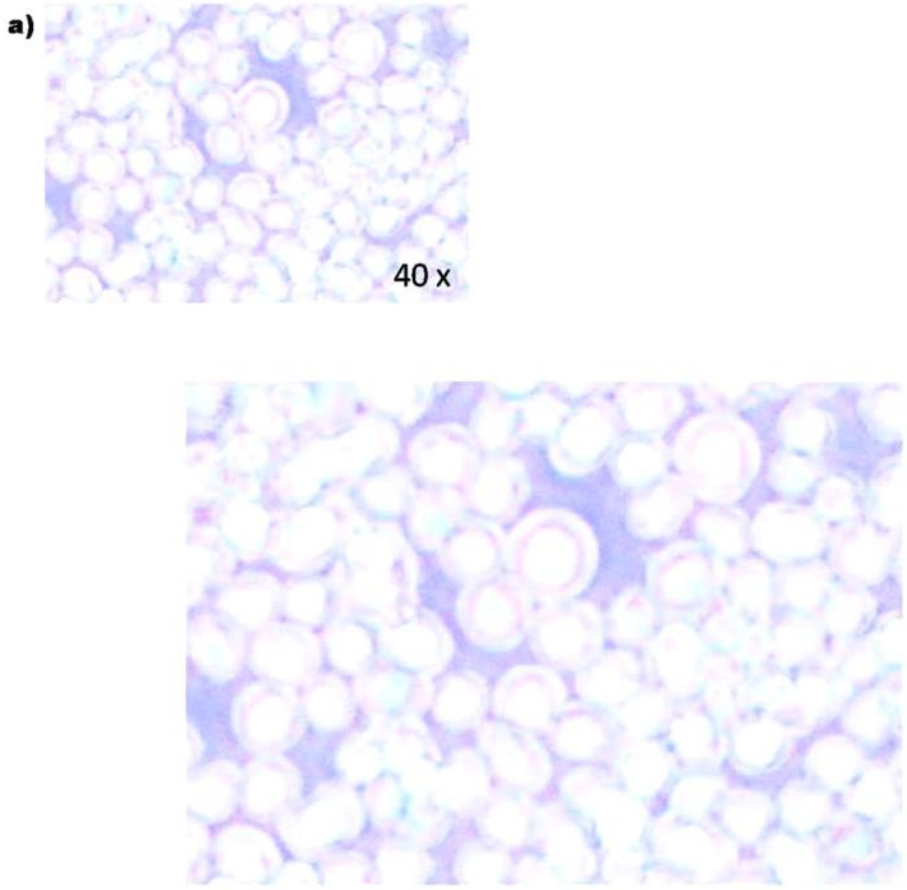

Figure 5.11 The effect of various conditions on THP-1 monocyte maturation and differentiation. Condition I: THP-1 Pro-monocytes in culture

THP-1 cells were left untreated as pro-monocytes during routine cell culture. Photographs of cell cultures were taken under a light microscope using a 40 x magnification. An enlarged view was generated with a graphic programme. Pictures are a representative view of one cell culture experiment. Cells are non-adherent and show a round and even morphology.

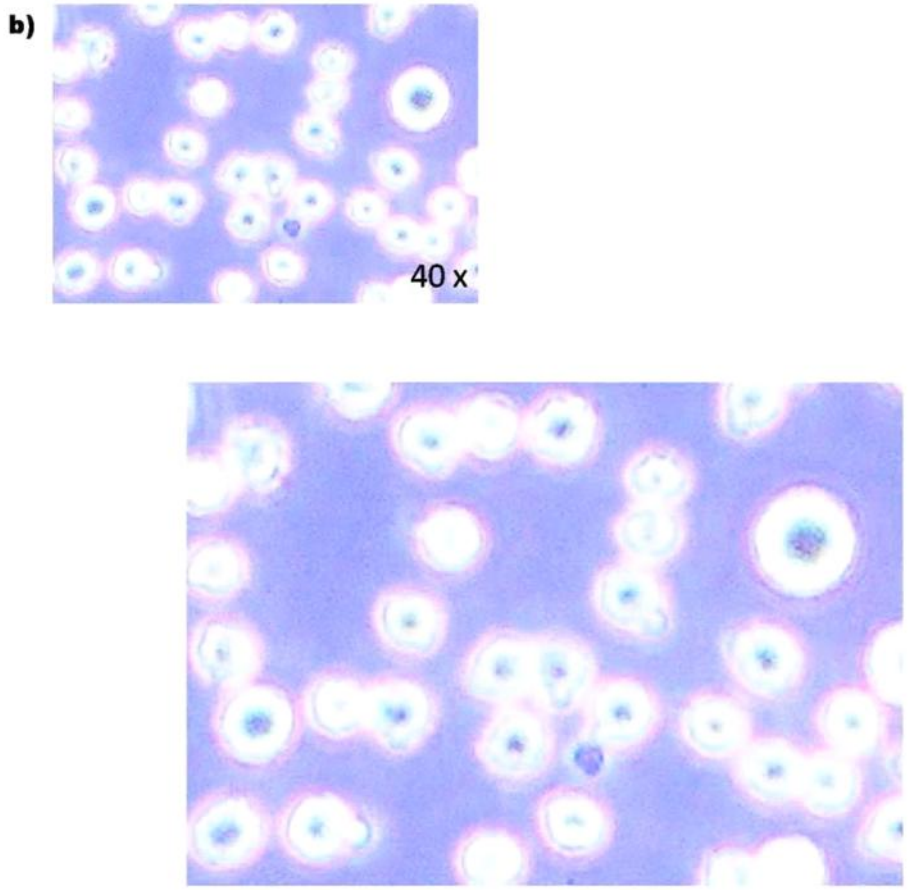

Figure 5.12 The effect of various conditions on THP-1 monocyte maturation and differentiation. Condition II: THP-1 monocytes after $VitD_3$ treatment

THP-1 cells were treated with $VitD_3$ for 72 h. Photographs of cell cultures were taken under a light microscope using a 40 x magnification. An enlarged view was generated with a graphic programme. Pictures are a representative view of one cell culture experiment. Cells start to adhere and increase in size, yet mostly retain the round, even morphology.

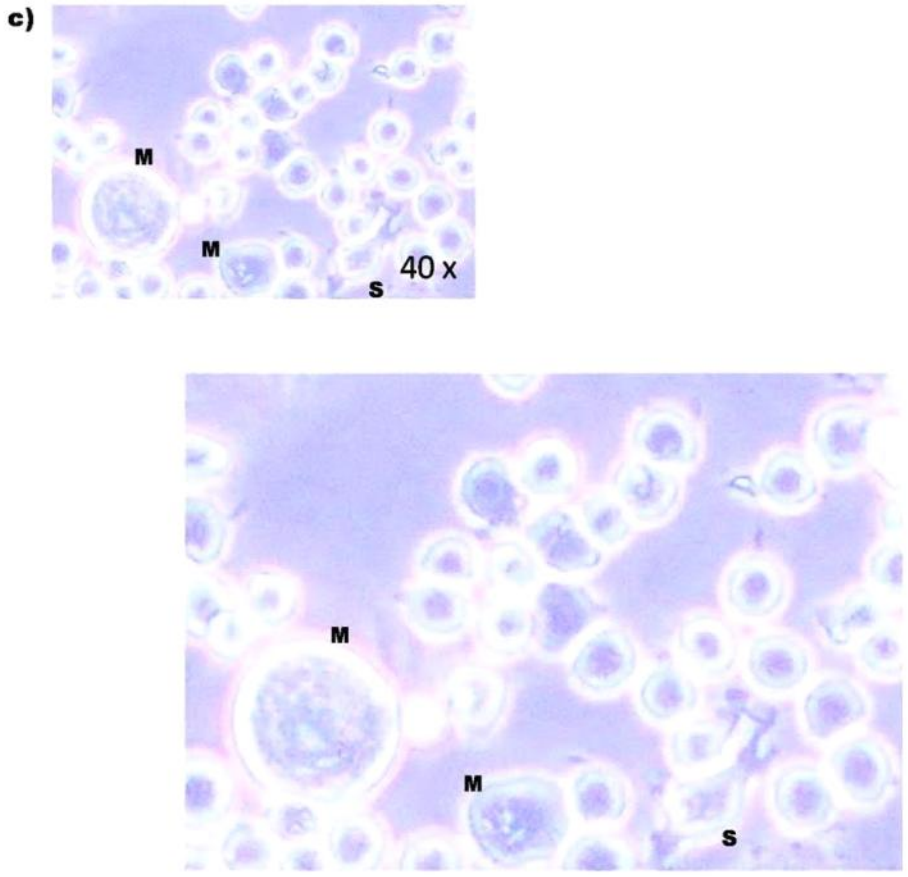

Figure 5.13 The effect of various conditions on THP-1 monocyte maturation and differentiation. Condition III: THP-1 monocytes after $VitD_3$ treatment and stimulation with leptin

THP-1 cells were pre-treated with $VitD_3$ for 48 h and then stimulated with 1000 ng/ml leptin for 24 h. Photographs of cell cultures were taken under a light microscope using a 40 x magnification. An enlarged view was generated with a graphic programme. Pictures are a representative view of one cell culture experiment. **M**: matured monocytes, **S**: stretched out monocyte. Cell adherence is increased and cell maturation and differentiation processes towards macrophages can be observed.

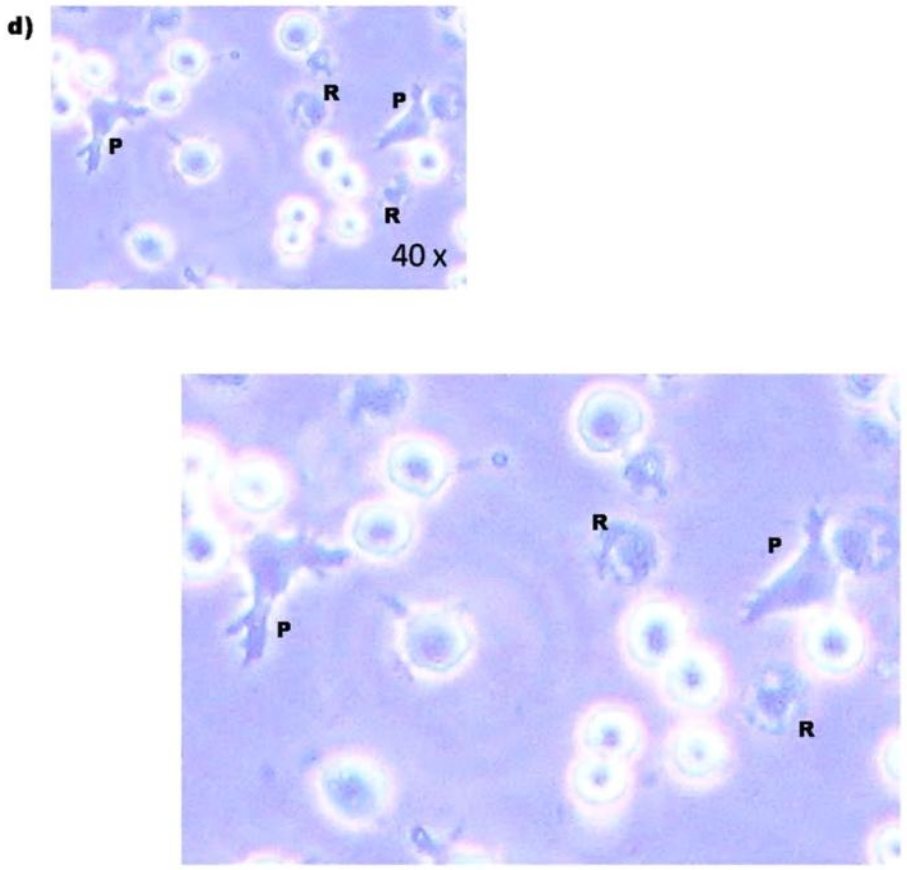

Figure 5.14 The effect of various conditions on THP-1 monocyte maturation and differentiation. Condition IV: THP-1 monocytes after $VitD_3$ treatment and stimulation with LPS

THP-1 cells were pre-treated with $VitD_3$ for 48 h and then stimulated with LPS from *E. coli* (100 ng/ml) for 24 h. Photographs of cell cultures were taken under a light microscope using a 40 x magnification. An enlarged view was generated with a graphic programme. Pictures are a representative view of one cell culture experiment. **P**: podosomes, **R**: granularity. Cell adherence is further increased, podosome formations and granluarity can be observed indicating differentiation processes towards dendritic and neutrophil-like cells.

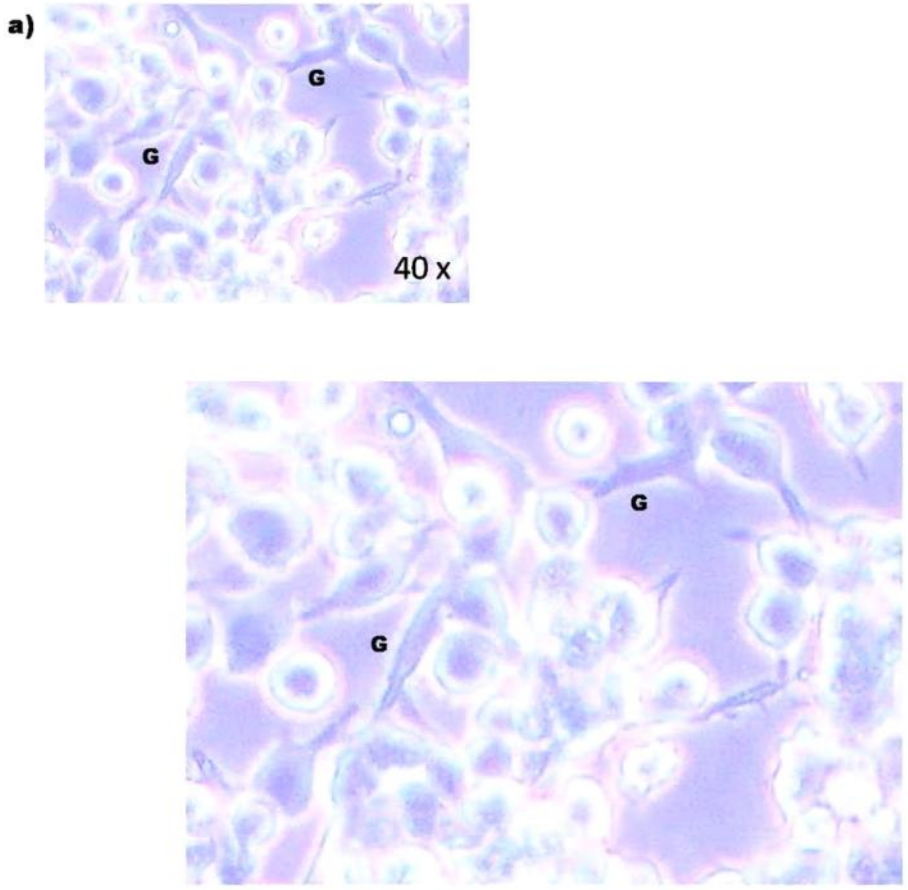

Figure 5.15 The effect of various conditions on THP-1 monocyte maturation and differentiation. Condition V: THP-1 monocytes after $VitD_3$ treatment and stimulation with PMA

THP-1 cells were pre-treated with $VitD_3$ for 48 h and then stimulated with PMA (50 ng/ml) for 24 h. Photographs of cell cultures were taken under a light microscope using a 40 x magnification. An enlarged view was generated with a graphic programme. Pictures are a representative view of one cell culture experiment. **G**: macrophages. All cells are adherent and several monocytes show a differentiation process towards macrophages.

5.3 Discussion

At the mRNA level, the transcription factors PU.1 and Sp1 are recognised as key elements in regulating TLR2 and TLR4 expression (Rehli *et al.*, 2000; Wang *et al.*, 2001; Haehnel *et al.*, 2002; Lin and Rikihisa, 2004). Leptin was shown to induce Sp1 phosphorylation in murine hepatic stellate cells (Lin *et al.*, 2006). However, although the adipokine potentially could influence PU.1 expression and activity through p38 and PI3K/Akt pathways (Rieske and Pongubala, 2001; Wang *et al.*, 2003), the present study is the first report to address a potential role for leptin in the regulation of PU.1.

Results from confocal and standard fluorescence microscopy obtained in the present study suggest that PU.1 protein expression is upregulated after 24 h of leptin stimulation in THP-1 and primary human monocytes. In contrast, Ito *et al.* (2005) show increased PU.1 protein expression after 4 and 8 h LPS or PMA stimulation in mouse mast cells, yet the expression was reduced to background levels at 24 h. Changes in PU.1 protein expression may vary between cell types and species. In addition, LPS or PMA may induce a different response over time than leptin. Apart from Ito *et al.* (2005), only Lodie *et al.* (1997) and Buras *et al.* (1995) investigated PU.1 protein expression under inflammatory conditions, finding no differences in the expression levels after 1 h LPS stimulation in mouse macrophages. No other timepoints were analysed in these two studies and possibly 1 h of LPS stimulation is too short to have any effect on PU.1 protein expression. Despite the findings by Ito *et al.* (2005), PMA stimulation of THP-1 monocytes in the present study indicated an upregulation of PU.1 after 24 h. However, results were not consistent between experiments and sometimes PMA appeared to induce a down- rather than an upregulation of the transcription factor. PMA is a photosensitive chemical and eventually was altered or degraded by exposure to light in

some instances, causing different cell responses. Regardless, a shift in THP-1 morphology from a monocyte to a macrophage-like cell type was observed every time after PMA stimulation, making a change in PMA activity somewhat questionable. Also, the fluorescence microscopy protocol was developed for the analysis of monocytes and it is possible that the advanced differentiation of monocytes into macrophage-like cells after PMA stimulation interfered with some technical component within the protocol.

Although upregulation of PU.1 protein by leptin potentially is an important element in leptin-induced changes of TLR expression, the main aim of the fluorescence microscopy experiments was to determine intracellular location and not to quantitatively assess expression of PU.1. Foster *et al.* (2007) demonstrated nuclear translocation of PU.1 after 1 h of LPS or PMA stimulation in THP-1 monocytes, yet this was not observed by Buras *et al.* (1995) in mouse macrophages. Nuclear translocation is generally an indication for activation of transcription factors, with the classic example of NF-κB release by IκB degredation and subsequent nuclear translocation (Baeuerle and Henkel, 1994). The overall increased fluorescence intensity after leptin stimulation in both nucleus and cytoplasm interfered with an accurate analysis of any translocation events, although at least in the THP-1 monocytes it appeared that PU.1 was more sequestered to the nucleus than in control cells. In the primary human monocytes staining intensity was increased drastically in both nucleus and cytoplasm after leptin stimulation, and it was not possible to differentiate between nuclear translocation and increased PU.1 protein expression. Furthermore, in contrast to findings by Foster *et al.* (2007) PMA failed to function as a positive control for nuclear translocation of PU.1 in the present study, likely due to some unknown technical factor in the fluorescence

microscopy protocol. Therefore, results obtained from fluorescence microscopy to evaluate PU.1 activity as an event of nuclear translocation remain inconclusive.

It should be noted that detection of PU.1 by fluorescence microscopy has yielded conflicting results in the past due to different antibodies. Thus, using antibodies either directed at the N-terminal or the entire region of PU.1, Buras *et al.* (1995) detect PU.1 in unstimulated mouse macrophages in both cytoplasm and nucleus, with a slightly more intense nuclear staining. However, after LPS stimulation, no PU.1 was detected in the nucleus by the N-terminal antibody, whereas the staining was the same as pre-stimulation when using the antibody directed at the full length protein. Conversely, using an antibody directed at the C-terminus only, Kwok *et al.* (2007) demonstrate PU.1 expression exclusively in the nucleus in unstimulated cells, despite working with the same mouse macrophage cell line (RAW 264.7) as Buras *et al.* (1995). Buras *et al.* (1995) speculate that LPS may either induce a conformational change of PU.1 or that other transcription factors are recruited together with PU.1 (as shown by Pongubala *et al.* (1992)) and mask the epitope of the antibody if this is only a terminal region and not a longer part of the protein. The present study used an antibody corresponding to aminoacids 1-135, thus covering an epitope from the N-terminal region and a larger part of the protein (total of 271 amino acids, PU.1 NCBI accession number NM_001080547). This might explain why a comparable distribution pattern of PU.1 was observed in the present study in THP-1 and primary human monocytes as was in the study of Buras *et al.* (1995) when using the antibody directed against full length PU.1 and possibly suggests that leptin-induced upregulation of PU.1 protein expression is a true finding and not an artefact. Nonetheless, results regarding leptin-induced upregulation of PU.1 protein expression should be confirmed by Western blot in future

studies. Further immediate investigation of antibody specifity was not possible since no blocking peptide is available for this antibody. However, the same antibody was also used in immunoprecipitation and produced a single band at the correct size for PU.1 (~ 40 kDa), which may be taken as an indication of antibody specifity.

PMA stimulation of THP-1 monocytes induced an upregulation of PU.1 mRNA, confirming recent findings by Suzuki *et al.* (2009). In contrast, leptin stimulation had no effect on PU.1 mRNA expression. Likewise, in a study on mouse macrophages Ishii *et al.* (2008) did not detect an effect of LPS on PU.1 mRNA expression. Although the different cell types and species may explain differences in the data obtained, it appears that only PMA is capable of inducing PU.1 mRNA expression in stimulation experiments. Since leptin had no effect on PU.1 mRNA expression, this could further challenge the results obtained from fluorescence microscopy in the present study, where leptin presumably induced an upregulation of PU.1 protein expression. However, a number of studies have demonstrated extensive post-transcriptional regulation of PU.1 mRNA, mainly affecting mRNA stability and translation. Thus, PU.1 mRNA was shown to be a direct target for the microRNA 155 in DCs, B cells and an erythroleukemia cell line (Hensold *et al.*, 1996; Vigorito *et al.*, 2007; Martinez-Nunez *et al.*, 2009). Overexpression of microRNA 155 in a THP-1 reporter cell line model resulted in reduced PU.1 protein expression, while PU.1 mRNA level remained stable (Martinez-Nunez *et al.*, 2009), indicating that microRNA 155 blocked PU.1 translation and that PU.1 was regulated at the level of translation and not transcription. Therefore, it is possible that by affecting microRNA 155, leptin could still influence PU.1 protein expression while at the same time having no effect on PU.1 mRNA expression. No studies are available to date on leptin and microRNA 155 expression, however studies

in adipocytes find significant correlations between circulating leptin concentrations and expression levels of other microRNAs (Takanabe *et al.*, 2008; Kloting *et al.*, 2009). It would therefore be interesting to analyse an effect of leptin on microRNA 155 expression to test this hypothesis.

PU.1 phosphorylation status is recognized as an important element for increased transcriptional activity (Pongubala *et al.*, 1993; Celada *et al.*, 1996) and at least five PU.1 phosphorylation sites are known (Lodie *et al.*, 1997). Since results from fluorescence microscopy for PU.1 nuclear translocation remained inconclusive, it was decided to investigate an effect of leptin on PU.1 phosphorylation status as an alternative to evaluate PU.1 activity. No phospho-PU.1 antibodies or phospho-PU.1 ELISAs are available to date and therefore an IP protocol for PU.1 was established to subsequently analyse total serine phosphorylation status of PU.1. However, although an IP protocol was developed successfully, it was not possible to solve technical problems with serine phosphorylation antibodies and Western blot procedures, resulting in a poor signal-to-noise ratio. This did not allow for any conclusions on serine phosphorylation status of PU.1 before and after leptin or LPS stimulation to be made. In future studies, the experiment possibly could be repeated using a commercially available Western blot blocking reagent certified to not interfere with phospho-antibodies. In addition, electrophoretic mobility shift assays (EMSAs) could be used to evaluate the effect of leptin on PU.1 DNA-binding capacity which is interpreted by some authors as an indication for increased PU.1 activity (Shackelford *et al.*, 1995; Carey *et al.*, 1996). However, it was demonstrated that both phosphorylated and dephoshorylated PU.1 bind DNA efficiently yet only the phosphorylated form displayed increased transcriptional activity (Pongubala *et al.*, 1993). It is therefore questionable if EMSAs would be a

suitable method for investigation of PU.1 activity. Alternatively, a combined experiment of siRNA knock down of PU.1 and subsequent analysis of TLR expression after leptin stimulation could directly associate an effect of leptin on TLR expression via PU.1, independent of PU.1 activity or expression status.

A study by Shibata *et al.* (2001) indicates that GM-CSF regulates TLR2 expression through PU.1 and thus the cytokine presents an interesting target for investigating an effect of leptin on this TLR transcription factor. To the best of my knowledge, only one study to date investigated an effect of leptin on GM-CSF expression. In this study, Gainsford *et al.* (1996) show that GM-CSF secretion was not affected by leptin in murine peritoneal macrophages. Likewise, the present report demonstrates that leptin has no effect on human THP-1 and primary monocyte GM-CSF secretion. Moreover, there was no constitutive GM-CSF secretion in monocytes and not only leptin, but neither *E. coli* nor *P. gingivalis* LPS did induce GM-CSF secretion. In fact, GM-CSF secretion was only observed after PMA stimulation. It is therefore unlikely that leptin alone would alter monocytic PU.1 and TLR2 expression through GM-CSF. GM-CSF is secreted either constitutively or during inflammatory conditions by a variety of cell types, including epithelial cells, T cells and macrophages (Shi *et al.*, 2006). The regulation of GM-CSF secretion is complex and it appears that every cell type reacts differently in different conditions (Lee *et al.*, 1990). For example, although no GM-CSF was detected in control cultures in the present study, Taupin *et al.* (1993) could detect constitutive GM-CSF production in their primary human monocyte cultures, which was enhanced by *E. coli* LPS stimulation. In contrast, another study detects low levels of constitutive GM-CSF production in a human myeloid cell line (K-562) yet this was not enhanced by LPS and only PMA induced a potent increase (Akin and Sonnenfeld,

1996). It is possible that the adherence status of monocytes is a determining factor in the regulation of GM-CSF secretion. Thus, after LPS-stimulation, adherent monocytes produced higher levels of GM-CSF than non-adherent monocytes (Lee *et al.*, 1990). However, monocytes used in the present study were mostly adherent after LPS stimulation and still no GM-CSF secretion was detected. Alternatively, the isolation procedure of primary human monocytes could impact on GM-CSF secretion. The physical isolation process of primary human monocytes with an adherence method can induce monocyte activation, leading to enhanced expression of pro-inflammatory cytokines (Rodenburg *et al.*, 1998). In contrast, isolation of primary human monocytes using antibodies and magnetic beads yields monocytes with cytokine expression patterns comparable to whole blood (Rodenburg *et al.*, 1998). In contrast to the above mentioned studies, the present report investigated GM-CSF secretion in monocytes isolated with CD14 antibodies and magenetic beads. It is therefore plausible to assume that the less activated state of monocytes isolated with this method account for some of the discrepancies to other findings.

Although leptin had no effect on GM-CSF protein expression, the present study shows for the first time a leptin-induced upregulation of GM-CSF mRNA expression. The upregulation was only significant at an earlier timepoint and declined over time. This is in contrast to the observed *E. coli* LPS effect on GM-CSF mRNA expression, which induced an increase over time. In addition, *E. coli* LPS-induced GM-CSF mRNA expression was drastically higher than GM-CSF mRNA expression induced by leptin. On the one hand, this raises the question as to whether the observed moderate transient effect of leptin would be of any biological significance, in particular when considering that no effect is observed at the protein level. On the other hand, drastic differences in

GM-CSF mRNA expression have been described for different LPS types as well. Thus, Barksby *et al.* (2009) found only a moderate increase in GM-CSF mRNA expression after *P. gingivalis* LPS stimulation in THP-1 monocytes, while at the same time *E. coli* LPS induced very high levels of GM-CSF mRNA expression. Possibly activation of different intracellular signalling pathways such as MyD88 dependent or independent signalling for TLR2 or TLR4, respectively, and leptin receptor signalling could account for differences in GM-CSF mRNA expression. Since the leptin effect on GM-CSF mRNA expression appears to be comparable to the effect of *P. gingivalis* LPS, it could be speculated that this effect may be of relevance in the inflammatory situation of periodontal disease where a combined leptin stimulation and *P. gingivalis* LPS challenge may induce higher GM-CSF mRNA expression than either stimulus alone. However, this assumption would need further evaluation in combination experiments of leptin and *P. gingivalis* LPS.

After stimulation of THP-1 monocytes with LPS or PMA, increased adherence and morphological changes such as cell elongation and formation of podosomes were observed. These changes have been acknowledged in other studies (Tsuchiya *et al.*, 1982; Baqui *et al.*, 1999; Foster *et al.*, 2007; van Helden *et al.*, 2008; Suzuki *et al.*, 2009) and represent a maturation and differentiation process of the THP-1 monocytes along the myeloid lineage. However, increased cell adherence and slight morphological changes were noted after leptin stimulation as well, an observation that has not been described before. Leptin induced an increase in cell size of THP-1 monocytes, a feature that is attributed to mature monocytes (Goasguen *et al.*, 2009). Additionally, cells grew elongated and started to resemble macrophages derived from THP-1 monocytes stimulated with PMA (Tsuchiya *et al.*, 1982). Leptin is known as an important regulator

of haematopoiesis and myeloid cell differentiation and *ob/ob* mice show several immune cell deficiencies (Claycombe *et al.*, 2008). The mechanisms of regulatory functions of leptin in haematopoiesis and differentiation are poorly characterized and so far mainly contributed to its anti-apoptotic effects (Lam *et al.*, 2006; Claycombe *et al.*, 2008). However, findings of the present study suggest an additional mechanism. PU.1 is essential in myeloid cell differentiation and maturation (Scott *et al.*, 1994; Shibata *et al.*, 2001; Bonfield *et al.*, 2003; Iwasaki *et al.*, 2005) and an important factor in the expression of typical macrophage cell surface receptors such as CD11b, GM-CSF and M-CSF receptor (Pahl *et al.*, 1993; Zhang *et al.*, 1994; Hohaus *et al.*, 1995). Thus, the leptin-induced upregulation of PU.1 may enhance maturation of THP-1 monocytes and could provide an explanation for the observed morphological changes. The finding of the present study that leptin induces monocyte maturation may be important in inflammatory conditions. In situations of increased leptin concentrations such as obesity and diabetes (Considine *et al.*, 1996; Sinha *et al.*, 1996) monocytes may be more likely to differentiate into macrophages during inflammation and this could potentially increase their response to PAMPs, possibly leading to an over reactive immune response which could impact on the pathogenesis of periodontal disease. It is important to note that in every stimulation only some cells and not all showed morphological changes. Possibly a longer incubation time or syncronization of cell cycles could induce more uniform changes. However, Suzuki *et al.* (2009) demonstrate that THP-1 monocytes consist of several subclones and unless isolated specifically, individual cells reach different stages of cell maturation and differentiation under various conditions. This could also explain why some THP-1 monocytes in the present study appeared to granularize after LPS stimulation while others were unaffected or developed podosomes, starting to resemble dendritic cells (Burns *et al.*, 2001). Cell granularization

could be an indication for apoptosis (Herman *et al.*, 2005), induced by toxic effects of LPS. Yet it could also be an indication for a differentiation process of THP-1 monocytes into neutrophil-like cells, which can be achieved with certain treatments (Momose *et al.*, 2003). The apoptotic or necrotic effects of LPS could be evaluated with Annexin V and propidium iodide using flow cytometry (Vermes *et al.*, 1995) in future studies, however investigations of LPS toxicity in a cell proliferation assay in the present study revealed no increased cell death after 24 h LPS stimulation (see 2.2.3.2).

In summary, findings of the present study indicate a role for leptin in PU.1 and GM-CSF expression. While an effect of leptin on GM-CSF at the mRNA and not the protein level unlikely plays a role in TLR expression, a leptin-induced upregulation of PU.1 protein expression could result in increased TLR expression and subsequently altered immune responses to PAMPs. In addition, leptin-stimulated PU.1 expression could impact on monocyte maturation and cell differentiation, resulting in a more inflammatory monocytic phenotype in conditions with higher leptin concentrations such as diabetes. However, more work is needed to completely undestand the effect of leptin on PU.1 activity and to provide a direct link between leptin, PU.1 and TLR expression.

Chapter 6 Leptin and adiponectin concentrations in patients with diabetes and periodontitis

Chapter 6 Leptin and adiponectin concentrations in patients with diabetes and periodontitis

6.1 Introduction

A number of studies support diabetes as a risk factor for increased incidence or severity of periodontal disease (Nelson *et al.*, 1990; Ryan *et al.*, 2003; Heitz-Mayfield, 2005; Moles, 2006). However, a mechanistic link between the two diseases is still obscure. For example, apparently no differences in the oral microflora between diabetics and non-diabetics with periodontal disease can be detected (Yuan *et al.*, 2001; Lalla *et al.*, 2006). Some studies suggest that, due to the hyperglycaemic state, physiological changes such as impaired vascular repair and modified collagen metabolism not only take place systemically but also in gingival tissues (Brennan, 1989; Tepper *et al.*, 2002; Bhatwadekar *et al.*, 2008). This likely interferes with tissue turnover and wound healing, contributing to an increased risk or exacerbation of periodontal disease in diabetes (Goova *et al.*, 2001; Ryan *et al.*, 2003). Additionally, the formation of AGEs in diabetes potentially results in excessive activation of pro-inflammatory mediators, which may contribute to the overall inflammation in the host response to periodontal pathogens (King, 2008). Indeed, circulating concentrations of pro-inflammatory mediators such as TNF-α, IL-6 or CRP are increased in diabetes (Pickup *et al.*, 2000; Pradhan *et al.*, 2001; Freeman *et al.*, 2002) and thus may alter the inflammatory environment in periodontal disease, however this remains to be investigated.

Importantly, AGEs are not the sole possible contributors to altered diabetic immune functions. Mediators of the immune system are derived from adipose tissue and not only include pro-inflammatory cytokines but also adipokines such as leptin and adiponectin which are closely associated with the amount of adipose tissue and also insulin levels

(Maffei *et al.*, 1995; Considine *et al.*, 1996; Hotta *et al.*, 2000; Kieffer and Habener, 2000; Yamauchi *et al.*, 2001). Due to a strong correlation with BMI, serum leptin concentrations are generally increased in obesity and diabetes in comparison to non-obese and non-diabetic individuals (Considine *et al.*, 1996; Sinha *et al.*, 1996). In contrast, serum adiponectin concentrations are decreased in obesity and diabetes (Arita *et al.*, 1999; Hotta *et al.*, 2000). Both adipokines are known to have various immunological functions. While leptin is associated with pro-inflammatory immune responses such as pro-inflammatory cytokine production and enhanced LPS-induced immune-responses in myeloid cells (Loffreda *et al.*, 1998; Santos-Alvarez *et al.*, 1999; Zarkesh-Esfahani *et al.*, 2001; Mattioli *et al.*, 2005), adiponectin has anti-inflammatory properties, suppressing LPS-induced NF-κB activation and pro-inflammatory cytokine production (Yokota *et al.*, 2000; Wulster-Radcliffe *et al.*, 2004; Ajuwon and Spurlock, 2005; Tsatsanis *et al.*, 2005; Yamaguchi *et al.*, 2005; Park *et al.*, 2008). Interestingly, however, depending on the adiponectin isoform, pro-inflammatory properties have been associated with this adipokine as well (Neumeier *et al.*, 2006; Haugen and Drevon, 2007).

Considering the immunological actions of leptin and adiponectin and the fact that they are detected at nanogram and microgram ranges in the circulation, surprisingly little is known about their concentration and role in periodontal disease or as a potential immunological link between diabetes and periodontal disease. A study by Karthikeyan and Pradeep (2007) revealed increasing serum leptin concentrations with an increase in periodontal tissue destruction in non-diabetic individuals, which may suggest a pro-inflammatory role of this adipokine in periodontal disease. In addition, a recent study showed decreased serum leptin concentrations after periodontal treatment (Kardesler *et*

al., 2010), supporting the findings of Karthikeyan and Pradeep (2007) for a pro-inflammatory role of leptin in periodontal disease. However, studies by Karthikeyan and Pradeep (2007) and by others also show a decrease in GCF leptin concentrations with increased periodontal tissue destruction in comparison to healthy sites, and this was interpreted as a "protective" role for leptin at local sites in the periodontium (Johnson and Serio, 2001; Bozkurt *et al.*, 2006; Karthikeyan and Pradeep, 2007). Except for one recent study in humans (Kardesler *et al.*, 2010), the potential role of leptin as an immunological link between diabetes and periodontal disease has only been addressed in animal models of experimental periodontitis and immune responses to *P. gingivalis* infection (Naguib *et al.*, 2004; Watanabe *et al.*, 2008). These animal studies found increased serum TNF-α concentrations in obese *fa/fa* rats or *db/db* mice with experimental periodontitis or *P. gingivalis* infection in comparison to lean controls (Naguib *et al.*, 2004; Watanabe *et al.*, 2008). However, both studies only used the *fa/fa* and *db/db* models for their known ability to develop obesity and diabetes. They failed to acknowledge a role for leptin in their experimental setup and immune responses and therefore results of these studies do not allow for any conclusions regarding leptin in diabetes and periodontal disease to be made. The study in humans (Kardesler *et al.*, 2010) identified no differences in serum leptin concentrations between diabetes patients with periodontitis and non-diabetic individuals with periodontitis, suggesting that leptin is not a major contributor in a relationship between diabetes and periodontitis in humans. Nonetheless, after periodontal treatment Kardesler *et al.* (2010) reported an increase in serum leptin concentrations in diabetes patients and a decrease in non-diabetic individuals, indicating that at least in some situations, leptin does appear to play a role in diabetes and periodontal disease.

Serum adiponectin concentrations in non-diabetic individuals with periodontal disease do not appear to be altered (Furugen *et al.*, 2008; Saito *et al.*, 2008) in comparison to periodontal health and no change after treatment for periodontal disease has been observed (Behle *et al.*, 2009). However, the study by Kardesler *et al.* (2010) describes increased serum adiponectin concentrations in diabetics with periodontitis in comparison to non-diabetic individuals with periodontitis. Additionally, the same study reported a decrease in serum adiponectin in diabetics and an increase in non-diabetics after periodontal treatment (Kardesler *et al.*, 2010). In contrast, Matsumoto *et al.* (2009) showed increased serum adiponectin concentrations after periodontal treatment in diabetics. Although contradictory, the results from the studies by Kardesler *et al.* (2010) and Matsumoto *et al.* (2009) suggest a potential role for adiponectin in a link between diabetes and periodontal disease, however the different findings between the studies need to be clarified. Also, GCF adiponectin concentrations in periodontal disease or diabetes could provide an important contribution for the role of this adipokine in local periodontal inflammation, however no studies have investigated GCF adiponectin to date.

Interestingly, in some studies treatment of periodontal disease in diabetes has resulted in improved HbA1c and reduced serum TNF-α concentrations (Iwamoto *et al.*, 2001; Kiran *et al.*, 2005), and in non-diabetic individuals, periodontal treatment has resulted in decreased serum CRP and IL-6 concentrations (D'Aiuto *et al.*, 2004). These findings indicate that apart from diabetes increasing the risk for periodontal disease, periodontal disease in turn could impact on the diabetic status, implying a bi-directional relationship between the two diseases. Yet, a meta-analysis of 10 studies did not detect significant changes in the glycaemic control of diabetes patients after treatment for periodontal

disease (Janket *et al.*, 2005) and not all studies reported reduced serum TNF-α or CRP concentrations (Dag *et al.*, 2009; Jastrzebski *et al.*, 2009). However, both leptin and adiponectin are closely associated with insulin concentrations (Kieffer and Habener, 2000; Combs *et al.*, 2004) and correlations between HbA1c and the adipokines have been described in diabetes (Moriya *et al.*, 1999; Fernandez-Real *et al.*, 2004; Radwan *et al.*, 2005). Moreover, both leptin and adiponectin concentrations have been linked to inflammatory markers such as CRP (Putz *et al.*, 2004; Abdella *et al.*, 2005), further supporting a potential role of these adipokines as an immunological link between diabetes and periodontal disease.

Clearly, more research is needed to investigate the effect of leptin and adiponectin on periodontal status in diabetes and periodontal disease and to evaluate their potential as therapeutic targets. The aim of the present study, therefore, was to compare serum leptin and adiponectin concentrations and GCF adiponectin concentrations between patients with T2DM and non-diabetic controls with or without periodontal disease. In addition, adipokine concentrations were monitored after periodontal treatment in T2DM patients and non-diabetic controls. Finally, the relationship of serum leptin and adiponectin with HbA1c and hsCRP concentrations was determined and the potential of the adipokines to predict periodontal status was evaluated.

6.2 Results

6.2.1 Analysis of serum leptin and adiponectin concentrations and of GCF adiponectin concentrations before periodontal treatment

Serum and GCF samples were obtained from T2DM patients and non-diabetic controls with or without periodontal disease as described in 2.8.3. Concentrations of leptin and adiponectin in serum and of adiponectin in GCF were determined with ELISAs. Leptin

concentrations in GCF were not determined due to a limited amount of sample volume availability. Serum leptin and adiponectin concentrations are known to differ between genders (Nystrom *et al.*, 1997; Arita *et al.*, 1999). Accordingly, data were tested for gender differences and treated separately for statistical analysis between different conditions when a significant gender difference was detected.

6.2.1.1 Analysis of serum leptin concentrations in T2DM patients and non-diabetic controls with or without periodontal disease before periodontal treatment

A significant gender difference was detected in serum leptin concentrations in both T2DM patients and non-diabetic controls (Figure 6.1). Female T2DM patients had 148 % higher serum leptin concentrations than male patients with T2DM ($p < 0.001$), having 36.53 ± 23.69 (mean + SD) and 14.73 ± 11.9 ng/ml serum leptin concentrations, respectively. In the control group, females had 68 % higher serum leptin concentrations than males ($p < 0.01$), having 16.47 ± 11.63 and 9.81 ± 7.02 ng/ml serum leptin concentrations, respectively. In addition, T2DM patients had higher serum leptin concentrations than non-diabetic controls (Figure 6.1). 50 % ($p < 0.01$) and 122 % ($p < 0.001$) higher serum leptin concentrations were detected in male and female T2DM patients, respectively, in comparison to controls.

Figure 6.2 shows the leptin concentrations, according to periodontal status, in male T2DM patients and non-diabetic controls. In T2DM patients with gingivitis, serum leptin concentrations were significantly higher than in periodontal health, showing a 145 % increase from 6.08 ± 2.91 to 14.89 ± 8.56 ng/ml ($p < 0.05$). Serum leptin in male T2DM patients with periodontitis reached a concentration of 15.89 ± 14.25 ng/ml, indicating a 160 % increase compared to health. However, after Bonferroni-Holm

correction of p values for multiple comparisons the critical p value was 0.025 and therefore this increase was only detected as a trend ($p = 0.045$). There was no significant difference in serum leptin concentrations between gingivitis and periodontitis in male T2DM patients ($p = 0.664$). No significant differences in serum leptin concentrations according to periodontal status were observed in non-diabetic male controls. In this group, serum leptin concentrations were 5.87 ± 1.58, 9.17 ± 5.86 and 10.5 ± 7.75 ng/ml for periodontal health, gingivitis and periodontitis, respectively. No significant difference in serum leptin concentrations was detected between periodontal healthy male T2DM patients and periodontal healthy non-diabetic controls ($p = 0.953$, Figure 6.2). However, a significant difference was observed in serum leptin concentrations between male T2DM patients and non-diabetic controls with gingivitis. T2DM patients with gingivitis had 60 % higher serum leptin concentrations than non-diabetic controls with gingivitis ($p < 0.05$). A trend for higher serum leptin concentrations ($p = 0.065$) in male T2DM patients with periodontitis was observed in comparison to non-diabetic controls with periodontitis.

Figure 6.3 shows the leptin concentrations, according to periodontal status, in female T2DM patients and non-diabetic controls. No significant differences in serum leptin concentrations were detected in T2DM patients or in non-diabetic controls according to periodontal status. However, according to the periodontal status all female T2DM patients had higher serum leptin concentrations than their non-diabetic counterparts. In periodontal health, serum leptin concentrations were 119 % higher ($p < 0.05$) in T2DM patients than in non-diabetic controls, increasing from 13.45 ± 6.16 to 29.41 ± 13.95 ng/ml. In gingivitis, serum leptin concentrations were 177 % higher ($p < 0.01$) in T2DM patients than in non-diabetic controls, increasing from 14.08 ± 9.9 to 39.04 ± 23.25

ng/ml. In periodontitis, serum leptin concentrations were 102 % higher ($p < 0.05$) in T2DM patients than in non-diabetic controls, increasing from 19.48 ± 14.7 to 39.42 ± 31.17 ng/ml.

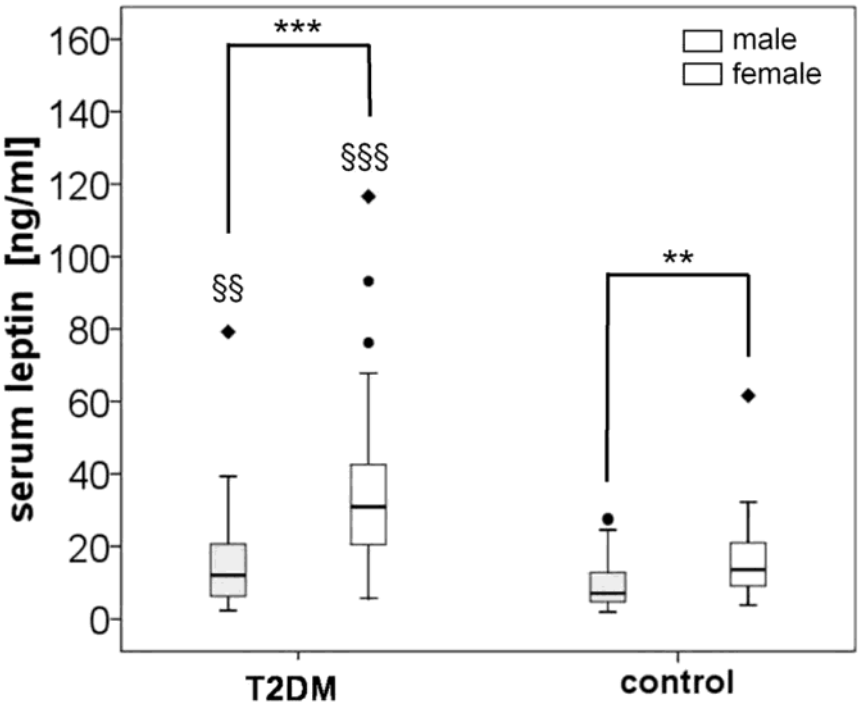

Figure 6.1 Serum leptin concentrations in males and females with T2DM and in non-diabetic controls

Box plots of serum leptin concentrations in 97 T2DM patients (64 males, 33 females) and 70 non-diabetic controls (43 males, 27 females). Statistics: ANOVA, Student's *t*-test $^{**}p < 0.01$, $^{***}p < 0.001$ (male versus female within diabetes or control group); $^{\S\S}p < 0.01$ and $^{\S\S\S}p < 0.001$ (diabetes versus non-diabetes within males or within females). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

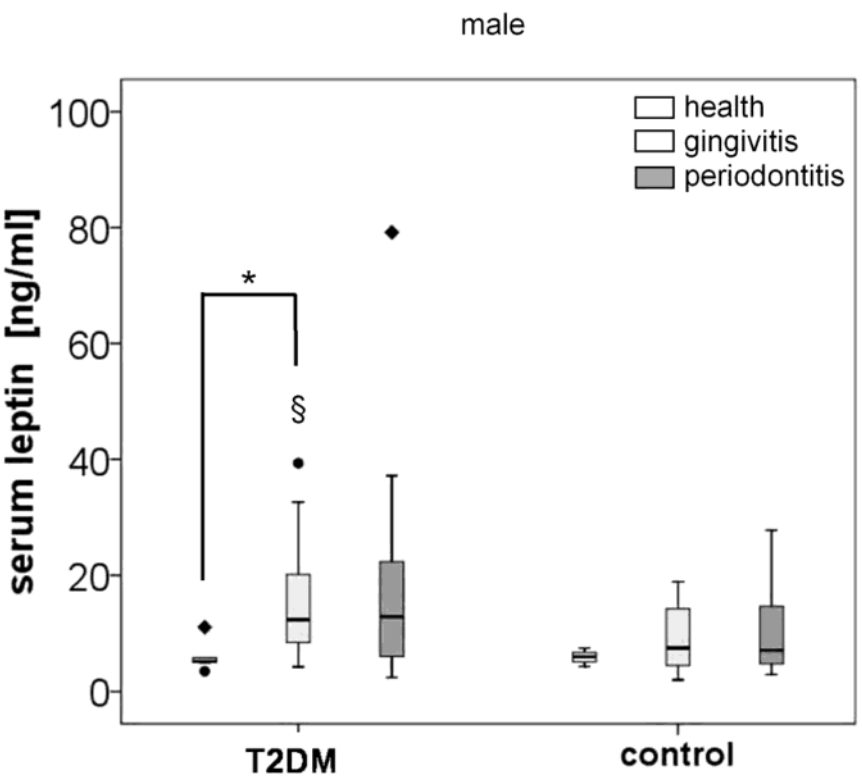

Figure 6.2 Male serum leptin concentrations according to periodontal status in T2DM and non-diabetic controls

Box plots of serum leptin concentrations in 64 male T2DM patients (periodontal health $n = 5$; gingivitis $n = 25$; periodontitis $n = 34$) and 43 non-diabetic male controls (periodontal health $n = 3$; gingivitis $n = 12$; periodontitis $n = 28$). Statistics: ANOVA, Student's t-test $^{*}p < 0.05$ (according to the periodontal status within diabetes or control); $^{\S}p < 0.05$ (diabetes versus non-diabetes within the corresponding periodontal status). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

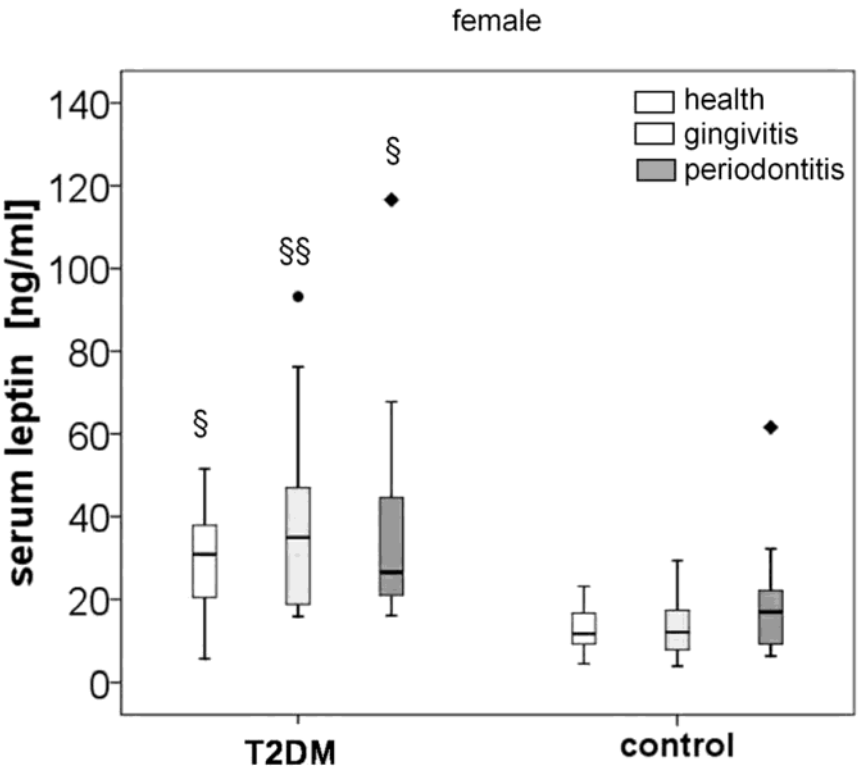

Figure 6.3 Female serum leptin concentrations according to periodontal status in T2DM and non-diabetic controls

Box plots of serum leptin concentrations in 33 female T2DM patients (periodontal health $n = 9$; gingivitis $n = 14$; periodontitis $n = 10$) and 27 non-diabetic female controls (periodontal health $n = 9$; gingivitis $n = 5$; periodontitis $n = 13$). Statistics: ANOVA, Student's t-test $^{\S}p < 0.05$; $^{\S\S}p < 0.01$ (diabetes versus non-diabetes within the corresponding periodontal status). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.1.2 Analysis of serum adiponectin concentrations in T2DM patients and non-diabetic controls with or without periodontal disease before periodontal treatment

A significant gender difference was detected in serum adiponectin concentrations in both T2DM patients and non-diabetic controls (Figure 6.4). Female T2DM patients had 40 % higher serum adiponectin concentrations than male patients with T2DM ($p < 0.01$), having 3.37 ± 1.87 and 2.49 ± 1.46 µg/ml serum adiponectin concentrations, respectively. In the control group, females had 41 % higher serum adiponectin concentrations than males ($p < 0.001$), having 3.76 ± 1.19 and 2.67 ± 0.88 µg/ml serum adiponectin concentrations, respectively. A trend was observed for higher serum adiponectin concentrations in non-diabetic controls than in T2DM patients ($p = 0.097$ and $p = 0.073$ for males and females, respectively).

Figure 6.5 shows the adiponectin concentrations, according to the periodontal status, in male T2DM patients and non-diabetic controls. No significant differences were detected within the different groups according to periodontal status. Periodontal healthy male T2DM patients and non-diabetic controls had 2.34 ± 1.09 and 2.54 ± 1.06 µg/ml serum adiponectin, respectively. Male T2DM patients and non-diabetic controls with gingivitis had 2.61 ± 1.86 and 2.6 ± 1.22 µg/ml serum adiponectin, respectively. Male T2DM patients and non-diabetic controls with periodontitis had 2.42 ± 1.16 and 2.71 ± 0.73 µg/ml serum adiponectin, respectively.

Figure 6.6 shows the serum adiponectin concentrations, according to the periodontal status, in female T2DM patients and non-diabetic controls. No significant differences were detected within the different groups according to periodontal status. Periodontal healthy female T2DM patients and non-diabetic controls had 2.59 ± 0.66 and 3.83 ±

1.46 µg/ml serum adiponectin, respectively. Female T2DM patients and non-diabetic controls with gingivitis had 3.83 ± 1.84 and 3.68 ± 1.2 µg/ml serum adiponectin, respectively. Female T2DM patients and non-diabetic controls with periodontitis had 3.43 ± 2.51 and 3.75 ± 1.01 µg/ml serum adiponectin, respectively.

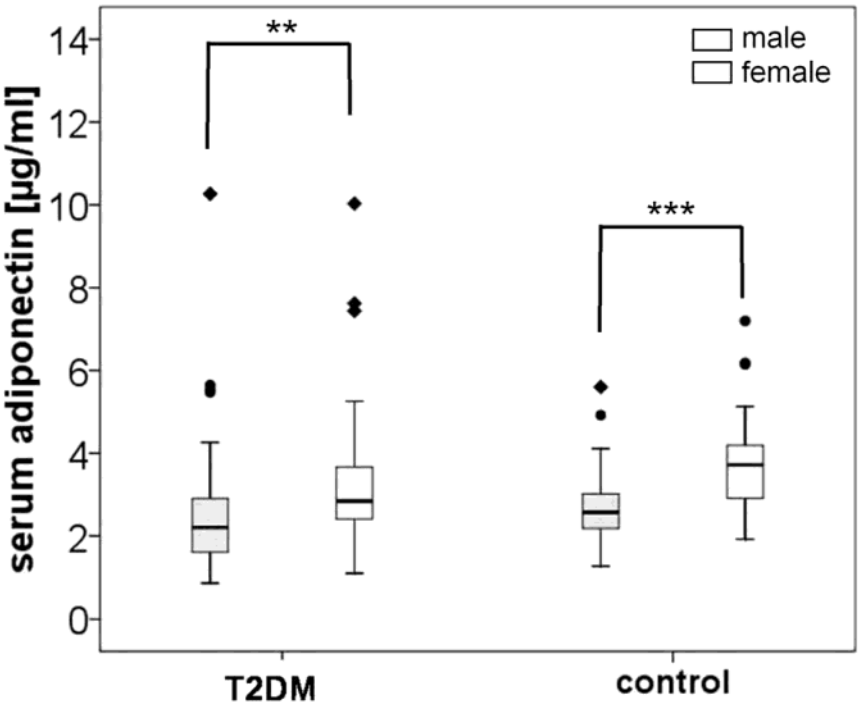

Figure 6.4 Serum adiponectin concentrations in males and females with T2DM and in non-diabetic controls

Box plots of serum adiponectin concentrations in 97 T2DM patients (64 males, 33 females) and 76 non-diabetic controls (44 males, 32 females). Statistics: ANOVA, Student's *t*-test $^{**}p < 0.01$, $^{***}p < 0.001$ (male versus female within diabetes or control group). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

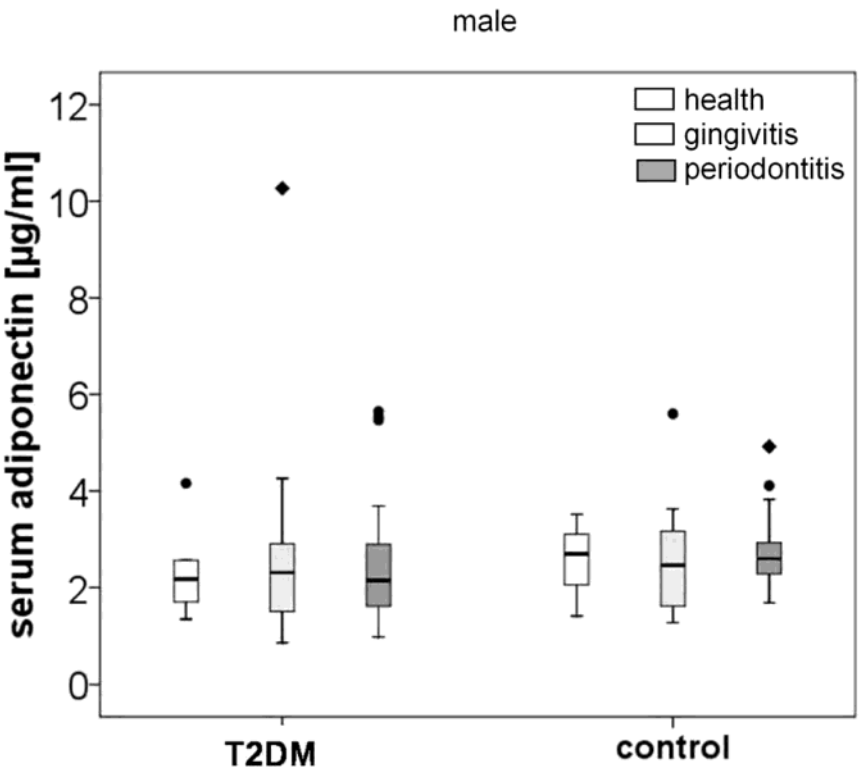

Figure 6.5 Male serum adiponectin concentrations according to periodontal status in T2DM and non-diabetic controls

Box plots of serum adiponectin concentrations in 64 male T2DM patients (periodontal health $n = 5$; gingivitis $n = 25$; periodontitis $n = 34$) and 44 non-diabetic male controls (periodontal health $n = 3$; gingivitis $n = 12$; periodontitis $n = 29$). Statistics: ANOVA, n.s. ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

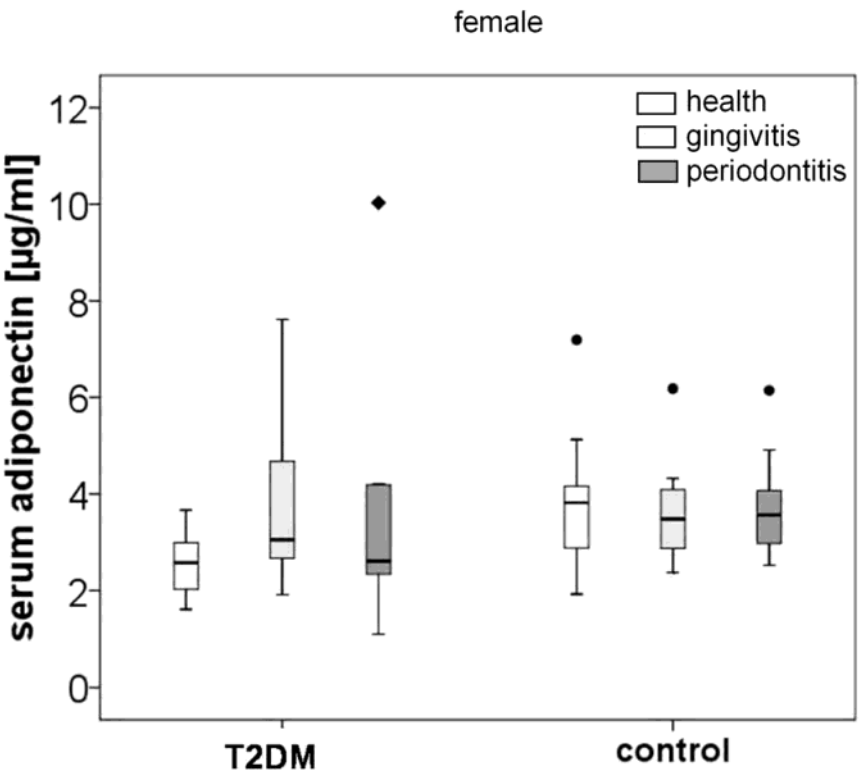

Figure 6.6 Female serum adiponectin concentrations according to periodontal status in T2DM and non-diabetic controls

Box plots of serum adiponectin concentrations in 33 female T2DM patients (periodontal health $n = 9$; gingivitis $n = 14$; periodontitis $n = 10$) and 32 non-diabetic female controls (periodontal health $n = 11$; gingivitis $n = 8$; periodontitis $n = 13$). Statistics: ANOVA, n.s. ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.1.3 Analysis of GCF adiponectin concentrations in T2DM patients and non-diabetic controls with or without periodontal disease before periodontal treatment

No significant gender difference was detected in GCF adiponectin concentrations in T2DM patients ($p = 0.275$) or non-diabetic controls ($p = 0.572$). Figure 6.7 shows that non-diabetic controls had 36 % higher GCF adiponectin concentrations than T2DM patients ($p < 0.01$), reaching a concentration of 1.7 ± 1.32 and 1.25 ± 1.23 ng/ml for non-diabetic controls and T2DM patients, respectively.

Figure 6.8 shows the GCF adiponectin concentrations, according to periodontal status, in T2DM patients and non-diabetic controls. In T2DM patients with gingivitis or periodontitis GCF adiponectin concentrations were significantly higher than in periodontal health, showing a 180 % ($p < 0.01$) and 206 % ($p < 0.001$) increase from 0.47 ± 0.29 to 1.32 ± 1.22 ng/ml and to 1.44 ± 1.33 ng/ml for gingivitis and periodontitis, respectively. No significant difference in GCF adiponectin concentrations was observed between gingivitis and periodontitis in T2DM patients ($p = 0.417$). In non-diabetic controls, GCF adiponectin concentrations in periodontitis were 192 % higher ($p < 0.001$) than in periodontal health, increasing from 0.75 ± 0.44 to 2.19 ± 1.3 ng/ml. No difference was observed in GCF adiponectin concentrations between non-diabetic controls with periodontal health and gingivitis ($p = 0.126$). However, the observed GCF adiponectin concentration of 1.31 ± 1.35 ng/ml in non-diabetic controls with gingivitis was 40 % lower ($p < 0.05$) than in non-diabetic controls with periodontitis. GCF adiponectin concentrations were 60 % higher ($p < 0.05$) in periodontal healthy non-diabetic controls than in periodontal healthy T2DM patients. No significant difference in GCF adiponectin concentrations was observed between T2DM patients and non-diabetic controls with gingivitis ($p = 0.869$). In contrast, non-

diabetic controls with periodontitis had 92 % higher GCF adiponectin concentrations than T2DM patients with periodontitis ($p < 0.01$). It is interesting to note that although a reasonable number of subjects ($n \geq 14$) are in each group, the spread of the data is larger in subjects with gingivitis and periodontitis than in periodontal health, supporting a relationship of periodontal status to GCF adiponectin concentrations.

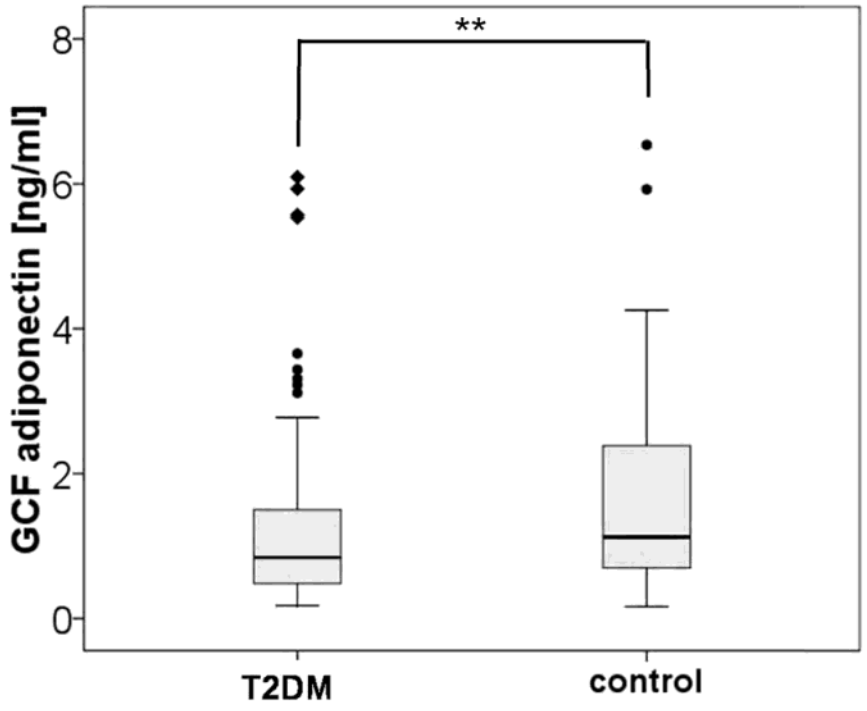

Figure 6.7 GCF adiponectin concentrations in subjects with T2DM and in non-diabetic controls

Box plots of GCF adiponectin concentrations in 98 T2DM patients and 76 non-diabetic controls. Statistics: Student's *t*-test $**p < 0.01$. ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

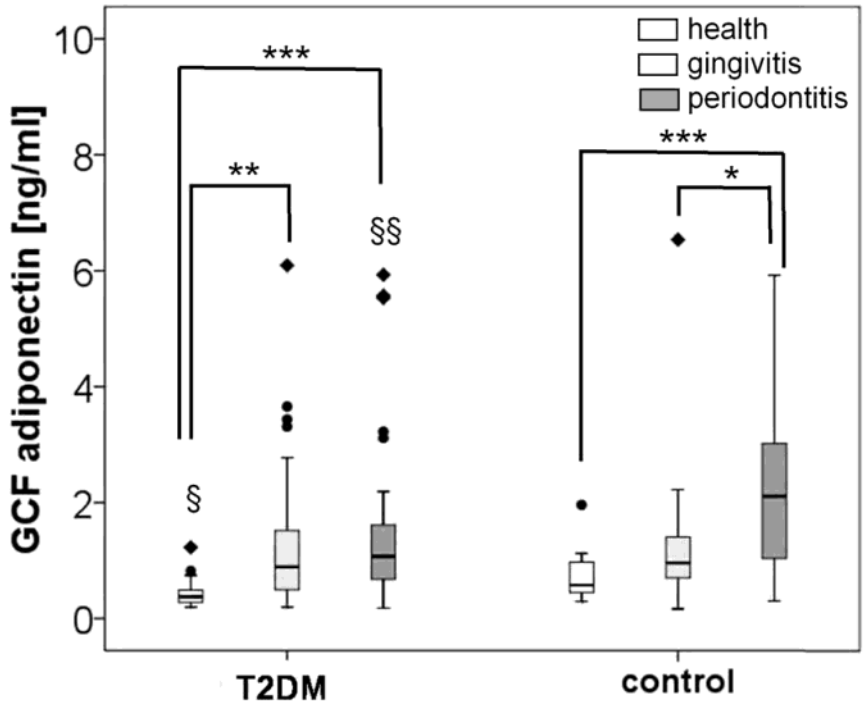

Figure 6.8 GCF adiponectin concentrations according to periodontal status in T2DM and non-diabetic controls

Box plots of GCF adiponectin concentrations in 98 T2DM patients (periodontal health n = 14; gingivitis n = 39; periodontitis n = 45) and 76 non-diabetic controls (periodontal health n = 14; gingivitis n = 20; periodontitis n = 42). Statistics: ANOVA, Student's t-test $^{**}p < 0.01$, $^{***}p < 0.001$ (according to the periodontal status within diabetes or control); $^{\S}p < 0.05$ and $^{\S\S}p < 0.01$ (diabetes versus non-diabetes within the corresponding periodontal status). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.2 Analysis of serum leptin, serum adiponectin and GCF adiponectin concentrations in periodontitis patients after periodontal treatment

In T2DM patients and non-diabetic controls with periodontitis, concentrations of serum and GCF adipokines were followed up after treatment for periodontal disease at month 3 and month 6. Blood and GCF samples were taken and analysed with ELISAs using the same procedure as for pre-treatment measurements (see 2.3).

No significant differences in serum leptin and serum adiponectin concentrations were detected in T2DM patients or non-diabetic controls with periodontitis after periodontal treatment at month 3 and month 6 in comparison to pre-treatment values (Table 6.1, Table 6.2).

GCF adiponectin concentrations decreased significantly after periodontal treatment in both T2DM patients and non-diabetic controls (Figure 6.9). Compared to pre-treatment values, a 36 % decrease ($p < 0.01$) in GCF adiponectin levels from 1.44 ± 1.33 to 0.92 ± 0.64 ng/ml at month 3 and a 28 % decrease ($p < 0.05$) to 1.04 ± 1.0 ng/ml at month 6 was observed after periodontal treatment of T2DM patients with periodontitis. In non-diabetic controls with periodontitis, GCF adiponectin concentrations decreased by 51 % from 2.19 ± 1.3 before periodontal treatment to1.08 ± 0.9 ng/ml at month 3 after periodontal treatment ($p < 0.05$). GCF adiponectin concentrations in non-diabetic controls at month 6 after periodontal treatment decreased to 1.21 ± 0.41 ng/ml, however the difference to pre-treatment GCF adiponectin concentrations was only observed as a trend ($p = 0.082$). No significant differences in GCF adiponectin concentrations were observed between month 3 and month 6 after periodontal treatment in either T2DM patients ($p = 0.9$) or non-diabetic controls ($p = 0.453$).

	T2DM serum leptin [ng/ml]		Control serum leptin [ng/ml]	
	male	**female**	**male**	**female**
pre-treatment	*n = 34* 15.89 ± 14.25	*n = 10* 39.42 ± 31.17	*n = 28* 10.5 ± 7.75	*n = 13* 19.48 ± 14.7
Month 3	*n = 28* 13.74 ± 7.76	*n = 7* 42.05 ± 31.65	*n = 24* 9.52 ± 6.96	*n = 10* 21.52 ± 12.69
Month 6	*n = 25* 14.86 ± 9.32	*n = 7* 39.65 ± 35.48	*n = 19* 9.82 ± 7.17	*n = 5* 26.06 ± 15.46

Table 6.1 The effect of periodontal treatment on serum leptin concentrations in T2DM and non-diabetic controls with periodontitis

The table shows mean ± SD serum leptin concentrations in patients with periodontitis and T2DM and in non-diabetic controls with periodontitis. Measurements were taken pre-treatment and post-treatment at 3 and 6 months. Statistics: repeated measures ANOVA and paired samples *t*-test for T2DM patients, n.s.; Friedman test and Wilcoxon Mann-Whitney test for non-diabetic controls, n.s.

	T2DM serum adiponectin [µg/ml]		Control serum adiponectin [µg/ml]	
	male	**female**	**male**	**female**
pre-treatment	*n = 34* 2.42 ± 1.16	*n = 10* 3.43 ± 2.51	*n = 29* 2.71 ± 0.73	*n = 13* 3.75 ± 1.01
Month 3	*n = 28* 2.55 ± 1.07	*n = 7* 3.75 ± 1.67	*n = 25* 2.53 ± 0.65	*n = 10* 3.84 ± 0.9
Month 6	*n = 25* 2.84 ± 1.39	*n = 7* 3.75 ± 1.76	*n = 16* 2.83 ± 0.97	*n = 4* 4.04 ± 1.07

Table 6.2 The effect of periodontal treatment on serum adiponectin concentrations in T2DM and non-diabetic controls with periodontitis

The table shows mean ± SD serum adiponectin concentrations in patients with periodontitis and T2DM and in non-diabetic controls with periodontitis. Measurements were taken pre-treatment and post-treatment after 3 and 6 months. Statistics: repeated measures ANOVA and paired samples *t*-test, n.s.

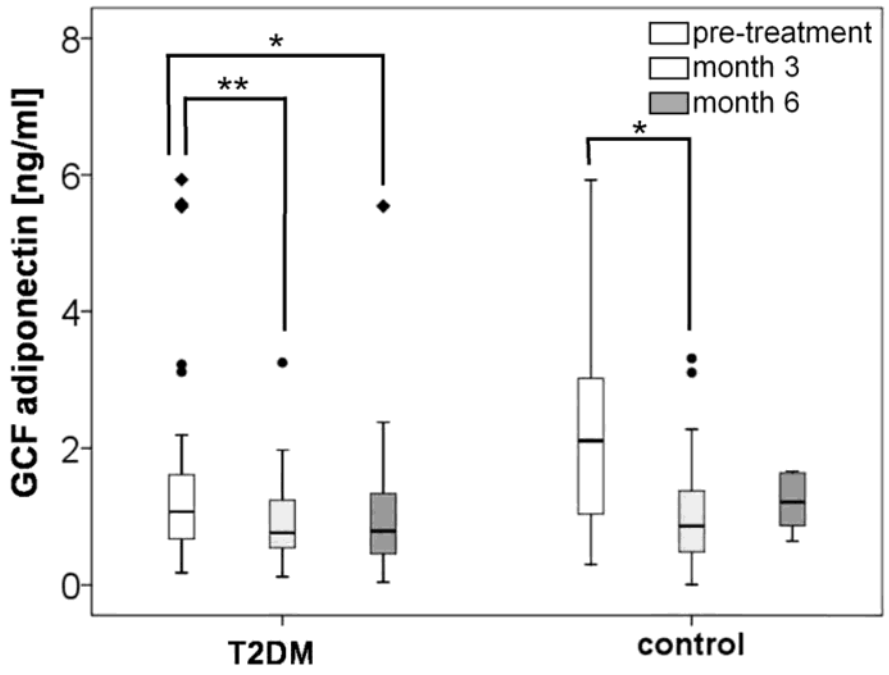

Figure 6.9 The effect of periodontal treatment on GCF adiponectin concentrations in T2DM and non-diabetic controls with periodontitis

Box plots of GCF adiponectin concentrations of patients with periodontitis and T2DM and of non-diabetic controls with periodontitis. Measurements were taken pre-treatment (T2DM $n = 45$; control $n = 42$) and after 3 (T2DM $n = 34$; control $n = 22$) and 6 months (T2DM $n = 33$; control $n = 6$) treatment for periodontal disease. Statistics: repeated measures ANOVA, paired samples t-test $^{*}p < 0.05$, $^{**}p < 0.01$ (compared to pre-treatment values within diabetes or within control group). ◆ outlier more than 3 times the IQR from the box boundaries, ● outlier more than 1.5 but less than 3 times the IQR from the box boundaries.

6.2.3 The relationship of serum adipokines with clinical parameters of glycaemic control and inflammation

In addition to measuring adipokines in serum, blood samples of T2DM patients and non-diabetic controls were also analysed for HbA1c and hsCRP concentrations by the Haematology and Clinical Biochemistry labs of the Royal Victoria Infirmary (see 2.8.3). HbA1c and CRP were analysed for correlations with leptin and adiponectin to investigate a possible relationship of the adipokines with markers of glycaemic control (HbA1c) and inflammation (hsCRP). Correlations were not split up into different groups according to diabetic or periodontal status, however gender differences in serum leptin and adiponectin concentrations were taken into account.

A statistically significant positive correlation of serum leptin with HbA1c was detected in both male ($p < 0.05$) and female ($p < 0.01$) subjects (Figure 6.10), % of HbA1c increased with increasing concentrations of serum leptin. Likewise, a positive correlation between serum leptin and hsCRP was detected with serum hsCRP concentrations increasing with increasing serum leptin concentrations in both male ($p < 0.001$) and female ($p < 0.01$) subjects (Figure 6.11).

A statistically significant negative correlation of serum adiponectin with HbA1c was detected in male ($p < 0.01$) subjects, where the percentage of HbA1c decreased with increasing concentrations of serum adiponectin (Figure 6.12). No correlation of serum adiponectin with HbA1c was detected in female subjects ($p = 0.323$, Figure 6.12). A negative correlation of serum adiponectin with hsCRP was detected in male ($p < 0.01$) subjects, with serum hsCRP concentrations decreasing with increasing concentrations of serum adiponectin (Figure 6.13). No correlation of serum adiponectin with hsCRP was detected in female subjects ($p = 0.211$, Figure 6.13).

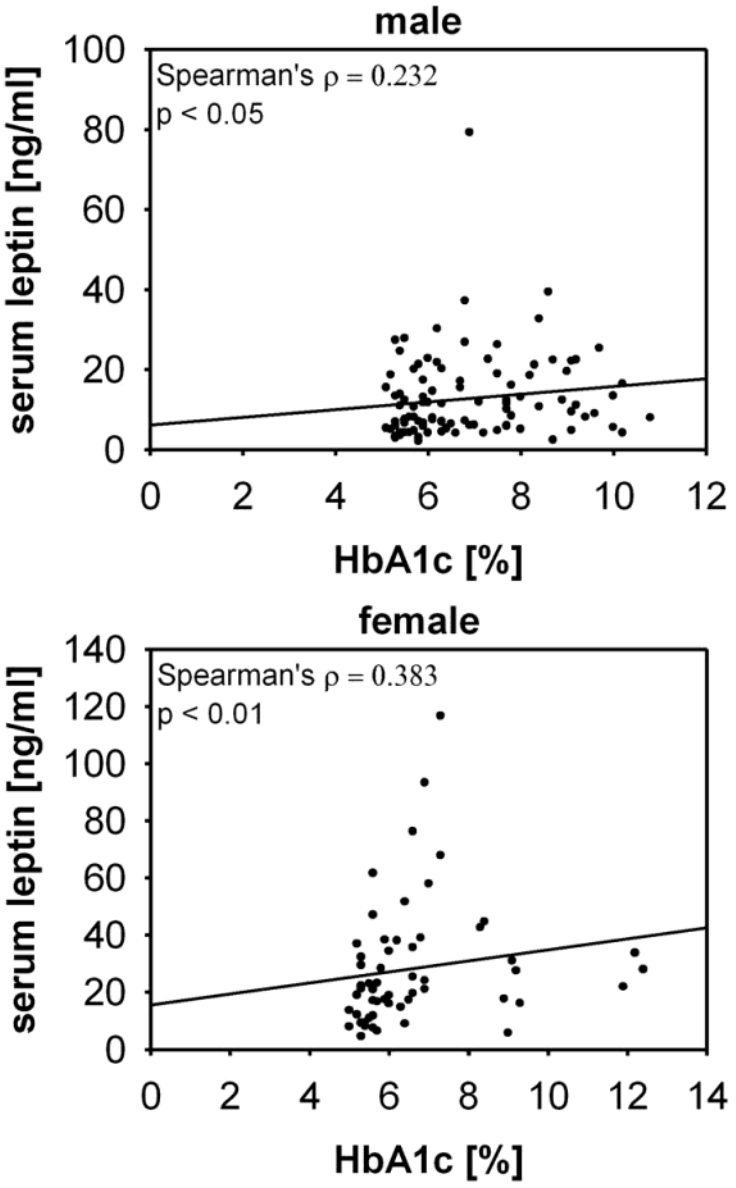

Figure 6.10 The relationship of serum leptin with HbA1c in males and females

Graphs show Spearman correlations of serum leptin concentrations with percentage of HbA1c in males (n = 101) and females (n = 56) (not differentiated according to diabetic or periodontal status).

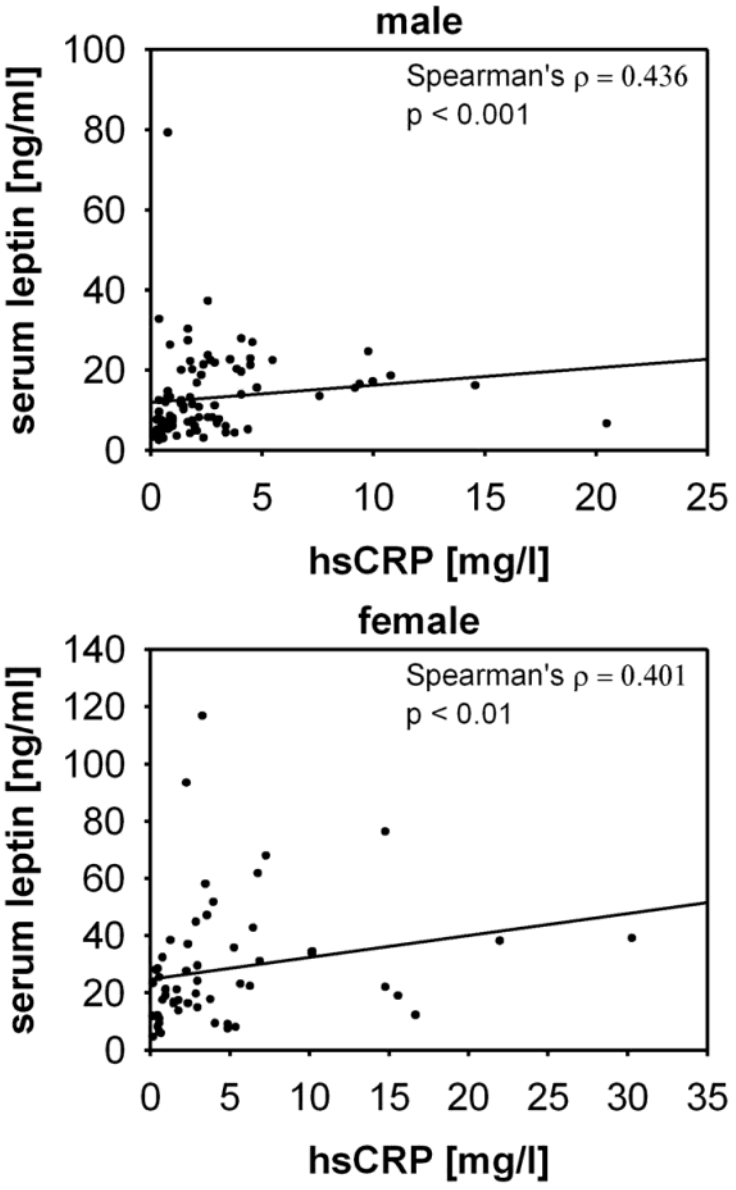

Figure 6.11 The relationship of serum leptin with hsCRP in males and females

Graphs show Spearman correlations of serum leptin concentrations with serum concentrations of hsCRP in males (n = 89) and females (n = 56) (not differentiated according to diabetic or periodontal status).

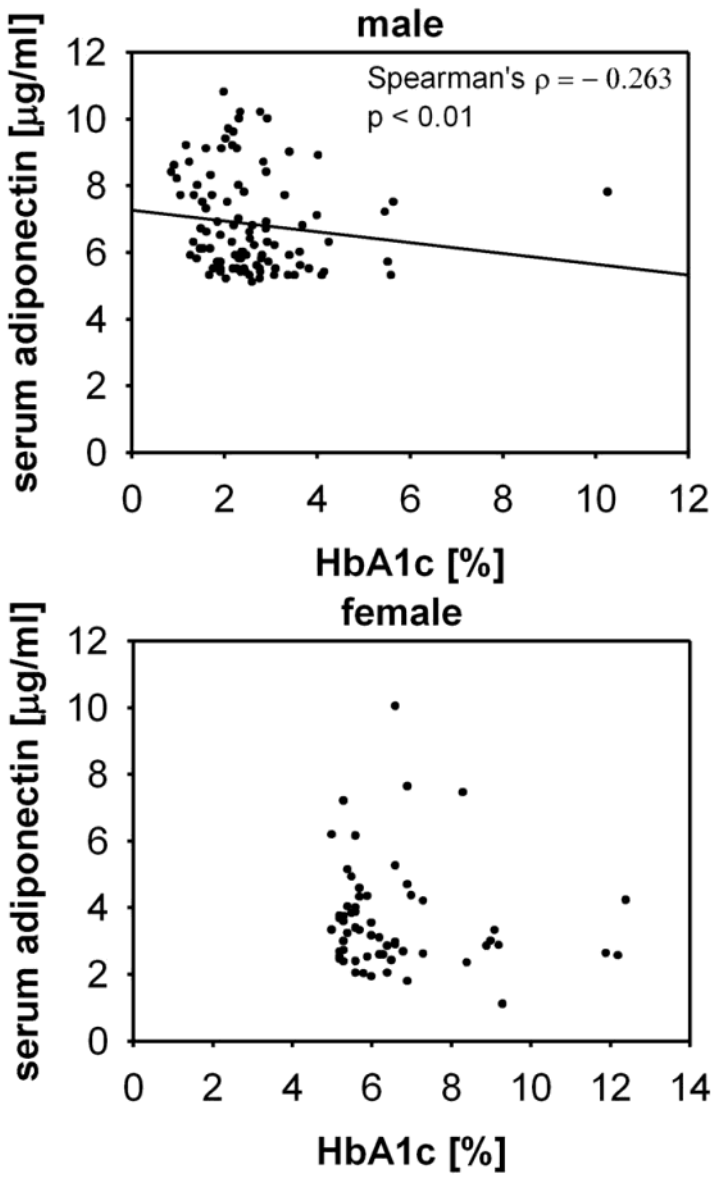

Figure 6.12 The relationship of serum adiponectin with HbA1c in males and females

Graphs show Spearman correlations of serum adiponectin concentrations with percentage of HbA1c in males (n = 102) and females (n = 60) (not differentiated according to diabetic or periodontal status).

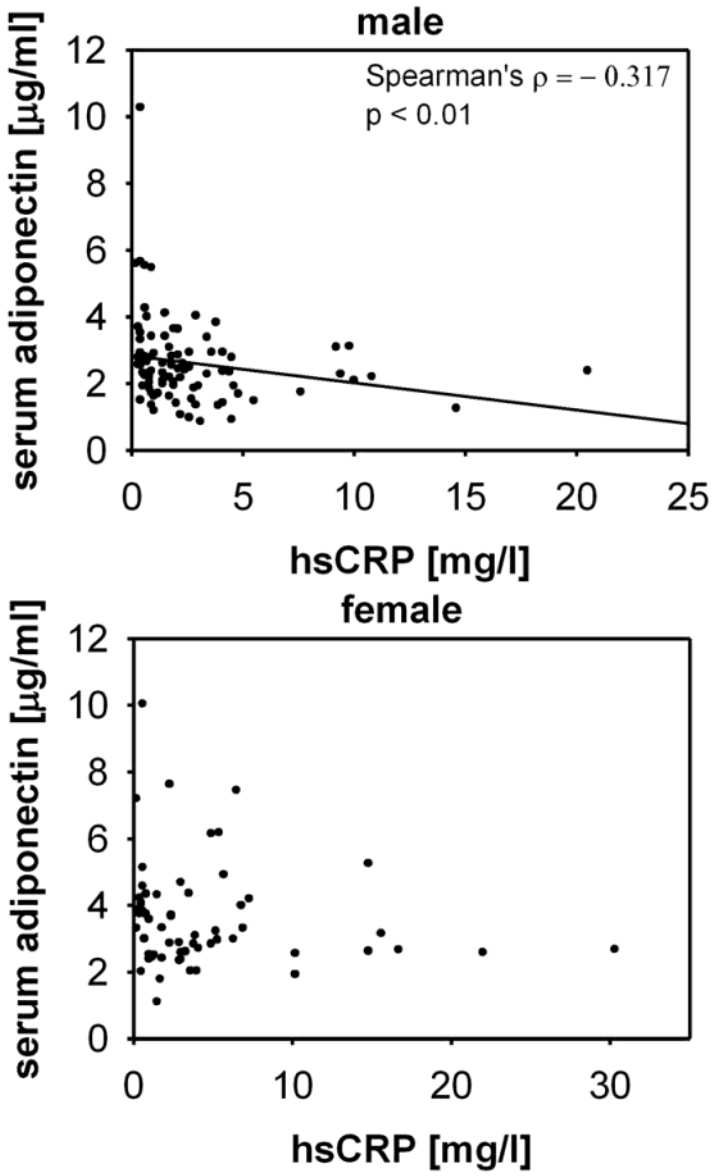

Figure 6.13 The relationship of serum adiponectin with hsCRP in males and females

Graphs show Spearman correlations of serum adiponectin concentrations with serum concentrations of hsCRP in males (n = 90) and females (n = 60) (not differentiated according to diabetic or periodontal status).

6.2.4 Investigation of the impact of BMI on periodontal status, adipokine concentrations and markers of glycaemic control and inflammation

Being secretory products of the adipose tissue, adipokines likely are closely associated with BMI. Therefore, differences in BMI according to diabetic or periodontal status were analysed to assess a possible impact of BMI on the obtained adipokine concentrations before periodontal treatment. Additionally, the relationship of BMI to HbA1c and hsCRP was investigated. Finally, the impact of adipokines on periodontal status was determined after taking BMI into account.

6.2.4.1 The effect of gender, diabetic and periodontal status on BMI in T2DM patients and non-diabetic controls

No significant gender difference in BMI was detected in T2DM patients ($p = 0.449$), however non-diabetic males had a 12 % higher BMI than non-diabetic females ($p < 0.01$, Table 6.3). Additionally, male T2DM patients had a 12 % ($p < 0.01$) and female T2DM patients had a 29 % ($p < 0.001$) higher BMI than non-diabetic male and female controls (Table 6.3).

After correction of p values for multiple comparisons with Bonferroni-Holm, no significant differences in BMI according to periodontal status were detected in T2DM patients. However, although no overall gender difference was detected in this group, significant differences in BMI were observed according to periodontal status when gender was taken into account (Table 6.3). Male T2DM patients with gingivitis or periodontitis had a 31 % higher ($p < 0.01$) or 29 % higher ($p < 0.01$) BMI than male T2DM patients who were periodontally healthy. In contrast, no significant differences in BMI according to periodontal status were detected in female T2DM patients. No

significant differences in BMI according to the periodontal status were detected in either male or female non-diabetic controls (Table 6.3).

When considering periodontal status, male T2DM patients with periodontitis had a 10 % ($p < 0.05$) and female T2DM patients with periodontitis a 36 % ($p < 0.01$) higher BMI than the corresponding non-diabetic controls with periodontitis (Table 6.3). In the gingivitis group, male T2DM patients had an 18 % higher BMI than male non-diabetic controls ($p < 0.01$). However, a higher BMI in female T2DM patients with gingivitis in comparison to non-diabetic controls with gingivitis was only observed as a trend ($p = 0.054$). No significant difference in BMI was observed between male T2DM patients with periodontal health and the corresponding non-diabetic control group ($p = 0.937$). In contrast, female T2DM patients with periodontal health had a 31 % higher BMI than periodontally healthy non-diabetic female controls ($p < 0.01$).

	T2DM BMI [kg/m²]		Control BMI [kg/m²]	
	male	**female**	**male**	**female**
Health	*n = 5* 24.9 ± 4.46¶¶	*n = 9* 32.01 ± 5.33**	*n = 3* 25.13 ± 2.36	*n = 11* 24.52 ± 4.3
Gingivitis	*n = 25* 32.7 ± 5.04**	*n = 12* 32.23 ± 7.09	*n = 14* 27.75 ± 3.74	*n = 8* 26.8 ± 3.06
Periodontitis	*n = 35* 32.22 ± 5.07*	*n = 28* 34.2 ± 5.59***	*n = 11* 29.19 ± 5.21	*n = 13* 25.22 ± 3.57
Total	*n = 65* 31.84 ± 5.34**	*n = 34* 32.81 ± 6.09***	*n = 43* 28.5 ± 4.76§§	*n = 32* 25.37 ± 3.71

Table 6.3 The BMI according to diabetic and periodontal status

The table shows mean ± SD BMI for patients with T2DM (male n = 65, female n =34) and for non-diabetic controls (male n = 43, female n = 32) before periodontal treatment, according to periodontal status. Statistics: ANOVA, Student's *t*-test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to non-diabetic controls within the corresponding gender group; $^{¶}p < 0.01$ compared to the other periodontal status groups within the column; $^{§§}p < 0.01$ compared to females within the total control group.

6.2.4.2 The relationship of BMI with adipokines, HbA1c and hsCRP

Pre-treatment measurements of adipokines and markers of glycaemic control (HbA1c) and inflammation (hsCRP) were investiaged for correlations with BMI. Correlations were not split according to diabetic or periodontal status, however gender differences in serum leptin and adiponectin concentrations were taken into account.

A statistically significant positive correlation of BMI with serum leptin was detected in both male ($p < 0.001$) and female ($p < 0.001$) subjects (Figure 6.14). Serum leptin concentrations increased with an increase in BMI. In contrast, a negative correlation of BMI with serum adiponectin concentrations was detected. Adiponectin concentrations increased with a decrease in BMI in both male ($p < 0.05$) and female ($p < 0.05$) subjects (Figure 6.15). No significant correlation between BMI and GCF adiponectin was detected (Spearman's $\rho = -0.049$, $p = 0.523$; $n = 172$, graph not shown).

A statistically significant positive correlation of BMI with HbA1c and hsCRP was detected and both the percentage of HbA1c ($p < 0.001$) and the concentration of hsCRP ($p < 0.001$) increased with an increase in BMI.

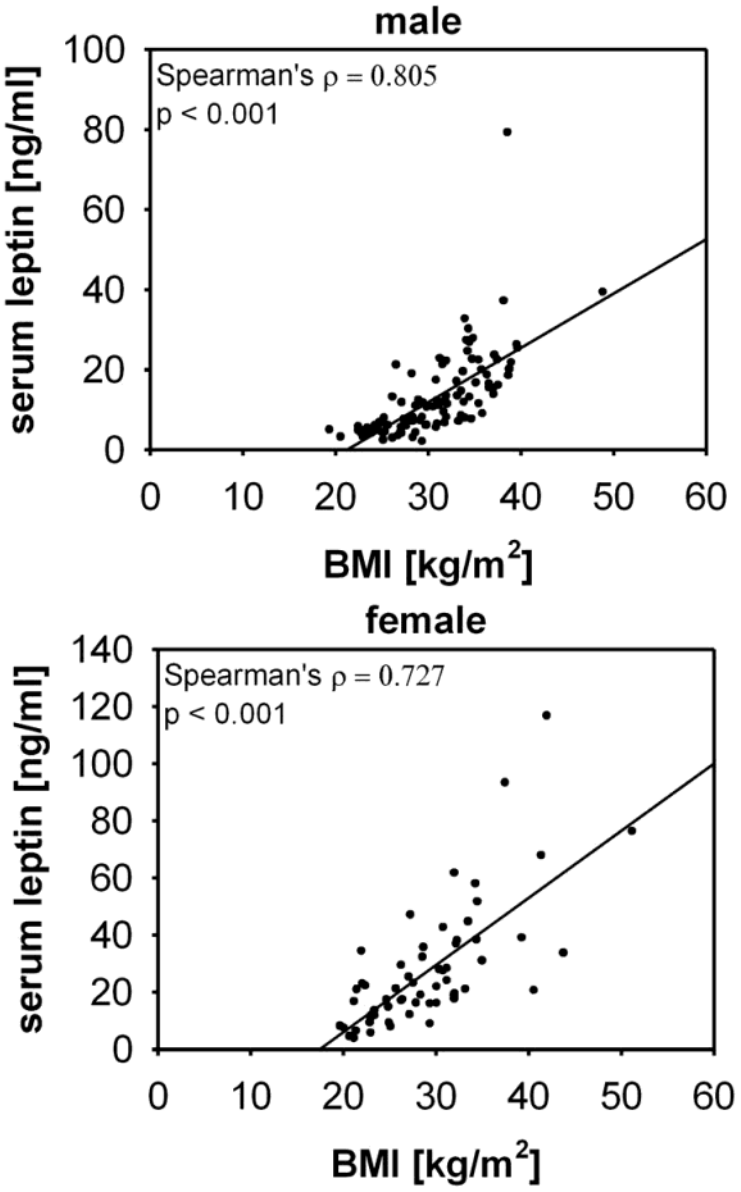

Figure 6.14 The relationship of BMI with serum leptin in males and females

Graphs show Spearman correlations of BMI with serum leptin concentrations in males (n = 105) and females (n = 60) (not differentiated according to diabetic or periodontal status).

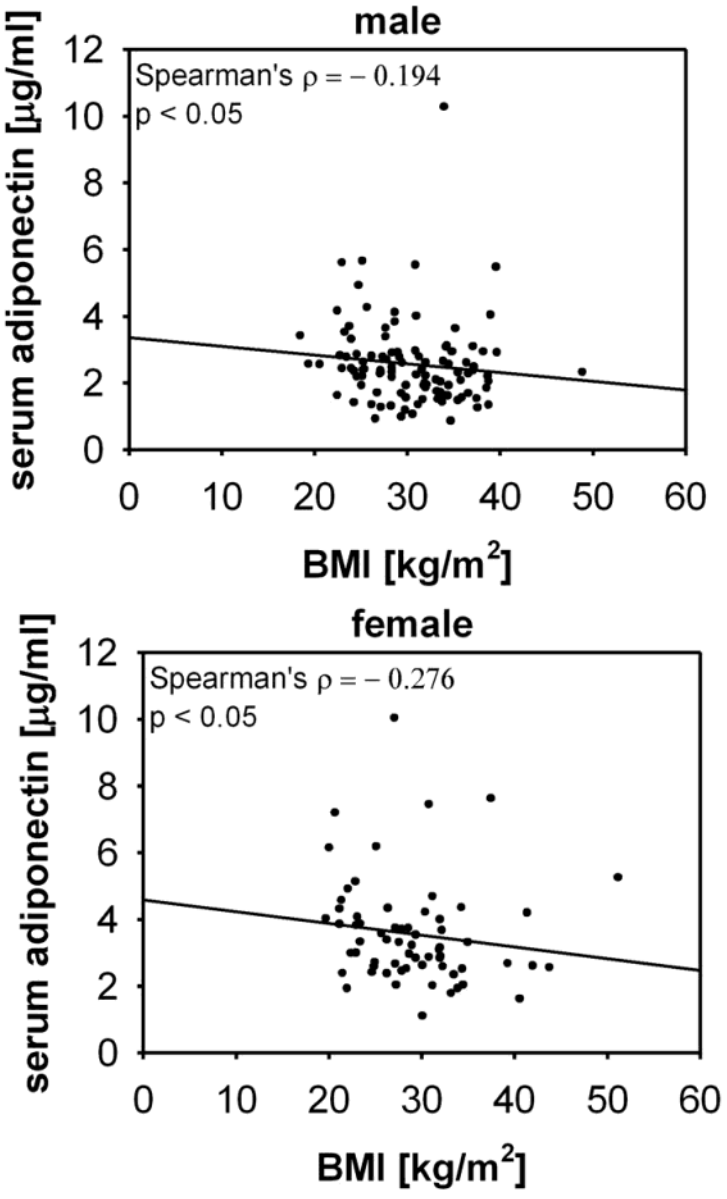

Figure 6.15 The relationship of BMI with serum adiponectin in males and females

Graphs show Spearman correlations of BMI with serum adiponectin concentrations in males (n = 106) and females (n = 65) (not differentiated according to diabetic or periodontal status).

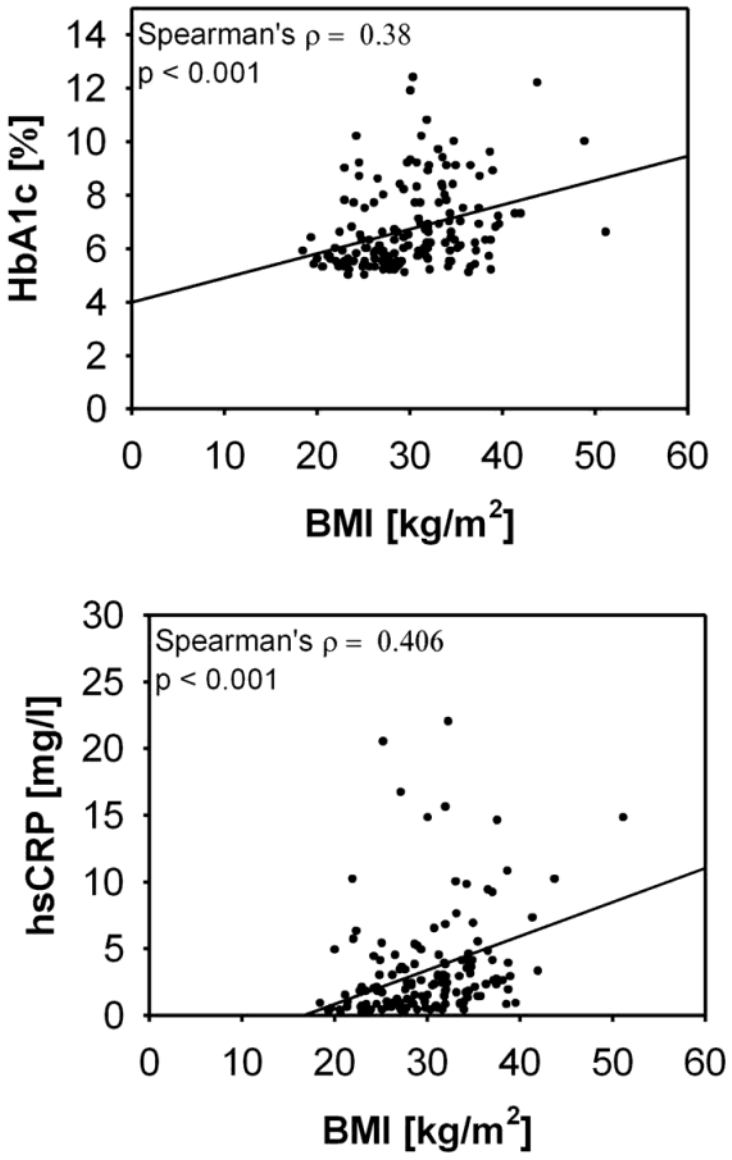

Figure 6.16 The relationship of BMI with HbA1c and hsCRP

Graphs show Spearman correlations of BMI with HbA1c (n = 160) and hsCRP (n = 149) (not differentiated according to diabetic or periodontal status).

6.2.4.3 BMI as predictor of adipokine concentrations and adipokines and BMI as predictors of the periodontal status

The potential of BMI as a predictor of serum leptin and adiponectin concentrations and of GCF adiponectin concentrations was assessed in linear regression models. Serum leptin or adiponectin or GCF adiponectin were assigned as the dependent variables, BMI was assigned the independent variable. The potential of serum leptin, GCF adiponectin and BMI in predicting gingivitis or periodontitis was assessed in multinomial logistic regression models. No differences in serum adiponectin according to periodontal status were observed (see 6.2.1.2) and therefore this parameter was not included in the analysis. Periodontal status (periodontal health, gingivitis or periodontitis) was assigned as the dependent variable, using periodontal health as the reference category. Serum leptin and BMI are closely associated (see 6.2.4.2) and thus would not be truly independent covariates. Therefore, these parameters were analysed individually in two separate models. Both BMI and GCF adiponectin were included as covariates in one single model as no association between these two parameters was observed (see 6.2.4.2). All tests were conducted on measurements taken before periodontal treatment and groups were not divided according to diabetic status. However, gender differences in serum adipokine concentrations were taken into account.

In linear regression models, BMI was determined as a significant predictor of serum leptin concentrations in both males ($p < 0.001$) and females ($p < 0.001$). A trend in both males ($p = 0.088$) and females ($p = 0.087$) for BMI as a predictor for serum adiponectin concentrations was noted, however BMI was not a predictor for GCF adiponectin concentrations ($p = 0.652$).

In multinomial logistic regression models, both BMI and serum leptin were identified as predictors of the periodontal status in males but not in females. BMI was a significant predictor of gingivitis ($p < 0.01$) and periodontitis ($p < 0.01$) in males but not in females ($p = 0.187$ and 0.406 for gingivitis and periodontitis, respectively). When including leptin in the model, leptin was a significant predictor of gingivitis ($p < 0.05$) and periodontitis ($p < 0.05$) in male subjects. Leptin was not a predictor of periodontal status in females ($p = 0.13$ and 0.345 for gingivitis and periodontitis, respectively). Both GCF adiponectin and BMI were significant predictors for gingivitis ($p < 0.01$ for both GCF adiponectin and BMI) and periodontitis ($p < 0.001$ for GCF adiponectin and $p < 0.01$ for BMI). Therefore, GCF adiponectin can be regarded as an independent predictor of gingivitis or periodontal disease, even when BMI is included in the model. Furthermore, in contrast to BMI, prediction of the periodontal status by GCF adiponectin does not differ between genders. Thus, GCF adiponectin is likely a more suitable predictor for gingivitis or periodontitis than BMI in females, because, in females, BMI failed to predict the periodontal status.

6.3 Discussion

Diabetes and periodontal disease are now both recognised as inflammatory conditions (Kornman *et al.*, 1997; King, 2008) and a number of studies support diabetes as a risk factor for increased incidence or severity of periodontal disease (Nelson *et al.*, 1990; Ryan *et al.*, 2003; Heitz-Mayfield, 2005; Moles, 2006). With the increase of obesity and diabetes in modern Western societies and developing countries (Kopelman, 2000), revealing immunological links between the two diseases to identify new immune mediators as possible targets for therapeutic treatments is therefore becoming an important issue. The adipokines leptin and adiponectin have various immunological functions, including an effect on immune responses to LPS (Loffreda *et al.*, 1998; Ajuwon and Spurlock, 2005) which could give them a key role in immune responses to periodontal pathogens. In addition, their concentrations are altered in diabetes (Sinha *et al.*, 1996; Hotta *et al.*, 2000) and therefore these adipokines are an interesting target as a potential immunological link between diabetes and periodontal disease.

The present study reveals a number of significant differences in serum leptin concentrations according to the periodontal and diabetic status of the study participants. As reported previously (Sinha *et al.*, 1996), both male and female T2DM patients had higher serum leptin concentrations than non-diabetic controls. Also, the present study shows for the first time that serum leptin concentrations increase in male T2DM patients with gingivitis and periodontitis compared to periodontal health, while no differences in serum leptin concentrations according to periodontal status were detected in female T2DM patients. Neither male nor female non-diabetic controls showed any differences in serum leptin concentrations according to periodontal status, which is in contrast to findings by Karthikeyan and Pradeep (2007) who reported increased serum leptin

concentrations with increased periodontal tissue destruction. However, it is important to note that as demonstrated in the present study, a strong gender difference in serum leptin concentrations exists. This gender difference was described before in other studies (Nystrom *et al.*, 1997; Perkins and Fonte, 2002) and is likely due to hormonal interferences as leptin has a strong negative correlation with testosterone (Nystrom *et al.*, 1997). Karthikeyan and Pradeep (2007) did not acknowledge the gender differences and combined data from males and females. This almost certainly interferes with a correct interpretation of serum leptin concentrations and their relationship to periodontal status in their study. Possibly, the association of serum leptin concentrations with periodontal status in male but not in female T2DM patients could be explained by gender related differences in life style. For example, on a population wide basis, males generally have a poorer oral health behaviour than females, including worse oral hygiene or less regular dental checkups (Albandar, 2002).

With the exception of periodontally healthy male subjects, serum leptin concentrations were consistently higher in both male and female T2DM patients than in non-diabetic controls when compared according to periodontal status. In contrast, a recent study did not detect any significant differences in serum leptin concentrations between T2DM patients and non-diabetic controls with periodontitis (Kardesler *et al.*, 2010). However, again this study did not differentiate between the genders and the combined data from males and females will have compromised interpretation of the study findings.

Taken together, investigations of serum leptin concentrations in this study generally indicate that regardless of the periodontal status, both male and female T2DM patients have higher serum leptin concentrations than non-diabetic controls. In addition, serum leptin concentrations in non-diabetic controls are not associated with the periodontal

status. Likewise, serum leptin concentrations in female T2DM patients are not associated with periodontal status. In contrast, serum leptin concentrations increase in male T2DM patients with gingivitis and periodontitis. It is tempting to speculate that due to the gender difference, at least in males, leptin may have a direct role in the increased prevalence and severity of periodontal disease in diabetes. Indeed, regression analysis revealed leptin to be a significant predictor of gingivitis and periodontitis in males but not in females, potentially indicating that the increased serum leptin concentrations in male T2DM patients directly impact on periodontal immune responses. Possibly, at least in males, serum leptin could therefore be considered as a biomarker for periodontal disease. This may be of particular interest when considering the general overall poorer oral health behaviour in males (Albandar, 2002) and may help to identify "at risk" groups for developing periodontal disease.

However, one should be careful not to draw premature conclusions regarding serum leptin concentrations without considering differences in the BMI. In agreement with findings of several studies (Maffei *et al.*, 1995; Considine *et al.*, 1996) and as shown in the present study, leptin has a strong positive correlation with BMI, and linear regression analysis in the present study determined BMI as a predictor of serum leptin concentrations in both males and females. Although a gender difference for serum leptin concentrations was detected in both T2DM patients and non-diabetic controls, only non-diabetic male subjects had a higher BMI than the non-diabetic females. There was no gender-related difference in BMI in T2DM patients. This likely emphasizes again the effect of testosterone on leptin concentrations (Nystrom *et al.*, 1997), which appears to overpower any BMI-related effects. Strikingly, apart from this gender-related difference, analyses of BMI measurements according to diabetic or periodontal status

exactly mimmick the findings for serum leptin concentrations. Thus, as observed for serum leptin concentrations, male and female T2DM patients had a significantly higher BMI than non-diabetic controls and with exception of male periodontally healthy subjects, this remained true when analysed according to the periodontal status. Likewise, as observed for serum leptin concentrations, in comparison to periodontal health BMI increased in male but not in female T2DM patients with gingivitis and periodontitis and no differences according to periodontal status were found in male or female non-diabetic controls. Moreover, BMI was determined as a significant predictor of gingivitis and periodontitis in males but not in females, which again exactly replicates the findings for serum leptin concentrations. The strong association of serum leptin with BMI therefore makes it impossible to distinguish between true effects of changes in serum leptin concentrations on periodontal status or the alternative that leptin merely is a surrogate measure for changes in BMI and itself has no direct impact on diabetes and risk for periodontal disease.

It should be noted that several studies have reported a decrease in GCF leptin concentrations with increased periodontal tissue destruction in comparison to healthy sites (Johnson and Serio, 2001; Bozkurt *et al.*, 2006; Karthikeyan and Pradeep, 2007), which were interpreted as a local protective role of leptin and might be independent of BMI differences between groups. However, again these studies do not acknowledge any gender differences and except for Karthikeyan and Pradeep (2007) do not match their study group according to BMI. It is not known to date if GCF leptin concentrations are effected by gender or BMI and any conclusions drawn from these studies therefore are disputable. Within the scope of the present study, it was not possible to analyse GCF leptin concentrations due to the limited amount of sample available. To gain insight into

local events in the periodontium, this should be addressed in future studies. Furthermore, to reveal any true effects of both serum and GCF leptin on periodontal status, any future studies should be on a BMI-matched study group.

Although the present study did not detect any significant differences in serum leptin concentrations between T2DM patients and non-diabetic controls after periodontal treatment, Kardesler *et al.* (2010) reported an increase in serum leptin concentrations in T2DM patients and a decrease in non-diabetic individuals after periodontal treatment. The authors did not offer an explanation for the increased serum leptin concentrations seen in T2DM patients after periodontal treatment and again the total neglect of gender differences in this study makes this finding somewhat questionable. The effect of periodontal treatment on serum leptin concentrations should therefore be evaluated in future studies in a BMI-matched study group and with consideration of gender differences.

Comparable to leptin, female subjects also had higher serum adiponectin concentrations than male subjects, regardless of the diabetic status. This gender difference has been reported previously (Arita *et al.*, 1999) and is a result of a negative correlation of adiponectin with testosterone (Bottner *et al.*, 2004). Although diabetes is associated with decreased serum adiponectin concentrations (Hotta *et al.*, 2000), a reduction in serum adiponectin in T2DM patients in comparison to non-diabetic controls was only observed as a trend in the present study. However, adiponectin is strongly associated with insulin (Hotta *et al.*, 2000) and diabetes medication has been reported to alter adiponectin concentrations, activating the adiponectin promoter and thereby leading to increased adiponectin expression (Okamoto *et al.*, 2006). Possibly a similar interference was encountered in the present study. In addition, as reported by others (Arita *et al.*,

1999; Merl *et al.*, 2005), the present study identified that adiponectin showed a weak negative correlation with BMI and linear regression analysis revealed a trend for BMI to predict serum adiponectin concentrations in both males and females. In comparison to leptin, the association of serum adiponectin with BMI does not appear to be as strong as that between leptin and BMI. This is further emphasized by the fact that in contrast to leptin, serum adiponectin concentrations did not differ according to periodontal status in either T2DM patients or non-diabetic controls or between the groups. Comparisons of non-diabetic individuals with and without periodontitis by Furugen *et al.* (2008) and Saito *et al.* (2008) are in agreement with the findings of the present study. In contrast to findings of the present study, Kardesler *et al.* (2010) noted increased serum adiponectin concentrations in T2DM patients with periodontitis in comparison to non-diabetic controls with periodontitis. However, as mentioned before, Kardesler *et al.* (2010) do not acknowledge any gender differences. Moreover, they neglected to correct for the considerable necessary dilution (1:2000) to assay serum adiponectin samples and thus any conclusions regarding serum adiponectin concentrations in diabetes and periodontitis from this study remain questionable. No other studies have reported on serum adiponectin concentrations in periodontal disease and results of the present study do not provide any evidence to support a role for serum adiponectin as an immunological link between diabetes and periodontal disease. Also, periodontal treatment did not affect serum adiponectin concentrations and this is in agreement with the study of Behle *et al.* (2009) on periodontal treatment and serum adiponectin concentrations. Nonetheless, Matsumoto *et al.* (2009) show increased serum adiponectin concentrations after periodontal treatment in T2DM patients while Kardesler *et al.* (2010) report a decrease. In addition, Kardesler *et al.* (2010) described increased serum adiponectin concentrations in non-diabetic individuals after periodontal

treatment. Possibly the different results between the studies are explained by differences in periodontal treatment. While the present study used non-surgical treatment, Matsumoto *et al.* (2009) provided specific antimicrobial treatment. It is not clear exactly what periodontal treatment was done by Kardesler *et al.* (2010) and it is likely that other factors such as the study group selection or treatment times could impact on serum adiponectin concentrations as well.

A number of correlations between serum adipokines and BMI with well established markers of glycaemic control and inflammation were investigated in the present study. Thus, irrespective of gender, leptin showed a positive correlation with HbA1c and hsCRP. A bidirectional feedback loop between serum leptin and insulin concentrations was proposed by Kieffer and Habener *et al.* (2000) and a study by Fischer *et al.* (2002) demonstrated a positive association of serum leptin with insulin and insulin resistance in T2DM patients. In contrast, studies investigating a correlation between leptin and HbA1c showed a negative association (Moriya *et al.*, 1999) or no correlations (Ozata *et al.*, 2001; Buyukbese *et al.*, 2004) between serum leptin levels and HbA1c concentrations in T2DM. However, it should be noted that these studies evaluated fasting leptin and HbA1c concentrations at a controlled time-point, whereas serum samples of the present study were obtained under non-fasting conditions, at any time throughout the day. Leptin secretion follows a distinct diurnal pattern (Sinha *et al.*, 1996; Licinio *et al.*, 1997) and is also influenced by food consumption (Elimam and Marcus, 2002). It is therefore possible that different study designs potentially yield different outcomes for a correlation between leptin and HbA1c. Also, the correlation of leptin and HbA1c observed in the present study was weak ($\rho < 0.3$ and < 0.4 for males and females, respectively) and the difference to findings of other studies should not be

overrated. The positive correlation of leptin with hsCRP was stronger ($\rho > 0.4$) and is confirmed by several studies in the literature (Kazumi *et al.*, 2003; Shamsuzzaman *et al.*, 2004; Viikari *et al.*, 2007) and potentially supports the pro-inflammatory role of this adipokine. This is further acknowledged by Chen *et al.* (2006) who demonstrated a direct effect of leptin in inducing CRP secretion in human primary hepatocytes. As seen throughout the present study, exactly the same positive correlations with HbA1c and hsCRP were observed for BMI, again highlighting the strong association between leptin and BMI.

In contrast, serum adiponectin showed a negative, albeit weak correlation with HbA1c and hsCRP. The negative correlation between adiponectin and HbA1c confirms findings of a recent meta-analysis which acknowledged adiponectin as a direct predictor of HbA1c concentrations (Wagner *et al.*, 2009). In addition, comparable to leptin a regulatory relationship between adiponectin and insulin was demonstrated previously (Combs *et al.*, 2004), emphasizing the link between adiponectin and parameters of glycaemic control. The majority of studies in the literature revealed a negative correlation of serum adiponectin concentrations with serum CRP levels in various inflammatory conditions (Higashiura *et al.*, 2004; Shetty *et al.*, 2004; Piestrzeniewicz *et al.*, 2009), which is in agreement with findings of the present study and could implicate an anti-inflammatory role for this adipokine.

Interestingly, in the present study adiponectin correlations with both HbA1c and hsCRP were only significant in males and not in females. It is possible that the gender difference in adiponectin concentrations impacts on correlations with HbA1c or hsCRP, however no studies in the literature compared adiponectin correlations with markers of glycaemic control or inflammation between genders and this remains to be elucidated.

In addition, the weak correlation of serum adiponectin with HbA1c and hsCRP should not be overrated.

To the best of my knowledge the present study is the first to investigate GCF adiponectin concentrations. In contrast to serum adiponectin, there was no gender difference in GCF adiponectin concentrations, no association with BMI, and BMI was not a predictor of GCF adiponectin. Although the composition of GCF is related to that of serum, GCF content is also reflective of local inflammatory status and the fact that GCF adiponectin levels were not influenced by gender or BMI support the contention that GCF adiponectin concentrations depend more on local tissue status than system status. This is further supported by the findings of significant differences in GCF adiponectin concentrations that, in contrast to serum adiponectin, were identified according to diabetic and periodontal status.

T2DM patients had lower GCF adiponectin concentrations than non-diabetic controls. This indeed might be explained by the strong association of adiponectin with insulin (Hotta *et al.*, 2000) and could be a result of the overall lower serum adiponectin concentrations observed in the T2DM patients. However, when analysed according to the periodontal status, this was not valid for all groups any longer. While GCF adiponectin concentrations from periodontal healthy and periodontal diseased T2DM patients remained lower than their non-diabetic counterparts, no difference in GCF adiponectin concentrations between T2DM patients with gingivitis and non-diabetic controls with gingivitis was detected. In comparison to periodontal health, GCF adiponectin concentrations consistently increased in T2DM patients with gingivitis and periodontitis but in the non-diabetic controls only individuals with periodontal disease had higher GCF adiponectin concentrations than periodontally healthy individuals.

There was no difference in GCF adiponectin concentrations between periodontal health and gingivitis in the non-diabetic controls.

Gingivitis can lead into periodontitis, however although both diseases involve inflammation of periodontal tissues, periodontitis is clearly distinguished from gingivitis by the occurring bone and attachment loss (Pihlstrom *et al.*, 2005). Adiponectin plays a role in the regulation of bone resorption (Yamaguchi *et al.*, 2008) and a negative correlation of serum adiponectin concentrations with bone mineral density has been described in both genders (Richards et al., 2007; Basurto et al., 2009). In contrast, adiponectin can also stimulate osteoblast proliferation (Berner *et al.*, 2004; Luo *et al.*, 2005) and actually osteoblasts have been identified as a local source of adiponectin (Berner *et al.*, 2004). The homeostasis of bone metabolism in the periodontium is altered in periodontal disease but not in gingivitis (Schwartz *et al.*, 1997) and possibly this provides an explanation for the increased GCF adiponectin concentrations in non-diabetic controls with periodontitis in comparison to periodontally healthy individuals and non-diabetic controls with gingivitis. In T2DM patients the increased GCF adiponectin concentrations in periodontal disease in comparison to periodontal health might also be explained by the altered bone metabolism. However, this does not explain the increased GCF adiponectin concentrations in T2DM patients with gingivitis in comparison to periodontal healthy individuals. Yet considering the close association of adiponectin with insulin (Combs *et al.*, 2004), it is possible that either the diabetic state itself or diabetes treatment can impact on GCF adiponectin concentrations and this may cause the differences in comparison to the non-diabetic controls. Alternatively, the observed increase in GCF adiponectin concentrations could simply be an effect of an increased periodontal

epithelial surface area (PESA) (Nesse *et al.*, 2008). The epithelial surface area increases with the severity of periodontal disease due to increasing probing depths and attachment loss (Nesse *et al.*, 2008). Therefore, an increased surface area in periodontal disease could lead to an increase of serum adiponectin secretion or accumulation into the GCF. However, this does not explain why GCF adiponectin concentrations in T2DM patients were lower than in non-diabetic controls and also does not address the particular differences seen in the gingivitis group. Nonetheless, a correlation of GCF adiponectin concentrations with PESA data should be analysed in future studies to evaluate the potential of GCF adiponectin simply being a byproduct of increased PESA in periodontal disease.

In contrast to serum adiponectin, periodontal treatment had a marked effect on GCF adiponectin concentrations in both T2DM patients and non-diabetic controls. Periodontal treatment significantly reduced GCF adiponectin concentrations after 3 months and levels remained low after 6 months. Again, it is possible that PESA decreased with periodontal treatment and this in turn caused the decrease in GCF adiponectin. However, 3 months of periodontal treatment does appear as a too short time to have such a large impact on the PESA and it is entirely plausible that indeed GCF adiponectin plays a role in periodontal disease and is affected by treatment. Other explanations such as daily or seasonal changes in GCF adiponectin concentrations are unlikely to be of importance as the study group was recruited and treated throughout two years. Importantly, multinomial regression analysis revealed GCF adiponectin as an independent predictor for gingivitis and periodontitis and unlike BMI and leptin this was true for both genders. Therefore, GCF adiponectin concentrations potentially are a suitable biomarker for early detection of periodontal disease with the advantage of

being completely independent of confounding factors such as gender and BMI. The strong impact of periodontal treatment on GCF adiponectin concentrations also suggests a potential of using this adipokine as a therapeutic target and indeed adiponectin was successfully applied as an insulin sensitizer in diabetes treatment in animal models (Diez and Iglesias, 2003). However, the adipokine awaits approval in human clinical trials and its potential as a therapeutic target in periodontal disease will need to be addressed in future studies.

The question remains as to whether GCF adiponectin has beneficial or detrimental effects in the periodontium and whether it would be a key factor in the increased risk for periodontal disease observed in people with diabetes. Generally, adiponectin is regarded as an anti-inflammatory cytokine, suppressing LPS-induced NF-κB activation and pro-inflammatory cytokine production (Yokota *et al.*, 2000; Wulster-Radcliffe *et al.*, 2004; Ajuwon and Spurlock, 2005; Tsatsanis *et al.*, 2005; Yamaguchi *et al.*, 2005; Park *et al.*, 2008). Therefore, the reduced GCF adiponectin concentrations in diabetes potentially could alter immune-responses to periodontal pathogens and thereby increase the risk for periodontal disease. However, if this was the case higher GCF adiponectin concentrations should provide a protective effect yet increased GCF adiponectin concentrations are observed in both T2DM patients and non-diabetic controls with periodontal disease. Importantly, adiponectin has not only anti-inflammatory properties, it has also been associated with pro-inflammatory properties (Neumeier *et al.*, 2006; Haugen and Drevon, 2007). Adiponectin exists in a number of different isoforms and different isoforms have been linked with different functions and pro-or anti-inflammatory properties. For example, Neumeier *et al.* (2006) demonstrated a suppressive effect of LMW adiponectin on LPS-induced activation of NF-κB and IL-6

production in human monocytes while HMW had no effect. Furthermore, only LMW but not HMW adiponectin induced IL-10 production yet HMW adiponectin induced the production of IL-6 (Neumeier *et al.*, 2006). It is tempting to speculate that not only the overall GCF adiponectin concentrations change according to periodontal and diabetic status, but that the different conditions occur in line with a shift in the proportions of the different isoforms in total adiponectin. Indeed, such an adiponectin isoform shift has been observed in other studies. For example, obesity is associated with a lower HMW adiponectin (Schraw *et al.*, 2008) and a shift from LMW to HMW was noted during weight loss (Engl *et al.*, 2007). Differences in the GCF adiponectin isoform proportions in diabetes or periodontal disease could therefore have different effects on immune responses to periodontal pathogens and could provide a possible explanation for an increased risk for periodontal disease in diabetes. However, the different GCF adiponectin isoform concentrations and their effect on periodontal immune responses remain to be investigated in future studies.

In summary, the findings of the present study reveal the possibility that serum leptin plays a role in diabetes and periodontal disease, however the results need to be confirmed in a study with BMI-matched individuals. Furthermore, the present study does not support a significant role for serum adiponectin in diabetes and periodontal disease. In contrast, GCF adiponectin clearly differs according to diabetes and periodontal disease status and could be a potential link between the two diseases. In addition, the decrease in GCF adiponectin after periodontal treatment potentially makes this adipokine a therapeutic target for periodontal disease in diabetes.

Chapter 7 General discussion

Obesity is widely accepted as a major public health problem (Ogden *et al.*, 2006; Hyde, 2008) and a recent UK health survey projects a 98 % increase in the rate of obesity-related diabetes by 2050 (Brown *et al.*, 2010). Diabetes is associated with an increased incidence or severity of periodontal disease (Nelson *et al.*, 1990; Ryan *et al.*, 2003; Heitz-Mayfield, 2005; Moles, 2006). Given the predicted future increase in diabetes, this likely impacts on the future prevalence of periodontal disease and therefore understanding the mechanistic connections between the two diseases becomes of increasing importance for oral health care. Altered adipokine concentrations during obesity and diabetes potentially play a role in an immunological link between diabetes and periodontal disease. However, although pro-inflammatory adipokines such as TNF-α or IL-6 are clearly involved in both diseases (Nishimura *et al.*, 2007), the role of other adipokines such as leptin or adiponectin is largely unknown.

Results from *in vitro* experiments of the present study support a potential role for leptin in an immunological link between diabetes and periodontal disease. It was shown for the first time that leptin enhanced monocytic TNF-α secretion induced by LPS from the periodontal pathogen *P. gingivalis*. Furthermore, leptin increased the expression of TLR2 in monocytes, possibly through an upregulation of the TLR transcription factor PU.1. In addition, monocyte maturation and differentiation was observed after leptin stimulation. Together, these findings suggest that increased leptin concentrations during diabetes potentially pre-condition monocytes for an over reactive immune response towards periodontal pathogens, thereby increasing the risk for periodontal disease.

Yet, *in vivo* the situation appears to be more complex. As expected, serum leptin concentrations were increased in T2DM patients in comparison to non-diabetic controls. However, while leptin concentrations in male T2DM patients increased with gingivitis and periodontitis, no difference according to periodontal status was detected in female T2DM patients. Likewise, leptin was a predictor of the periodontal status only in males but not in females. In addition, differences in BMI between groups further complicated the *in vivo* situation. Thus, differences in BMI measurements exactly mimicked differences in leptin concentrations, making it impossible to distinguish between true effects of leptin on the periodontal status or simply a role for leptin as a bystander due to changes in BMI. The only other study to investigate serum leptin concentration in T2DM and periodontitis to date did not detect any significant differences in serum leptin concentrations between T2DM patients and non-diabetic controls with periodontitis (Kardesler *et al.*, 2010). However, the authors neither acknowledged gender or BMI differences, making any findings questionable. Studies of leptin levels in rheumatoid arthritis, a disease with a comparable inflammatory progression as periodontal disease, are inconsistent. Although some studies report increased serum leptin concentrations with increased disease severity (Bokarewa *et al.*, 2003; Toussirot *et al.*, 2005; Otero *et al.*, 2006; Targonska-Stepniak *et al.*, 2008), others detect no differences in serum leptin concentrations between subjects with and without rheumatoid arthritis (Anders *et al.*, 1999; Hizmetli *et al.*, 2007; Wislowska *et al.*, 2007).

Controversial findings between human studies, gender differences and the added complexity of the BMI show the difficulties of translating *in vitro* findings for leptin to the situation *in vivo* and highlight the importance of further investigating the potential role of leptin in diabetes and periodontal disease in a more extensive experimental

approach. This could possibly be achieved by repeating the clinical part of the present report with a BMI-matched study group, recruitment of specifically males or females and focusing on obtaining both serum and GCF leptin concentrations to assess a relationship between local and systemic changes in leptin concentrations. Apart from investigating leptin concentrations in diabetes and periodontal disease in a BMI-matched study population, animal models may provide a useful tool to further determine the role of leptin *in vivo*. A number of animal models have been used to investigate periodontal disease in obesity and diabetes (Pontes Andersen *et al.*, 2007). Of these, the *db/db* or *ob/ob* mice and the *fa/fa* rats are the most common ones. In general, studies in these animals show increased severity of periodontal disease in diabetes in comparison to non-diabetic controls (Ryan *et al.*, 1999; He *et al.*, 2004; Liu *et al.*, 2006). However, due to the lack of the leptin receptor (*db/db* mice, *fa/fa* rats) or the lack of leptin (*ob/ob* mice) findings of these experiments do not allow us to draw any conclusions about leptin and its potential role as a link between diabetes and periodontal disease. If anything, one could assume that leptin would have a protective role against periodontal disease in diabetes. Indeed, leptin is important in leukocyte development (Claycombe *et al.*, 2008) and in mounting an appropriate immune response against pathogens (Yang *et al.*, 1997; Loffreda *et al.*, 1998; Lord *et al.*, 1998). Thus, the lack of leptin or the inability to respond to leptin could impact on immune responses to periodontal pathogens and explain an increased risk for periodontal disease. Yet, leptin concentrations in diabetes are increased and while the complete lack of leptin may lead to an insufficient immune response to periodontal pathogens, too high leptin concentrations may induce an over reactive response.

A study by Pontes Andersen *et al.* (2006) used the type 2 diabetic Goto-Kakizaki (GK) rats as an alternative animal model to investigate diabetes and periodontal disease. The GK rats do not have a deficiency in leptin or the leptin receptor and spontaneously develop diabetes as a consequence of prolonged inbreeding (Goto *et al.*, 1976). Pontes Andersen *et al.* (2006) show increased periodontal disease in the diabetic animals in comparison to wild-type controls, confirming results achieved in the *db/db* and *fa/fa* models. However, the study fails to measure serum leptin concentrations and animals were not BMI matched. Interestingly, BMI of animals with diabetes and periodontal disease was actually lower than BMI of non-diabetic controls, which is in contrast to the situation generally observed in humans. Although periodontal disease was induced experimentally, Pontes Andersen *et al.* (2006) also observed a natural increase in spontaneous periodontal disease in diabetic animals in comparison to wild-type controls. This finding, together with the spontaneous diabetes development without implications for leptin, would make the GK rats a suitable model to further investigate the role of leptin in periodontal disease. In future studies, experiments from Pontes Andersen *et al.* (2006) should therefore be repeated using BMI matched animals to determine changes in leptin concentrations in diabetes and periodontal disease. Pontes Andersen *et al.* (2006), as well as undertaking studies in other animal models, exclusively used male animals to investigate diabetes and periodontal disease. However, the present study reveals a strong gender difference in leptin concentrations between males and females. Additionally, the present study only detects differences in serum leptin concentrations according to the periodontal status in males and not in females. A number of studies report gender differences in immune responses such as mitogen-induced T cell proliferation, mixed lymphocyte reactions and psychological stress-induced changes in leukocyte cell numbers and phagocytic capacity (Weinstein *et al.*,

1984; De la Fuente *et al.*, 2004; Stefanski and Gruner, 2006). It would therefore be desirable to not only investigate the role of leptin in diabetes and periodontal disease in the male GK model, but to repeat experiments in female GK rats.

The potential role of leptin to pre-condition monocytes for a more pro-inflammatory immune response towards periodontal pathogens could possibly be investigated with a different approach in rodent models. Circulating monocytes could be isolated from wildtype animals and cultured in conditions with high leptin concentrations. Monocytes could then be transferred back into monocyte-depleted wildtype animals and experimental periodontitis could be induced. Transfer of wildtype untreated monocytes would be used in a control group. In case that leptin pre-conditions monocytes for a more pro-inflammatory immune response towards periodontal pathogens, animals with leptin-treated monocytes should exhibit more severe periodontal disease than animals with untreated monocytes. This experimental approach would allow for direct conclusions for the effect of leptin on monocytes in periodontal disease independent from any interference of the BMI or the complete lack of leptin responses as seen in the *ob/ob*, *db/db* and *fa/fa* models.

The effect of leptin on monocyte immune responses and differentiation may actually even be of greater relevance in periodontal disease than determined in previous studies and in the present report. The present study reveals a leptin-induced upregulation of TLR2 expression and morphological changes in leptin-stimulated monocytes were observed. Yet, preliminary data from our laboratory also reveal a leptin-induced upregulation of CD16 expression in monocytes (Birch *et al.*, 2010 unpublished). The $CD14^{low}CD16^{+}$ monocytes are a mature monocyte subset, which is characterised by increased TLR2 expression and TNF-α production in response to LPS when compared

to $CD14^{+}CD16^{-}$ monocytes (Belge *et al.*, 2002). Importantly, it was demonstrated that the percentage of $CD14^{low}CD16^{+}$ monocytes is increased in periodontal disease (Nagasawa *et al.*, 2004). However, a relationship between changes in serum leptin concentrations in diabetes and periodontal disease and the percentage of circulating $CD14^{low}CD16^{+}$ monocytes remains to be elucidated.

Additionally, the effect of leptin on TLR2 expression should be further investigated *in vitro*. Using siRNA, knock-out of TLR transcription factors such as PU.1 and Sp1 could provide a direct mechanistic link between leptin and altered monocyte TLR2 expression. Alternatively, gene expression arrays may provide a useful tool to further identify target genes of leptin in myeloid cells. DNA microarrays have been applied in previous studies to investigate the effect of leptin on gene expression patterns in adipose tissue of *ob/ob* and other obesity models, as well as in humans (Soukas *et al.*, 2000; Lopez *et al.*, 2003; Taleb *et al.*, 2006). Other studies focused on gene expression patterns after leptin treatment in cancer or the hypothalamus (Fenton *et al.*, 2008; Perera *et al.*, 2008; Tung *et al.*, 2008), however no studies to date have analysed changes in gene expression patterns after leptin stimulation in immune cells. It should be noted that understanding of leptin signalling mechanism is not only relevant in a potential immunological link between diabetes and periodontal disease, but can be applied to a much wider field. Thus, since its discovery as a "satiety" hormone (Flier and Maratos-Flier, 1998; Friedman and Halaas, 1998), leptin was a target for obesity treatment in humans. However, although recombinant leptin was successful in treating human leptin-deficiency induced obesity, it had only marginal effects on humans with diet-induced obesity (Heymsfield *et al.*, 1999). It appears that humans with diet-induced obesity are unresponsive to increased leptin concentrations due to hypothalamic leptin resistance

and today, obesity research focuses on improving leptin sensitivity rather than using the adipokine itself for obesity treatment (Ozcan *et al.*, 2009). However, recently leptin also became a potential target for treatment of anorexia nervosa, congenital lipoatrophy or exercise-induced hypothalamic amenorrhea (Kelesidis *et al.*, 2010), yet more research is needed to fully understand leptin signalling and underlying mechanisms of leptin resistance in humans.

In addition to leptin, the present study investigated adiponectin concentrations in diabetes and periodontal disease. In contrast to leptin, no differences in serum adiponectin concentrations according to periodontal status were detected. However, a different picture emerged for GCF adiponectin concentrations. Not only did T2DM patients have lower GCF adiponectin concentrations than non-diabetic controls, GCF adiponectin concentrations actually increased in T2DM patients with gingivitis and periodontitis and in non-diabetic controls with periodontitis. Importantly, the present study also determined that GCF adiponectin concentrations were not affected by BMI or gender, allowing for broader conclusions to be made than for leptin. In contrast to serum leptin and adiponectin concentrations, GCF adiponectin concentrations were clearly affected by periodontal treatment. In both T2DM patients and non-diabetic controls, a significant decrease in GCF adiponectin was detected after treatment of periodontal disease. The treatment effect on GCF adiponectin concentrations suggests a potential for adiponectin as a therapeutic target in periodontal disease. Depending on the isoform, both pro-and anti-inflammatory properties have been associated with adiponectin. For example, LMW adiponectin suppressed LPS-induced activation of NF-κB and IL-6 production and induced the production of IL-10 in human monocytes (Neumeier *et al.*, 2006). In contrast, HMW adiponectin induced IL-6 production in

human monocytes but had no effect on the production of IL-10 (Neumeier *et al.*, 2006). In addition, a number of studies describe a role for adiponectin in bone homeostasis (Berner *et al.*, 2004; Luo *et al.*, 2005; Yamaguchi *et al.*, 2008), which likely is of particular relevance in periodontal disease. In support for a role of adiponectin in periodontal disease bone homeostasis, a study by Yamaguchi *et al.* (2007) shows that globular adiponectin inhibits osteoclast formation induced by the periodontal pathogen *Aggregatibacter actinomycetemcomitans* LPS in a mouse macrophage cell line. However, prior to further evaluation of adiponectin as a therapeutic target, it should be determined which GCF adiponectin isoform concentrations change in diabetes and periodontal disease or indeed which isoform is affected by periodontal treatment. Additionally, the role of different adiponectin isoforms on LPS-induced immune responses to periodontal pathogens such as *P. gingivalis* should be investigated *in vitro*. This could possibly be accomplished using a comparable experimental setup with human monocytes as described for the investigation of leptin in LPS-induced immune responses in the present study.

In conclusion, findings of the present study contribute to the general knowledge of the role of leptin in the immune system, in particular its role in monocyte differentiation and immune responses towards periodontal pathogens. Furthermore, the present report highlights the importance of studying adipokines in periodontal disease and diabetes. Specifically, leptin and adiponectin show potential as a relevant immunological link between diabetes and periodontal disease, with the consideration of adiponectin as a future therapeutic target for periodontitis treatment.

Chapter 8 Future work

A number of findings in the present report revealed opportunities for further research and this could be addressed in future studies.

1. Investigation of leptin intracellular signalling events with JAK2, PI3K/Akt and p38 inhibitors to determine mechanisms for the LPS-enhancing effect of leptin in monocyte TNF-α expression.

2. Further evaluation of an effect of leptin on TLR4 expression in primary human monocytes and analysis of TLR1 and TLR6 expression in leptin-stimulated THP-1 and primary human monocytes.

3. Analysing the effect of leptin on TLR2 expression in THP-1 monocytes after siRNA knock down of PU.1 to investigate a direct link between leptin, PU.1 and TLR2 expression.

4. Investigation of the effect of leptin on CD16 expression in primary human monocytes and correlation of serum leptin concentrations with the $CD14^{low}CD16^{+}$ monocyte subpopulation in T2DM patients with and without periodontal disease.

5. Analysis of adiponectin isoforms in serum and GCF of patients with T2DM before and after treatment of periodontal disease and investigation of the effect of adiponectin isoforms on monocyte immune responses to *P. gingivalis* LPS *in vitro*.

Chapter 9 References

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