

The role of IL-18 in cross-susceptibility between periodontal disease and diabetes mellitus

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Thesis submitted in partial fulfilment of the requirements of the regulations for the degree of Doctor of Philosophy

> Newcastle University School of Dental Sciences & Institute of Cellular Medicine May 2011

Table of Contents

Table of Cont	ents		I
List of Figure	s		V
Listof Tables.			VII
Acknowledge	ments		VIII
List of Abbre	viations		IX
Abstract			XIV
Chapter 1	Introd	luction	1
1.1	Periodo	ontal disease: aetiology and pathogenesis	
1.2	Diabete	es Mellitus: aetiology and pathogenesis	7
1.3	The rel	ationship between periodontal disease and diabetes	
1.4	Adipos	e tissue, adipokines, inflammation and immunity	
	1.4.1	The cellular components of adipose tissue	19
	1.4.2	Adipose tissue in the oral cavity	
	1.4.3	Leptin	
	1.4.3.1	Leptin receptors (ObR)	
	1.4.3.2	Leptin, immunity and inflammation	
	1.4.3.3	Leptin, diabetes and periodonial disease	
1.5	IL-18 a	nd periodontal disease	
	1.5.1	Structure and gene regulation of IL-18	
	1.5.2	The IL-18 receptor and mechanism of action	
	1.5.3	The proIL-18 synthesis and IL-18 processing	
	1.5.3.1	The inflammasomes	
	1.5.3.2	Pattern recognition receptors (PRKs)	
	154	Biological roles of II -18 and clinical relevance	
	1.5.1	II -18 in diabetes and obesity	45
	1.5.5	II -18 in periodontal diseases	46
16	Aime		
1.0	AIIII5	•••••••••••••••••••••••••••••••••••••••	
Chapter 2	Mater	ials and Methods	51
2.1	Cell cul	lture	51
	2.1.1	THP-1 monocytes	51
	2.1.1.1	Recovery of THP-1 monocytes	53
	2.1.1.2	Cell culture of THP-1 monocytes	
	2.1.1.3 2 1 1 4	Vitamin D3 treatment of THP-1 monocytes	
	2.1.1.4	Freezing of THP-1 monocytes	
	2.1.2	Isolation and culture of primary human monocytes	
	2.1.2.1	Magnetic bead method for isolation of monocytes	55
	2.1.2.2	Assessing purity of human primary monocytes	56
2.2	Reagen	ts	61
	2.2.1	LPS	61
	2.2.2	Leptin	61
	2.2.3	Stimulation of monocytes with LPS or leptin	61
	2.2.4	Testing for endotoxin contamination	

	2.2.4.1 2.2.4.2	Evaluation of mitogenic or cytotoxic effects of reagents The mitogenic and cytotoxic effect of leptin and LPS	62 65
	2.2.5	ATP and inhibitors	67
2.3	Enzym	e-linked immunosorbent assays (ELISA)	71
	2.3.1	Development of IL-18 ELISA	71
	2.3.2	IL-8, IL-6, IL-1 β and TNF- α ELISA	72
2.4	mRNA	analysis	79
	2.4.1	Analysis of mRNA expression	79
	2.4.2	RNA extraction	79
	2.4.3	The cDNA synthesis by reverse transcription	80
	2.4.4	The conventional RT-PCR	81
	2.4.5	Real-Time RT-PCR	83
2.5	Determ	nination of caspase-1 activity	
2.6		ioprecipitation and Western blot	89
	2.6.1	Lysis of cells	89
	2.6.2	Bradford protein determination assay	89
	2.6.3	Immunoprecipitation	92
	2.6.4	western blot analysis	92
2.7	ATP de	etermination assay	94
2.8	Subject	t population	96
	2.8.1	Clinical assessments and periodontal treatment	98
	2.8.2	Sample collection	99
2.9	Statisti	cal analyses	101
	2.9.1	Analysis of cell culture data	101
	2.9.2	Analysis of clinical data	101
Chantar 3	Analy	sis of II 18 concentrations in type 2 diabetes	
Chapter 3	and ne	priodontal disease	103
2.1			103
3.1	Introdi Results	1Ction	103
3.4	3 2 1	II -18 FI ISA Development	107
	3.2.1.1	Developing the ELISA to increase sensitivity	1107
	3.2.1.2	Intra-and inter-assay variation of IL-18 ELISA	115
	3.2.1.3	Validation of IL-18 ELISA for serum samples	118
	3.2.1.4	Spike/recovery assay	118
	3.2.2	IL-18 in serum and GCF samples from T2DM and	101
	2 2 2 1	periodontal disease	121
	3.2.2.1	Analysis of serum and GCF IL-18 concentrations in T2DM subjects and non-diabetic controls with and without periodontal	121
	3000	Analysis of serum II -18 concentrations in T2DM subjects and	121
	3.2.2.2	non-diabetic controls with periodontitis after periodontal treatment	126
	3.2.3	The relationship between serum IL-18 and clinical	100
		parameters of 12DM	128
3.3	Discuss	sion	132

Chapter 4	An i	nvestigation into the effect of leptin on IL-18	
	expre	ssion and secretion in monocytes141	
4.1	Introd	Introduction141	
4.2	Result	s145	
	4.2.1	Investigation of the effect of leptin on IL-18 release in	
	1 2 2	human monocytes	
	4.2.2	The effect of leptin on IL-18 mRNA expression in THP-1	
	121	Interestigation of the effect of leptin on II -18 II -6 and II -	
	4.2.4	8 secretion in THP-1 monocytes 154	
	4.2.5	Investigation of the effect of leptin on II -18 expression in	
	11210	THP-1 monocytes	
	4.2.6	Leptin enhances IL-18 release in <i>P. gingivalis</i> LPS treated	
		cells but not in <i>E.coli</i> LPS treated cells	
4.3	Discus	ssion	
	Discus		
Chapter 5	5 Analy	vsis of caspase-1 inflammasome activation in	
_	leptin	-stimulated monocytes171	
5 1	- Introd	luction 171	
5.1	Result	171 rs 178	
	5.2.1	The investigation of the effect of leptin on the expression	
	01211	of the inflammasome components	
	5.2.2	The effect of leptin on the caspase-1 activity	
	5.2.3	Investigation of the effect of leptin on caspase-1 activation 184	
	5.2.4	The effect of leptin on caspase-1 p20 subunit	
	5.2.5	The inhibition of leptin-induced IL-18 release by caspase-	
		1 inhibitor (Ac-YVAD) 192	
	5.2.6	The analysis of IL-18 processing after leptin stimulation 194	
5.3	Discus	ssion	
	- · ·		
Chapter of) An 11	nvestigation into signalling mechanisms that	
	regula	ate leptin-induced IL-18 release in monocytes 203	
6.1	Introd	luction	
6.2	Result	s	
	6.2.1	Analysis of ObR mRNA expression in THP-1 monocytes 206	
	6.2.2	The effect of JAK-2 inhibitor on leptin-induced IL-18	
	())	release	
	6.2.3	The effect of ATP on IL-18 release in monocyte treated	
	674	The determination of endogenous ATD production in	
	0.2.4	leptin treated monocytes 215	
	625	The effect of P2X7R inhibitor (PPADS) on lentin-induced	
	0.2.3	II -18 release	
	D!	21/	
6.3	Discus	ssion	

Chapter 7 Gene	eral discussion	
Future work		
References	••••••	

List of Figures

Figure 1.1 The basic structure of inflammasome	39
Figure 1.2 The activation of the NLRP3 inflammasome	43
Figure 2.1 The morphological characteristics of THP-1 monocyte cell line	52
Figure 2.2 The purity of monocytes isolated by magnetic bead method	59
Figure 2.3 TNF-α production in human primary monocytes isolated by magnetic bead method.	60
Figure 2.4 Standard curve of the Cell Titer 96 cell proliferation assay	64
Figure 2.5 The effect of leptin on cell proliferation in THP-1 monocytes after 6 h	66
Figure 2.6 The cytotoxicity of caspase-1 inhibitor Ac-YVAD on THP-1 monocytes after 6 h	69
Figure 2.7 The cytotoxicity of JAK-2 inhibitor (AG490) and P2X7 receptor inhibitor PPADS on THP-1 monocytes after 3 h	r 70
Figure 2.8 Standard curve of the IL-8 ELISA	75
Figure 2.9 Standard curve of the IL-1β ELISA	76
Figure 2.10 Standard curve of the IL-6 ELISA	77
Figure 2.11 Standard curve of the TNF- α ELISA	78
Figure 2.12 The basic principle of PCR analysis	84
Figure 2.13 The standard curve of caspase-1 assay	88
Figure 2.14 The standard curve of Bradford protein determination assay	91
Figure 2.15 The standard curve of ATP determination assay	95
Figure 2.16 A summary of study flow chart	100
Figure 3.1 The standard curve of IL-18 ELISA	109
Figure 3.2 Each graph shows a different CAb concentration paired with a different DAb concentration.	111
Figure 3.3 A representative standard curve of IL-18 ELISA	113
Figure 3.4 Serum IL-18 concentrations between non-diabetic control subjects and T2DM subjects	123
Figure 3.5 Serum IL-18 concentrations according to periodontal status in non-diabet control subjects and T2DM subjects	tic 124
Figure 3.6 The effect of periodontal treatment on serum IL-18 concentrations in nor diabetic control subjects and T2DM subjects	ı- 127
Figure 3.7 The relationship of serum IL-18 with HbA1c	129
Figure 3.8 The relationship of serum IL-18 with hsCRP	130
Figure 3.9 The relationship of serum IL-18 with BMI	131
Figure 4.1 Kinetics of IL-18 secretion in response to <i>E.coli</i> LPS and leptin	148

Figure 4.2	Dose response of IL-18 secretion to leptin stimulation in THP-1 monocytes
Figure 4.3	Leptin induced IL-18 production in primary monocytes
Figure 4.4	The effect of leptin on IL-18 mRNA expression in THP-1 monocytes 152
Figure 4.5	IL-18 mRNA expression by THP-1 monocytes after stimulation with <i>E.coli</i> LPS and leptin
Figure 4.6	The effect of leptin on IL-1β secretion in THP-1 monocytes
Figure 4.7	The effect of leptin on IL-6 secretion in THP-1 monocytes
Figure 4.8	The effect of leptin on IL-8 secretion in THP-1 monocytes157
Figure 4.9	The effect of leptin on IL-1 β mRNA expression in THP-1 monocytes 159
Figure 4.1	0 IL-1β mRNA expression by THP-1 monocytes after stimulation with <i>E.coli</i> LPS and leptin
Figure 4.1	Pre-treatment monocyte with leptin enhances IL-18 release in response to <i>P.gingivalis</i> LPS but not to <i>E.coli</i> LPS
Figure 5.1	Effect of leptin on mRNA expression of caspase-1 inflammasome components in THP-1 monocytes
Figure 5.2	NLRP3 mRNA expression in THP-1 monocytes after stimulation with <i>E.coli</i> LPS and leptin
Figure 5.3	Leptin as well as <i>E.coli</i> LPS and <i>P.gingivalis</i> LPS up-regulate caspase-1 activity
Figure 5.4	Western blot analysis for caspase-1 p10 subunit187
Figure 5.5	Immunoprecipitation/Western blotting for caspase-1 p10 subunit
Figure 5.6	Western blot analysis for an active caspase-1 p20 subunit191
Figure 5.7	IL-18 secretion in leptin treated cells is significantly blocked by caspase-1 inhibitor (Ac-YVAD)
Figure 5.8	The effect of leptin on IL-18 processing in human monocytes 195
Figure 6.1	The ObR mRNA expression in THP-1 monocytes
Figure 6.2	The effect of JAK-2 inhibitor on leptin-induced IL-18 release
Figure 6.3	IL-18 secretion in leptin treated cells is significantly enhanced by ATP213
Figure 6.4	The effect of ATP on leptin-induced IL-18 release in primary monocytes 214
Figure 6.5	Leptin induces the production of endogenous ATP in THP-1 monocytes. 216
Figure 6.6	The effect of ATP inhibitor (PPADS) on <i>E.coli</i> LPS-induced IL-18 release
Figure 6.7	The effect of ATP inhibitor (PPADS) on leptin-induced IL-18 release 220
Figure 7.1	The schematic diagram of proposed effect of leptin on IL-18 production. 234

List of Tables

Table 1.1 Summary of the immunological role of leptin 26
Table 1.2 The role of IL-18 in periodontal disease 47
Fable 2.1 Working concentrations and specifications for flow cytometry antibodies 58
Fable 2.2 Antibody working concentrations and standard curve detection ranges for ELISAs 74
Fable 2.3 List of primers used in the RT-PCR analyses 82
Fable 2.4 Demographics of the study population
Table 3.1 The sensitivity of IL-18 ELISA from three independent ELISAs
Fable 3.2 Intra-assay variation of IL-18 ELISA 116
Fable 3.3 Inter-assay variation IL-18 ELISA 117
Table 3.4 Spike/recovery and linearity of IL-18 ELISA for human serum samples 120
Table 3.5 The GCF IL-18 concentrations in T2DM patients and non-diabetic controls with different periodontal status

Acknowledgements

I would sincerely like to express my gratitude to my supervisors Dr. John Taylor and Prof. Philip Preshaw not only for their constructive advice and discussions but also for their support and encouragement throughout my academic programme. I also wish to thank the clinical team Rebecca Wassall, Susan Bissett, Hannah Fraser and Kerry Stone for their contribution in the collection of clinical data and samples.

I would like to extend my grateful thanks to Dr. Jared Thornton and Dr. Katrin Jeadicke for their helpful technical discussions especially about Western blot analysis and other experimental setup. In addition, thanks are also due to Dr. Amy Anderson, Dr.Rachel Harry from the musculoskeletal group for the help in isolation of primary monocytes. Furthermore, I would like to acknowledge David McGeeney who is a statistician in School of Mathematics & Statistics for his useful advice on the statistical analysis.

I am very thankful to my colleagues and friends in the Oral Biology Research lab, namely Aisha, Katrin, Rana, Lesley, Chris, Emma, Jared, Helen, Wiz, Ryan and Rob for their support and making such a great working environment. Without them, I could not have completed this project. I would also like to offer my regards and blessings to all of those in Oral Biology department who supported me in any respect during the completion of the project.

I would like to express my deepest appreciation to Anandhamahidol foundation for the scholarship. Special thanks go to P'POP and P'Manida for all the financial support.

Finally, I would like to gratefully thank my parents, brothers and sister for all their endless love, caring and support. Thank you very much for believing in me and encouraging me to continue my education.

List of Abbreviations

ADA	American Diabetes Association
AGEs	advanced glycation end-products
AIM	absent in melanoma
AP-1	activator protein-1
ASC	apoptosis-associated speck-like protein containing a CARD
ATMs	adipose tissue macrophages
BAT	brown adipose tissue
BMDMs	bone marrow-derived macrophages
BMI	body mass index
BSA	bovine serum albumin
CAb	capture antibody
CARD	caspase-recruitment domain
CCLs	CC-chemokine ligands
CNTF	ciliary neurotrophic factor
ConA	concanavalin A
COX	cyclooxygenase
CRP	C-reactive protein
Ct	cycle threshold
DAb	detection antibody

DAMPs	danger associated molecular patterns
DCs	dendritic cells
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assays
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
GCF	gingival crevicular fluid
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
HbA1c	haemoglobin A1c
hBD	human beta-defensin
ICAM-1	intracellular adhesion molecule-1
ICE	IL-1β converting enzyme
ICSBP	interferon consensus sequence-binding protein
IFN	interferon
IGIF	IFN-γ inducing factor
IKK	inhibitor of kB kinase
IL	Interleukin
IL-18BP	IL-18 binding protein

IL-18BPa	IL-18 binding protein antagonist
IL-18R	IL-18 receptor
IL-1Ra	IL-1R antagonist
IQR	inter-quartile range
IRAK	IL-1 receptor-activating kinase
IRS	insulin receptor substrate
JAK	Janus kinases
JNK	Jun N-terminal kinase
LAL	Limulus amebocyte lysate
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
LRRs	leucine-rich repeats
MAMPs	microbe-associated molecular patterns
МАРК	mitogen-activated protein kinase
МСР	monocyte chemoattractant protein
MMPs	matrix metalloproteinases
MSU	monosodium urate
NF-κB	nuclear factor-ĸB
NHANES	National Health and Nutrition Examination Study
NIK	NF-κB binding kinase
NK cells	natural killer cells

NLRs	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
NOS	nitrix oxide synthase
ObR	leptin receptor
P2X7R	P2X7 receptor
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGE	prostaglandin E
РНА	phytohemagglutinin
PI3K	phosphatidylinositol 3-kinase
PMNs	polymorphonuclear neutrophils
PPADS	pyridoxal phosphate-6-azo tetrasodium salt hydrate
PRRs	pattern recognition receptors
PYD	pyrin domains
RAGEs	receptor for advanced glycation end-products
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIG-I	retinoic acid inducible gene-I
RLRs	RIG-I-like receptors
ROS	reactive oxygen species
RT-RCR	Reverse transcription polymerase chain reaction
SD	standard deviation

STAT	signal transducers and activators of transcription
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
Th	T helper cell
TLRs	Toll-like receptors
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
Treg	regulatory T cells
VitD3	vitamin D3
WAT	white adipose tissue
WHO	World Health Organization

Abstract

IL-18 is a cytokine with a number of important actions central to innate and adaptive immune responses. There is some evidence that IL-18 is elevated in gingival crevicular fluid (GCF) and associated with the severity of periodontal disease. In addition, the proinflammatory adipokine leptin is elevated in type 2 diabetes mellitus (T2DM) and obesity. The action of leptin may be one factor which underpins the cross-susceptibility between these disorders and chronic inflammatory diseases such as periodontal disease. However, the relationship between IL-18 and leptin in periodontal diseases still remains to be determined. Therefore, the objective of this study was to determine the serum concentrations of IL-18 in periodontal disease subjects with and without T2DM. In addition, the present study aimed to investigate how IL-18 is regulated by leptin in human monocytes, as well as the extent to which caspase-1 plays a role in this pathway.

The present study demonstrated for the first time that serum IL-18 levels were significantly elevated in T2DM patients with periodontitis compared to periodontally healthy individuals. The levels of serum IL-18 are positively correlated with HbA1c and BMI but not with hsCRP levels. *In vitro*, leptin was found to significantly increase IL-18 secretion by THP-1 and primary human monocytes but had no effect on IL-18 mRNA expression. Activation of JAK-2 tyrosine kinase is a primary event in leptin receptor (ObRb) signalling and co-incubation of monocytes with the JAK-2 inhibitor AG490 significantly reduced leptin-induced IL-18 release. Interestingly, addition of exogenous ATP (a known inflammasome-activating signal) significantly enhanced IL-18 release in leptin-stimulated monocytes. These data suggest that leptin stimulates IL-18 via JAK-2 signalling and this is the result of activation of IL-18 processing rather than IL-18 transcription. The present study provided further evidence to support this

hypothesis: leptin up-regulated caspase-1 activity as demonstrated by chromogenic peptide assays, Western blot analysis revealed that leptin modulated the levels of active caspase-1 p20 and the effect of leptin on IL-18 release was inhibited by a caspase-1 inhibitor (Ac-YVAD-cmk). Leptin alone was also found to stimulate endogenous ATP release from monocytes which suggests one possible mechanism for inflammasome activation by this adipokine.

In conclusion, the present study demonstrates a novel role for leptin in immune responses by monocytes. The up-regulation of serum IL-18 levels may be directly relevant to periodontal destruction in diabetic individuals. Leptin enhances IL-18 secretion via modulation of the caspase-1 inflammasome function and this process may contribute to the cross-susceptibility between T2DM and periodontal diseases.

Chapter 1 Introduction

Diabetes mellitus (DM) is a potential host response stressor in periodontal disease. Many studies have recognized that periodontitis is more prevalent and severe in diabetic patients (Page and Beck, 1997; Torrungruang et al., 2005; Mealey and Ocampo, 2007; Taylor and Borgnakke, 2008; Genco, 2009; Preshaw, 2009; Um et al., 2010). In fact, periodontal disease has been classified as the sixth complication of diabetes and it is widely accepted that diabetes is an important risk factor for periodontal disease (Southerland et al., 2006; Chavarry et al., 2009). The interplay between diabetes and periodontitis has become an area of intensive research. However, the precise pathological mechanisms underlying how diabetes contributes progression of periodontal disease are not fully understood.

Cytokines are central to the function of both innate and adaptive immune responses. It has been suggested that imbalance or inappropriate production of cytokines may underpins the cross-susceptibility between periodontal disease and diabetes (Barksby et al., 2007; Preshaw et al., 2007; Preshaw, 2009). Several studies supported that development of diabetes is mediated by inflammation and alteration in immune responses (Dandona et al., 2004; King, 2008; Preshaw, 2009). For example, the function of neutrophils is impaired in patients with diabetes (Graves et al., 2006) whereas monocytes exhibit an hyperresponsive activity with excessive production of proinflammatory mediators such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α) and IL-6 (Lalla et al., 2001). It has also been reported that IL-6 and TNF- α levels are elevated in serum of individuals with diabetes (Dandona et al., 2004; Sun et al., 2010). Interleukin-18 (IL-18), a member of IL-1 cytokine family, is a pro-inflammatory cytokine that is important in the regulation of T-cells. Uncontrolled production of IL-18 is associated with Th1-immunopathologies and destructive inflammatory disease (Dayer, 1999). IL-18 is elevated in obesity and metabolic syndrome (Hung et al., 2005), type 2 diabetes (Esposito et al., 2003b; Hivert et al., 2009) and inflammatory diseases such as rheumatoid arthritis (Moller et al., 2001) and periodontal disease (Miranda et al., 2006). In fact, it was demonstrated that IL-18 concentrations are higher than IL-1 β concentrations in GCF from periodontitis patients (Orozco et al., 2006). Recent studies demonstrated that the levels of GCF IL-18 were higher in patients with chronic periodontitis compared with patients with gingivitis (Figueredo et al., 2008b; Pradeep et al., 2009c). In addition, GCF IL-18 levels in subjects with periodontitis are decreased after periodontal therapy (Pradeep et al., 2009c). Moreover, a more recent study reported a significant positive correlation between serum IL-18 levels and periodontal inflammation and clinical attachment loss in patients who had periodontitis and coronary artery disease (Schallhorn et al., 2010).

Although many studies have demonstrated an individual relationship between IL-18 levels and diabetes or periodontal disease, the knowledge regarding the role of IL-18 in periodontal disease associated with diabetes is still limited. The purpose of the present study is therefore to investigate the immunological role of IL-18 in the cross-susceptibility between periodontal disease and diabetes.

1.1 Periodontal disease: aetiology and pathogenesis

Periodontal disease is a group of chronic inflammatory diseases that affect the supporting tissues of the dentitions. Epidemiological studies have shown that some forms (eg. gingivitis) of periodontal diseases in the adult can remain stable over many years and not lead to periodontal destruction, whereas other forms (eg. aggressive periodontitis), despite extensive treatment, continue to break down, leading ultimately to tooth loss (Seymour and Gemmell, 2001). Gingivitis and periodontitis are the two common forms of periodontal diseases. Gingivitis, a more stable form, is an inflammatory condition of the soft tissue surrounding the teeth (the gingiva) without the involvement of the attachment apparatus whereas periodontitis involves the deeper periodontial ligament, cementum and alveolar bone (Kinane, 2001). Periodontal diseases are a global public health problem (Albandar and Rams, 2002), and in particular periodontitis, which is a major cause of adult tooth loss (Papapanou, 1996).

Gingivitis was previously believed inevitable following the formation of microbial plaque on teeth. It is now accepted that certain individuals will be more susceptible than others to gingivitis and indeed periodontitis (Kinane and Bartold, 2007). The classic study in 1965 first described the development of gingivitis (Loe et al., 1965). The gingivitis lesion occurs within the first four days following the accumulation of dental plaque. Plaque bacteria release products which induce tissue inflammation directly and indirectly, leading to the formation of oedema, an increase in gingival crevicular fluid, an accumulation of polymorphonuclear leukocytes and loss of connective tissue (Page and Schroeder, 1976). The clinical signs of gingivitis become more apparent when

plaque is allowed to accumulate but the inflammatory changes can be resolved when adequate oral hygiene is resumed.

The pathogenesis of periodontitis is more complex than the pathogenesis of gingivitis. Gingivitis is a prerequisite for the development of periodontitis but not all patients with gingivitis will develop periodontitis. Advanced/severe periodontitis is reported in only a fraction of the population (10–15%) (Kinane and Attstrom, 2005; Kinane and Bartold, 2007) suggesting the importance of individual susceptibility to this disease.

The development of periodontitis is associated with a specific group of predominantly Gram-negative, anaerobic or microaerophilic bacteria. They colonize on the root surface in the subgingival area (Page and Kornman, 1997). The key periodontal pathogens in chronic periodontitis are *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* whereas those in localized aggressive periodontitis are *Aggregatibacter* (formerly termed *Actinobacillus*) *actinomycetemcomitans* (Van Dyke and Serhan, 2003).

Although bacteria are essential, the bacteria alone are not sufficient for the disease to occur. It is now well- recognized that host responses to the periodontal pathogens and their virulence factors play an important role in the pathogenesis of periodontitis (Gardy et al., 2009). After colonization on the gingival sulcus by periodontal bacteria, the bacteria release their products, for example, lipopolysaccharide (LPS) which is a well known virulence factor of gram-negative bacteria. LPS has the potential to interact with nearly all cell types in periodontal tissues including immune cells and resident cells and thereby initiating a defence mechanism. The initial immune response in periodontal disease is characterized by the action of the innate immune system which, in this context, consists of the gingival epithelium, fibroblasts, neutrophils, dendritic cells, and monocytes/macrophages (Teng, 2006). In fact, innate host recognition of LPS is a key

initiating event for the subsequent clearance of gram-negative bacteria from infected host tissues (Jain and Darveau, 2010). A group of receptors called pattern recognition receptors (PRRs) which include cell surface Toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs) are responsible for the detection of microbe-associated molecular patterns (MAMPs), i.e. LPS, and thereby leading to cellular activation. To date, a number of studies have demonstrated how periodontal pathogens, in particular *P. gingivalis* signal via TLRs (Pathirana et al., 2010; Taylor, 2010). However, little knowledge is yet known how NLRs sense oral bacteria. A recent study (Bostanci et al., 2009) revealed that the NLRP3 and NLRP2 mRNA expression are significantly increased in gingival tissues affected by periodontal disease compared to healthy ones. The same study also found that *P. gingivalis* culture supernatant up-regulates the NLRP3 mRNA expression in Mono-Mac-6 monocytic cells.

In the gingival epithelium, the binding of LPS to TLR on nearby cells induces the production of cytokines and chemokines resulting in the expression of adhesion molecules, increased permeability of gingival capillaries and chemotaxis of polymorphonuclear neutrophils (PMNs) through the junctional epithelium and into the gingival sulcus to phagocytose bacteria. One important component of innate immunity that plays a vital role in periodontal disease is monocytes. In response to inflammatory signals, monocytes can migrate quickly to sites of infection in the tissues and differentiate into macrophages which can effectively capture invading pathogens. The phagocytosis of bacteria by macrophages results in cytokine secretion and antigenpresentation to induce a more effective adaptive immunity (Teng, 2006; Liu et al., 2010).

Later on, if the plaque biofilm matures further, the pathogenic species developing in the periodontal pockets release an array of virulence factors, antigens or by products, particularly LPS, into the pocket junctional epithelium, blood vessels and deeper connective tissues of periodontium (Socransky and Haffajee, 1991; Slots and Ting, 1999). This leads to a chronic inflammatory response characterized by dysregulation of immune-bacteria interactions where the infected tissues/cells are overwhelmed by the persistent pathogens accompanied with continuous and/or excessive production of potent proinflammatory cytokines (i.e. IL-1 β , TNF- α , interferon-gamma (IFN- γ) and IL-6, etc) (Okada and Murakami, 1998; Preshaw, 2008; Liu et al., 2010).

Consequently, as the disease progresses to the more advanced stage(s), the specific cytokines and chemokines produced by innate immune response direct the host response towards a robust cell-mediated adaptive immunity. The dominant perivascular T-cell/macrophage infiltrate is observed in the connective tissues. If this T-cell response does not overcome the bacterial challenge, the disease proceeds to B-cell/plasma-cell dominated lesion. The production of antibodies by B-cell/plasma may be protective and control the infection, or may be nonprotective which leads to connective tissue destruction and bone loss (Gemmell et al., 2002; 2007).

The characteristics of chronic periodontitis is mediated by the B-cell/plasma cell response (Kinane and Bartold, 2007; Ohlrich et al., 2009). The immunohistological features of chronic periodontitis are characterized by an apical migration of plaque on the root surface, accompanied by subgingival calculus formation. At this stage, a predominance of plasma cell infiltrate with few macrophages is observed in connective tissue. There is an alteration in appearance and reduction in number of local fibroblasts with the formation of encapsulated fibrous band surrounding the body of the lesion. High levels of IL-1 and IL-6 produced from infiltrating cells lead to the production of matrix metalloproteinases (MMPs), especially by fibroblasts which, in turn, results in

further attachment loss and bone resorption (Gemmell and Seymour, 1998; Nishikawa et al., 2002; Smith et al., 2010).

Clearly, periodontal disease is a multi-factorial disorder. The primary cause of periodontal disease is plaque bacteria but the disease progression is modified by an individual's susceptibility (Kinane et al., 2007; Preshaw, 2008). A wide variety of determinants and factors, either environmental or acquired, e.g. smoking, diabetes, systemic diseases, genetic factors, microbial composition of dental plaque are known to influence the host response (Nunn, 2003; Kinane and Bartold, 2007; Kinane et al., 2007). Therefore, these factors could subsequently have certain effects on the disease initiation and progression (Gaspard, 1995; Grossi, 2000; Chavarry et al., 2009; Anner et al., 2010). Therefore, a complete understanding how diabetes contributes periodontal disease progression could be a key to establish rational diagnostic and therapeutic strategies for periodontal disease associated with diabetes.

1.2 Diabetes Mellitus: aetiology and pathogenesis

Diabetes mellitus is a hormonal disease characterized by changes in carbohydrate, protein, and lipid metabolism. The main feature of diabetes is an increase in blood glucose level (hyperglycemia), with results from either a defect in insulin secretion from the pancreas, a change in insulin action, or both (Mealey and Ocampo, 2007). A number of hormones affect glycemic homeostasis, but only insulin decreases blood glucose. Insulin is required for transport of glucose from the bloodstream into the cells. A quantitative decrease in insulin production or a qualitative reduction in insulin action results in an inability or diminished capacity to transport glucose into cells. Glucose is retained within the bloodstream, causing hyperglycemia. Sustained hyperglycemia

affects almost all tissues in the body and is associated with significant complications. Classically, diabetes presents as a triad of symptoms including polydypsia, polyphagia and polyuria. These symptoms are the direct result of hyperglycemia and the resultant osmotic imbalance. The five major complications of diabetes are retinopathy, nephropathy, neuropathy, circulatory abnormalities and altered wound healing. The periodontium is also a target for diabetic damage, and periodontal disease has been suggested to be the sixth classic complication of diabetics (Loe, 1993; Grossi and Genco, 1998; Iacopino, 2001).

Diabetes is a global public health problem and has a dramatic impact on the health care system not only due to high morbidity and mortality but also due to significant total medical costs (King, 2008). The prevalence of diabetes is significantly increasing annually. By 2030, The World Health Organization (WHO) estimates there will be approximately 366 million people in the United States who have diabetes (Smyth and Heron, 2006). Almost 6 million of these individuals are unaware that they have the disease, and therefore are unable to do what is needed to prevent long-term complications (Mealey and Oates, 2006).

The American Diabetes Association (ADA) classification of diabetes is based on the pathophysiology of each form of the disease (ADA, 2003). The two commonly encountered types of diabetes are type 1 (formerly insulin-dependent diabetes) and type 2 (formerly noninsulin-dependent diabetes). Type 1 diabetes mellitus (T1DM) is caused by autoimmune destruction of β -cell in the pancreatic islets of Langerhans. The autoimmune destruction of these cells results in insufficient in production of endogenous insulin. Type 1 diabetes most often occurs in children and young adults. Only about 5% to 10% of all patients with diabetes have type 1.

In contrast to type 1, type 2 diabetes usually occurs in adults. Because type 2 constitutes about 90-95 % of all diabetes cases, it is the form most commonly encountered in the dental clinic (ADA, 2003). Type 2 diabetes was previously named as noninsulin dependent diabetes as patients who have this disease do not need insulin treatment to survive. The primary cause of type 2 diabetes is insulin resistance which alters the production and utilization of insulin at the target cells. At the early stage, the body increases insulin production which results in hyperinsulinemia to overcome the insulin resistance and maintain normal glucose tolerance. As the condition progresses, the body can no longer be able to control any increases in metabolic load. There is a reduction in insulin production associated with peripheral insulin resistance and β -cell dysfunction (Rhodes, 2005). Eventually, insulin secretion is defective and insufficient to compensate for insulin resistance, leading to impaired glucose tolerance and frank type 2 diabetes. Diabetic individuals can remain undiagnosed for many years because the hyperglycemia appears gradually and many times without symptoms (DeFronzo and Ferrannini, 1991).

The acquired or environmental factors play a major role in pathogenesis of type 2 diabetes. The risk of developing this form of diabetes increases with age, diet, perinatal factors, lack of physical activity; and foremost among these is obesity (Mealey and Ocampo, 2007). Indeed, about one-third of obese individuals develop type 2 diabetes (Mooradian, 2001; Wild et al., 2004). A body mass index (BMI) over 25 kg/m² is defined as overweight, and a BMI of over 30 kg/m² is defined as obese. The incidence of obesity is growing rapidly in parallel with the trends observed for type 2 diabetes. The WHO has estimated that there are more than one billion adults who are overweight worldwide, with at least 300 million of them being obese (Wild et al., 2004). This is associated with increased high calorie food consumption and lack of physical activity which represent a sedentary lifestyle.

The concept that obesity is a proinflammatory state has been suggested to be causally involved in the development of insulin resistance and type 2 diabetes (Kralisch et al., 2007a; Shoelson et al., 2007; Shoelson and Goldfine, 2009; Kim, 2010a). It is now well understood that adipose tissue is not simply an energy storage depot but that it also actively plays an important role in immune-inflammatory process and thereby contributing to development of obesity associated with type 2 diabetes. The adipose tissue consists of a variety of cell types, including adipocytes, immune cells (macrophages and lymphocytes), preadipocytes, and endothelial cells. Adipocytes uniquely secrete adipokines, such as leptin, adiponectin and resistin that potently regulate glucose homeostasis (Zhang et al., 1994; Scherer et al., 1995). The discovery that an increased body mass index is associated with an increase in numbers of adipocytes and infiltrating macrophages provides a major mechanistic link how obesity mediates inflammation (Weisberg et al., 2003). Adipose tissue macrophages (ATMs) are a major source of proinflammatory cytokines (Olefsky and Glass, 2010). Activation of these tissue macrophages leads to the release of a variety of chemokines, which in turn recruit additional macrophages and further develop to chronic inflammatory state. Obese individuals demonstrate a chronic low-grade inflammation (Zeyda and Stulnig, 2009). For example, a variety of adipocyte-derived metabolites such as lipids, fatty acids and inflammatory cytokines are elevated in circulation of obese individuals (Pickup and Crook, 1998; Bergman and Ader, 2000). Free fatty acids contribute to insulin resistance by inhibiting glucose uptake, glycogen synthesis, and glycolysis, and by increasing hepatic glucose production (Bergman and Ader, 2000). In addition, adipocytes and ATMs display a pro-inflammatory activity and produce proinflammatory cytokines such as IL-1 β , TNF- α and IL-6 and chemokines such as IL-8

and monocyte chemoattractant protein (MCP)-1 (Hotamisligil et al., 1995; Mohamed-

Ali et al., 1997). Moreover, elevated levels of TNF- α and IL-6 are associated with features of insulin resistance in obese individuals (Pickup et al., 1997; Festa et al., 2000). Pro-inflammatory cytokines amplify the inflammatory response and underpin the development of insulin resistance and type 2 diabetes. In particular, TNF- α seems to play a key role in development of insulin resistance at the receptor level. TNF- α blocks autophosphorylation of the insulin receptor substrate- 1 (IRS-1) and decreases the tyrosine kinase activity of the insulin receptor while IL-6 is known to inhibit glucosestimulated insulin release. Increased levels of IL-6 also lead to increased production of TNF- α and C-reactive protein (CRP), which in turn, may also have an indirect impact on insulin resistance (Fernandez-Real and Ricart, 2003). Importantly, TNF-α, IL-6, IL- 1β signalling can activate inflammatory pathways within insulin target cells. This leads to activation of Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK) β . In an insulin-resistant state, JNK1 (Hirosumi et al., 2002) and IKK (Itani et al., 2002) signaling is upregulated in insulin-resistant skeletal muscle, fat, and other tissues. As a result, these serine kinases activate transcription factor targets, including activator protein 1 (AP1) and nuclear factor- κ B (NF- κ B), which then stimulate transcription of inflammatory cytokine genes (Nguyen et al., 2007). These serine kinases can also interfere the phosphorylation of insulin receptor substrate proteins and possibly other insulin signaling molecules, inhibiting normal insulin action and ultimately creating a state of cellular insulin resistance. High production of proinflammatory cytokines correlates with elevation of serum levels of the acute-phase reactants fibrinogen and CRP have been found in people with insulin resistance and obesity (Festa et al., 2000). Interestingly, activation of interleukin-18 has also been found to be involved in the pathogenesis of the metabolic syndrome (Hung et al., 2005; Troseid et al., 2010).

1.3 The relationship between periodontal disease and diabetes

The relationship between periodontal disease and diabetes has been well established. A strong body of evidence is now available to demonstrate that diabetes is a significant risk factor for gingivitis and periodontitis (Taylor, 2001; Mealey and Oates, 2006; Barksby et al., 2007; Mealey and Ocampo, 2007; Taylor and Borgnakke, 2008; Preshaw, 2009). Early research reports of diabetes and periodontal disease focused on the investigation of the relationship between a variety of clinical parameters of periodontal disease and the degree of glycemic control. In gingivitis, a number of studies demonstrated that diabetes significantly increases gingival inflammation (Cianciola et al., 1982; Ervasti et al., 1985; de Pommereau et al., 1992; Campus et al., 2005). A higher prevalence and severity of gingivitis was found in individuals with diabetes compared to non-diabetes control with similar plaque accumulation (Cianciola et al., 1982). Positive correlation between glycemic control and severity of gingival inflammation and periodontal destruction was demonstrated (Gusberti et al., 1983; Tervonen and Oliver, 1993; Karjalainen and Knuuttila, 1996; Tervonen et al., 2000; Tsai et al., 2002; Lu and Yang, 2004; Campus et al., 2005; Jansson et al., 2006). Poor metabolic control can increase the severity of gingival inflammation in diabetes individuals (Gusberti et al., 1983), whereas improvement in glycemic control may be associated with decreased gingival inflammation (Sastrowijoto et al., 1990; Karjalainen and Knuuttila, 1996). Poorly controlled diabetic patients had significantly greater gingival bleeding (Ervasti et al., 1985) and gingival inflammation (Cutler et al., 1999) than in either well-controlled diabetic subjects or non-diabetic controls. The number of bleeding sites decreased as glycemic control improved (Ervasti et al., 1985). In a longitudinal experimental gingivitis study (Salvi et al., 2005), a more severe development of gingival inflammation was observed in adult type 1 diabetic subjects than in non-diabetic controls, despite similar levels of plaque accumulation and similar bacterial composition of plaque, suggesting a hyperinflammatory gingival response in diabetes. These studies suggest that the presence of diabetes is often, but not always, associated with increased gingival inflammation and also suggest the important role of the level of glycemic control in this association.

In similar fashion, the risk for periodontitis is increased when diabetes is present (Papapanou, 1996; Taylor, 2001; Novak et al., 2006; Lalla et al., 2007a; Novak et al., 2008). Epidemiologic studies reported that greater prevalence, extent, or severity of periodontitis was seen in diabetic individuals than in non-diabetic subjects (Taylor, 2001; Taylor and Borgnakke, 2008). For example, in the early series of a large crosssectional study involved 2,878 Pima Indians, the prevalence and severity of attachment loss and bone loss was greater among diabetic subjects than among non-diabetic control subjects in all age groups (Shlossman et al., 1990). Similarly, a number of subsequent cross-sectional and case-control studies also demonstrated a greater risk of attachment loss and bone loss in diabetic individuals (Cutler et al., 1999; Tervonen et al., 2000; Campus et al., 2005; Novak et al., 2006; Lalla et al., 2007a). Furthermore, statistical analysis revealed that diabetic individuals had increased risk of having periodontitis by approximately threefold compared with non-diabetic control subjects (Shlossman et al., 1990; Emrich et al., 1991). The association between diabetes and periodontitis was further confirmed in longitudinal research (Nelson et al., 1990; Taylor et al., 1998). As research suggests that there is significant heterogeneity in the diabetic population, not all diabetic subjects are at risk developing periodontitis. It is evident that glycemic control status is an essential variable predicting the adverse effect of diabetes on periodontal health. A number of studies also support that poor glycemic control contributes to progression of periodontitis (Tervonen et al., 2000; Tsai et al., 2002; Lu and Yang, 2004; Campus et al., 2005; Jansson et al., 2006; Peck et al., 2006). Indeed, analysis of data from the National Health and Nutrition Examination Study (NHANES) III of USA concluded that adults with poorly controlled diabetes had a significantly higher prevalence of severe periodontitis than those without diabetes (odds ratio = 2.90); conversely, well-controlled diabetic subjects had no significant increase in the risk of periodontitis (Tsai et al., 2002). In addition, diabetes individuals with poor glycemic control are at risk to have more attachment loss, deeper periodontal pocket and bone loss than well-controlled subjects (Safkan-Seppala and Ainamo, 1992; Seppala et al., 1993; Tervonen and Oliver, 1993; Seppala and Ainamo, 1994; Guzman et al., 2003).

Taken collectively, the presence of diabetes significantly increases the risk for periodontal diseases, both gingivitis and periodontitis. The poorer diabetes control contributes to poorer periodontal health; however, individuals with well-controlled diabetes do not appear to have significant greater risk of periodontal disease than non-diabetic subjects.

Exploring the underlying mechanism how diabetes contributes to periodontal disease highlights the link between these two diseases. Although there is a large body of evidence explains the pathobiology of the interaction between diabetes and periodontal disease, the mechanisms that link these two diseases have yet to be fully understood. Early research focused on potential differences in the subgingival bacterial flora in people with diabetes as a likely explanation for increased periodontal destruction. However, several studies show few differences in the subgingival microbiota between periodontitis patients with diabetes and without diabetes (Sastrowijoto et al., 1989; Tervonen et al., 1994; Novaes et al., 1997). This lack of significant differences in primary bacteriologic agents of periodontal disease suggests that differences in host response may play a role in increased prevalence and severity of periodontal destruction seen in patients with diabetes.

As stated previously in this review, it is well documented that the immuneinflammatory response plays a key role in periodontal disease. Periodontal disease is characterized by the interaction between bacterial challenge and the host response involving networks of cytokines functioning in synergy. It has been suggested that periodontal disease and diabetes share a common pathogenesis as a chronic inflammatory condition and it is also suggested that diabetes is a potential host response modifier in periodontal disease (Southerland et al., 2006). An increasing number of studies suggest that alterations in host inflammatory response as a result of diabetes may lead to further dysregulation of immune-inflammatory responses in the periodontium, causing increased periodontal destruction (Nishimura et al., 1998; Salvi et al., 1998; Ryan et al., 2003; Mealey and Oates, 2006; Preshaw, 2009; Santos et al., 2010; Venza et al., 2010). Diabetes often involves an increased inflammatory response at the local and systemic level. For example, IL-6 and TNF- α levels are elevated in the plasma of obese patients and those with type 2 diabetes (Dandona et al., 2004). Hyperglycaemia also results in increased levels of IL-6, IL-12 and TNF- α .

Adipose tissue is a key component in pathophysiological connections between obesity, inflammation and insulin resistance which is a strong risk factor for development of type 2 diabetes. Thus, adipose tissue may also have an important role in mechanism that links diabetes and periodontal disease. Adipose tissue secretes a range of cytokines such as TNF- α and IL-6 and adipokines, such as leptin and adiponectin. In addition, adipose tissue consists of a variety of immune cells, including macrophages and lymphocytes. Macrophages are present in much higher numbers in adipose tissue of obese subjects

than in that of lean subjects and appear to be major sources of cytokines. In recent years, there has been an increasing number of studies investigating the role of adipokines in periodontal disease related to diabetes (Johnson and Serio, 2001; Bozkurt et al., 2006; Barksby et al., 2007; Karthikeyan and Pradeep, 2007b; a; Yamaguchi et al., 2007; Preshaw, 2009; Shimada et al., 2010).

Another mechanism that links diabetes and periodontal disease is the adverse effects of diabetes on the function of host defense cells, such as polymorphonuclear leukocytes, monocytes and macrophages (Lalla et al., 2001). An intact host immune-inflammatory response is critical for establishment and maintenance of periodontal health. In diabetes, the functions of polymorphonuclear leukocyte which include cell adherence, chemotaxis and phagocytosis are impaired. Defects in this first line defense against periodonthopathic microorganisms may decrease bacterial killing, allowing proliferation of pathogenic bacteria and increased periodontal destruction. Another major cell necessary for effective periodontal defense mechanisms is the monocyte/macrophage. In individuals with diabetes these cells may be hyperresponsive to bacterial antigens (Offenbacher, 1996; Salvi et al., 1997a; Salvi et al., 1998). This upregulation in turn could lead to significantly increased production of proinflammatory cytokines and mediators and further tissue damage.

A further important mechanism by which diabetes affects periodontal health is the formation of advanced glycation end-products (AGEs). In people with prolonged hyperglycaemia, structural proteins and matrix molecules become glycated, resulting in the formation of AGEs (Brownlee, 1994; Monnier et al., 1996). The level of AGEs is greatly increased in people with diabetes and hyperglycemia results in increased expression of receptor for advanced glycation end-products (RAGEs) (Mealey and Ocampo, 2007). Interestingly, formation of AGEs also occurs in the periodontium and

higher expression of the RAGEs was detected in the gingival tissues of type 2 diabetic subjects than of those nondiabetic controls (Schmidt et al., 1996; Katz et al., 2005). RAGEs are found on the surface of many cell types including smooth muscle cells, endothelial cells, neurons, monocytes, and macrophages (Schmidt et al., 1994a; Pietropaoli et al., 2010). The interaction between AGEs and RAGEs has significant effects at the cellular level, affecting cell-cell, cell-matrix, and matrix-matrix interactions, and enhancing inflammatory responses. Indeed, AGE formation is commonly thought to be a major link between various complications of diabetes (Mealey and Ocampo, 2007; Taylor and Borgnakke, 2008; Preshaw, 2009; Beisswenger, 2010). For example, engagement of AGEs and their receptor on endothelium and modification of vascular collagen contributes to macrovascular diabetic complications such as cardiovascular disease (Schmidt et al., 1994b). Furthermore, the interactions of AGEs on the cell surface of monocyte/macrophages induce increased cellular oxidant stress and activate the NF- κ B, altering the phenotype of the monocyte/macrophage and resulting in increased production of pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6 (Schmidt et al., 1999; Lalla et al., 2001; Lalla,

Taken all together, it can be concluded that diabetes increases the risk for periodontal disease compared to non-diabetics. This increased susceptibility most likely results from pathological processes that are common to both diseases and/or upregulated in the context of diabetes (Preshaw, 2009). Investigations on the role of inflammatory mechanisms in of periodontal disease related to diabetes should provide insight into the processes underlying the onset and progression of periodontal diseases and diabetes. The advance in understanding of the inflammatory basis for diabetes and its periodontal

2007), which contribute to further periodontal tissue destruction.

complications may prove valuable for introducing novel therapeutic approaches, alongside the currently used pharmacological and non-pharmacological interventions.

1.4 Adipose tissue, adipokines, inflammation and immunity

Obesity is a common disorder in which excess body fat has accumulated to such an extent that health may be negatively affected. In fact, the evidence of obesity and associated pathologies, particularly cardiovascular diseases and diabetes mellitus type 2, is dramatically increasing worldwide in both children and adults (Trayhurn and Wood, 2004; Tilg and Moschen, 2006; Zavalza-Gomez et al., 2008). In recent years, there has been an increasing interest in the role of adipose tissue as an active participant in controlling the body's physiological and pathological processes (Matarese et al., 2005; Kralisch et al., 2007b; Lago et al., 2007a; Ahima and Osei, 2008; Antuna-Puente et al., 2008; Fernandez-Riejos et al., 2008; Wozniak et al., 2009; Fernandez-Riejos et al., 2010). One of the central roles of adipose tissue is that of an active secretory organ, sending out and responding to signals that modulate appetite, energy expenditure, insulin sensitivity, endocrine and neurological systems, bone metabolism, and inflammation and immunity. Adipose tissue expresses and secretes a variety of products known as 'adipokines', including leptin, adiponectin, resistin and visfatin, as well as cytokines and chemokines such as TNF- α , IL-6 and MCP-1. The release of adipokines by either adipocytes or adipose tissue-infiltrated macrophages leads to a chronic subinflammatory state that could play a central role in the development of insulin resistance and type 2 diabetes, and could be a potential link between periodontal disease and diabetes (Pischon et al., 2007; Saito and Shimazaki, 2007; Preshaw, 2009; Shimada et al., 2010). This part of the review will focus on the role of adipose tissue and related adipokines in modulating inflammation and immunity.

1.4.1 The cellular components of adipose tissue

Adipose tissue can be divided into 2 major types: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT represents the vast majority of adipose tissue in the organism and is the site of energy storage, whereas the main role of BAT is non shivering thermogenesis. WAT is composed of both immune and non-immune cells, adipocytes being the most abundant. Recent studies have reported that macrophages play a crucial role as a resident immune cell in WAT, suggesting the links between adipose cells and the immune system (Weisberg et al., 2003; Xu et al., 2003; Curat et al., 2004; Olefsky and Glass, 2010; Wentworth et al., 2010). For example, Curat et al. (2004) demonstrated that 11% of the cells in the stroma-vascular fraction of human adipose tissues are macrophages. In fact, the number of macrophages present in WAT is increased in obesity and is directly correlated with both adipocyte size and body mass, with no significant differences present between subcutaneous and visceral WAT (Weisberg et al., 2003). In obese mice, WAT macrophages form multinucleated giant cells reminiscent of those present in granulomas, suggesting an activated phenotype (Xu et al., 2003). Moreover, Weisberg et al. (2003) reported that macrophages are the major source of TNF- α and contribute approximately 50% of WAT-derived IL-6. Taken together, obese individuals have an increased number of macrophages in WAT compared with that of lean persons, and these macrophages appear to be activated, both from a morphological (giant cells) and a functional (cytokine production) standpoint (Fantuzzi, 2005).

1.4.2 Adipose tissue in the oral cavity

The moist surface tissue lining over the oral cavity is called the oral mucosa. The two main tissue components of the oral mucosa are a stratified squamous epithelium, called the oral epithelium, and an underlying connective tissue layer, called the lamina propia. Similar to skin, oral mucosa contains sebaceous glands which represent a local adipose tissue depot in oral tissues. They are present in the upper lip and buccal mucosa in about three quarters of adults and have been described occasionally in the alveolar mucosa and dorsum of the tongue. Sebaceous glands appear as pale yellow spots and sometimes are called *Fordyce's spots*. In many regions, such as cheeks, lips, and parts of the hard palate, a layer of loose fatty or glandular connective tissue containing the neurovascular bundles that supply the mucosa separates the oral mucosa from underlying bone or muscle. The layer represents the submucosa in the oral cavity. Particularly in anterior region of hard palate, there are numerous of adipose tissue in the submucosa. Fatty connective tissue and sebaceous glands are the composition of lamina propia and submucosa in oral cavity, yet their function remains unclear. Some reports claim that sebaceous gland functions only as the protective barrier by producing sebum lubricates over the surface of oral mucosa so that it slides easily against the teeth (Ten Cate, 1998). However, there is no information regarding the role of adipose tissue in the physiological and pathological processes in oral tissue.

1.4.3 Leptin

WAT has been described as a multifunctional secretory organ for it is able to produce a myriad of adipokines and proinflammatory mediators. Adipokines are proteins synthesized and secreted mainly by adipocytes of WAT; however, it is important to note that these factors might be synthesized at other sites and participate in functions
unrelated to those within WAT (Fantuzzi, 2005; Tilg and Moschen, 2006; Lago et al., 2007a). Although adipose tissue has been found to produce more than 50 cytokines and other molecules, only leptin and adiponectin are primarily produced by adipocytes and can therefore be properly classified as adipokines. This part of the review will focus mainly on leptin and its role in inflammation and immunity.

Leptin is a 16 kDa non-glycosylated peptide hormone encoded by the gene *obese* (*ob*), the murine homologue of the human gene *LEP* (Zhang et al., 1994). Leptin is a member of the class I cytokine superfamily (Lago et al., 2007a). Structurally, leptin consists of a four α -helices bundle motif which is similar to other pro-inflammatory cytokines such as IL-6, IL-12 and granulocyte colony-stimulating factor. Fundamentally, leptin plays a primary role in control of appetite. There has been demonstrated that mice with a mutation in the leptin (ob/ob mice) or leptin receptor (db/db mice) gene, as well as human subjects with mutations in the same genes, are massively obese, suggesting a controlling role of leptin in body weight (Friedman, 2002; Oswal and Yeo, 2010). Leptin is mainly produced by the adipose tissue, and also circulating leptin levels are directly correlated with WAT mass. The production of leptin is regulated by food intake, eating-related hormones, and energy status. In addition, leptin levels are genderdependent (Blum et al., 1997; Castracane et al., 1998). The synthesis of leptin is inhibited by testosterone and increased by female sex hormones. The levels of leptin are therefore higher in women than in men even after adjusted for BMI. In recent years, leptin has been shown to play an important role in regulating the immune response, both in normal and pathological conditions (La Cava and Matarese, 2004; Otero et al., 2005a; Lago et al., 2007b; Lago et al., 2009; Fernandez-Riejos et al., 2010). Both ob/ob mice and db/db mice are not only obese but they also show the immune/endocrine deficiencies observed during starvation (Lord et al., 1998; Howard et al., 1999). Even in humans, it has been found that leptin levels are associated with immune response in malnourished infants, which have low plasma leptin and impaired immune responses (Palacio et al., 2002). Moreover, leptin signaling deficiency impairs humoral and cellular immune responses. The leptin receptor Ob-Rb is expressed by B and T lymphocytes, suggesting that leptin regulates directly the B and T cell responses (Busso et al., 2002). Leptin modulates immune responses at the development, proliferation, anti-apoptotic, maturation, and activation levels (Bennett et al., 1996; Howard et al., 1999; Stofkova, 2009). Furthermore, the production of leptin is affected by inflammatory state. A wide range of inflammatory mediators can increase and suppress the leptin levels (Gualillo et al., 2000; Lago et al., 2007a). Thus, leptin appears to function not only as an adipostatin, but also as a general signal of energy reserves involved in numerous functions, linking metabolism, reproduction, and many aspects of immunity and inflammation.

1.4.3.1 Leptin receptors (ObR)

Leptin has been described as a cytokine-like hormone with pleiotropic actions. It exerts its biological activities by binding to its receptors. The leptin receptors are encoded by the gene *diabetes* (*db*). As a result of the alternative splicing of the RNA transcript of *db* gene, there are at least six Ob-R isoforms: the soluble form Ob-Re, which lacks a cytoplasmic domain; four forms with short cytoplasmic domains (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf), which are considered to be transporting receptors; and the long form Ob-Rb appears to be the main form capable of transducing the leptin signal. Leptin's functional receptor (ObRb) is highly expressed in the hypothalamus where it mediates the effects of leptin on energy homeostasis (Fei et al., 1997). Interestingly, ObRb is also expressed in many cell types of innate and adaptive immunity including in neutrophils,

dendritic cells, lymphocytes and monocytes (Zarkesh-Esfahani et al., 2001; Mattioli et al., 2005). In vitro, THP-1 monocytes also express functional leptin receptors (Ob-Rb) (Gabay et al., 2001). However, the expression of the leptin receptor appears to be reduced in human peripheral blood mononuclear cells from obese individuals (Tsiotra et al., 2000).

Ob-R is a member of class I cytokine receptor family, which includes receptors for IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), granulocyte colony-stimulating factor (G-CSF) and gp130. Activation of the ObR-b initiates a cascade of signal transduction pathways. In particular, the JAK/STAT (Janus kinases/signal transducers and activators of transcription) pathway is thought to be one of major pathways activated by leptin (Ihle et al., 1995). The leptin receptor has three conserved tyrosines in its cytoplasmic domain, which correspond to positions Y985, Y1077, and Y1138. The activation of the leptin receptor predominantly activates JAK2 tyrosine kinase. Subsequently, JAK2 phosphorylates the three conserved tyrosines, including the Y1138, which serves as a docking site for STAT-3. STAT-3 becomes activated, homodimerizes, and translocates to the nucleus, resulting in induction of specific genes which regulate the specific body homeostasis such as appetite and energy balance (Bates and Myers, 2003). It has also been reported that leptin signalling pathways activate the transcription factors such as NF- κ B and activator protein-1 (AP-1) which in turn lead to activation and production of inflammatory cytokines and chemokines such as IL-1, IL-6, TNF- α and IL-8 in different cell types (Gonzalez et al., 2004; Tang et al., 2007; Tong et al., 2008). Conversely, leptin receptor is also upregulated by proinflammatory signals (Fernandez-Riejos et al., 2010).

In addition to the JAK-STAT pathway, the mitogen-activated protein kinase (MAPK), the p38, the IRS1, and the phosphatidylinositol 3-kinase (PI3K) pathways are also

important pathways that mediate leptin's action (Lago et al., 2007a; Fernandez-Riejos et al., 2010; Oswal and Yeo, 2010). For example, the stimulation of cultured human and murine chondrocytes with the combination of leptin and IFN- γ or IL-1 increased nitrix oxide synthase 2 (NOS2) activation via a mechanism involving JAK2, PI3K and p38 (Otero et al., 2003; Otero et al., 2005b). It is interesting to note that different specific leptin signalling pathways are required to mediate specific effects. Thus, the MAPK pathway seems to mediate anti-apoptotic effects in PBMCs (Najib and Sanchez-Margalet, 2002), whereas the PI3K pathway may be important in regulating glucose uptake (Bates et al., 2007).

1.4.3.2 Leptin, immunity and inflammation

Leptin exerts a pro-inflammatory role, while at the same time protecting against infection (Fantuzzi, 2005). For its protecting role, mice lacking leptin (ob/ob) or its functional receptor (db/db) exhibit a number of defects in both innate and adaptive immunity (Kimura et al., 1998; Mancuso et al., 2002). In addition, congenital leptin deficiency in humans leads to impaired immune cell function and increased susceptibility to infection or other diseases (Ozata et al., 1999), whereas reduced numbers of circulating CD4⁺ T cells and impaired T cell proliferation and cytokine release in patients with congenital leptin deficiency were reversed by daily subcutaneous injections of recombinant human leptin (Farooqi et al., 2002). On the other hand, leptin is associated with many immune-mediated pathologies as well as up-regulation of pro-inflammatory cytokine production (Fraser et al., 1999; Gualillo et al., 2000; Faggioni et al., 2001; Gabay et al., 2001; Palacio et al., 2002; Gonzalez et al., 2004; Karthikeyan and Pradeep, 2007a; Lago et al., 2007b). The summary of an immunological role of leptin is shown in Table 1.1.

Leptin has a numerous roles in innate immunity. Studies using leptin (ob/ob) or leptin receptor (db/db) knock-out mice demonstrated the obesity-related deficiency in macrophage phagocytosis and the expression of proinflammatory cytokines whereas exogenous leptin administration restores phagocytic function (Mancuso et al., 2002) and up-regulates the production of proinflammatory cytokine secretion, such as TNF- α , IL-6, and IL-12 (Gainsford et al., 1996; Loffreda et al., 1998). A study identified that several phenotypic abnormalities are present in macrophages from ob/ob mice, including activation of an oxidant-sensitive transcription factor, increased expression of IL-6 and cyclooxygenase (COX)-2 and increased COX-2-dependent production of prostaglandin E(PGE)₂ (Lee et al., 1999). Leptin also exerts its effect on chemokine production as leptin enhances CC-chemokine ligands (CCLs) expression in cultured murine macrophage, through activation of a JAK2-STAT3 pathway (Kiguchi et al., 2009).

Innate immunity	Adaptive immunity
Monocyte/macrophage	T lymphocytes
↑Proliferation	↑CD69, CD25 and CD71
↑Phagocytosis	↑Naive T cells
↑IL-6, ↑TNF-α	↓Memory T cells
Monocytes	↑Maturation of dendritic cells
↑Cytokine production	↑Th1 cytokines (IL-2, IFN-γ)
↑Caspase-1 mRNA	↓Th2 cytokine (IL-4)
↑Expression of activation makers	
Macrophages	B lymphocytes
↑IL-6, ↑COX2, ↑PGE2	↑Cytokine production
↑Chemokine production	
Neutrophils	
↑ Chemotaxis	
↑ ROS production	
NK cells	
↑Development and NK cell function	

Table 1.1 Summary of the immunological role of leptin

Furthermore, human leptin stimulates proliferation of human monocytes in vitro and also increases monocyte expression of activation markers CD25, HLA-DR, CD38, CD71, CD11b, and CD11c (Santos-Alvarez et al., 1999). It has been also noted that the cytokine stimulation produced by leptin is comparable to that produced by endotoxin LPS (Santos-Alvarez et al., 1999). Interestingly, Ahmed et al. (2007) found that leptin enhances expression of IL-1 β gene through the induction caspase-1 mRNA expression in bovine monocytes/macrophages. Moreover, the production of reactive oxygen species (ROS) is increased as a result of monocyte activation by leptin in vitro (Sanchez-Pozo et al., 2003). Similarly, leptin can induce the release of ROS and promote chemotaxis of neutrohils (Caldefie-Chezet et al., 2001). Leptin also affects natural killer (NK) cells development and function. Leptin receptor (db/db) deficient mice were found to have a reduction in the total number of NK cells in the liver, spleen, lung and peripheral blood (Tian et al., 2002). Leptin regulates NK cell function by increasing IL-2 and perforin gene expression via STAT3 pathway in human NK cell lines (Zhao et al., 2003).

The effect of leptin in adaptive immunity is also well studied. In contrast to macrophages/monocytes, leptin alone was not able to activate T lymphocytes (Martin-Romero et al., 2000). Leptin was found to enhance proliferation and activation of human circulating T lymphocytes when they are costimulated with other nonspecific immunostimulants such as phytohemagglutinin (PHA) or concanavalin A (ConA). Leptin increases the expression of early activation markers such as CD69, as well as the expression of late activation markers, such as CD25, or CD71 in both CD4+ and CD8+ T lymphocytes (Martin-Romero et al., 2000). Leptin also affects the proliferation of distinct lymphocyte subpopulations. Specifically, leptin induces proliferation of the

naive CD4⁺CD45RA⁺ T cells, but inhibits proliferation of the memory CD4⁺CD45RO⁺ T cells (Lord et al., 2002).

At the functional level, leptin increases production of Th1 cytokines (IL-2 and IFN- γ) while it suppresses production of Th2 cytokine (IL-4) in T-cell proliferation assay with mouse cells (Lord et al., 1998). Leptin also promotes functional and morphological changes (ie. \uparrow HLA-ABC and HLA-DR expression, \uparrow cytokine production) in human dendritic cells (DCs), mediating them towards Th1 response (Mattioli et al., 2005). Furthermore, the role of leptin on B cells has recently demonstrated (Agrawal et al., 2011). The study found that leptin induces secretion of inflammatory cytokines IL-6 and TNF- α and anti-inflammatory IL-10 by human B cells via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathways.

Taken collectively, leptin has a key role in the activation of the immune system, and it is a mediator of inflammation. The action of leptin may be one of the mechanisms responsible for the low-grade systemic inflammation that may be present in metabolic syndrome-associated chronic pathologies such as diabetes associated with obesity.

1.4.3.3 Leptin, diabetes and periodontal disease

Obesity induces an insulin-resistant state in adipose tissue, liver, and muscle and is a strong risk factor for the development of type 2 diabetes mellitus. Obesity-related insulin resistance may result from a combination of altered functions of insulin target cells and the accumulation of macrophages that secrete proinflammatory mediators (Olefsky and Glass, 2010). The release of cytokines or adipokines such as leptin and adiponectin by either adipocytes or adipose tissue-infiltrated macrophages leads to a chronic low-grade inflammatory state (Antuna-Puente et al., 2008; Tilg and Moschen,

2008). Indeed, inflammation is a key process underlying development of diabetes, and activation of proinflammatory pathways is known to interfere with insulin signalling and induce insulin resistance (Hotamisligil, 2006).

Many studies demonstrated that the increased leptin concentrations and gene polymorphism of leptin or leptin receptor are positively correlated with the higher risk of developing diabetes (McNeely et al., 1999; Nannipieri et al., 2006; Han et al., 2008; Welsh et al., 2009). For example, in a large sudy recruiting 5,672 patients, increased circulating levels of leptin were found to be strongly related to risk of diabetes (Welsh et al., 2009). Recent studies demonstrated a role of adipose tissue-derived cytokines and the development of insulin resistance (Zavalza-Gomez et al., 2008). For example, TNF- α can reduce insulin sensitivity by influencing the phosphorylation state of the insulin receptor (Mishima et al., 2001), and neutralization of TNF- α improves insulin sensitivity (Hotamisligil et al., 1995). Interestingly, it is known that leptin is capable of controlling TNF-a production and macrophages activation (Loffreda et al., 1998). In type 2 diabetes leptin levels are correlated with BMI and therefore are generally increased (Weisberg et al., 2003), whereas adiponectin levels are significantly decreased in diabetic subjects and improve after treatment with insulin-sensitizing agents (Kadowaki and Yamauchi, 2005). Furthermore, leptin signalling can activate inflammatory pathways which regulate insulin actions. For example, it has been reported that JNK activation can interfere insulin signaling through phosphorylation of serine residues of IRS-1 and there is a significant increase in total JNK activity in all tissues tested in leptin (ob/ob) deficient mice (Hirosumi et al., 2002). Moreover, leptin activation can induce phosphorylation of IRS (Tong et al., 2008) which is a common insulin signalling pathway. These findings suggest the interaction between leptin and insulin signalling. More recent review indicates that adipose tissue also contains a large number of T cells (Nishimura et al., 2009a). Interestingly, the CD8⁺ T cell fraction increased during the progression of obesity and the infiltration of adipose tissue by CD8⁺ T cells preceded the accumulation of macrophages. Therefore, it has been proposed that CD8⁺ T cells are essential for the maintenance of inflammatory responses in obesity-related insulin resistance and metabolic abnomalities (Nishimura et al., 2009b). However, in lean mice, regulatory T cells (Treg) and Th2 cells appear to restrict inflammatory responses in part via production of the antiinflammatory cytokine IL-10 (Feuerer et al., 2009; Winer et al., 2009). In obese adipose tissue tissue, the immune response is shifted to of CD8+ and Th1 dominated inflammatory response (Lumeng et al., 2009). Interestingly, leptin is found to induce Th1 response but inhibit Th2 response (Lord et al., 1998). As leptin is considered as a mediator of inflammation, leptin could potentially play part in diabetes pathogenesis either by enhancing proinflammatory cytokine production or activating host immune responses, in both innate and adaptive immunity.

In periodontal disease, the understanding of leptin role is still limited. Leptin levels decreased progressively in GCF as periodontal disease progressed (Johnson and Serio, 2001; Karthikeyan and Pradeep, 2007a). However, the plasma levels of leptin have been reported to increase in the greater periodontal destruction sites (Karthikeyan and Pradeep, 2007b). In vitro, leptin enhanced *Pvevotella intermedia* LPS-induced TNF- α production in a dose-dependent manner (Kim, 2010b). Recently, a proposed role for adipokines in cross-susceptibility between periodontal disease and type 2 diabetes mellitus has been reviewed (Preshaw et al., 2007; Preshaw, 2009). Elevated adipokine levels may lead to dysregulation of cytokine production in the inflammatory process and this may underpin the pathogenesis of periodontitis associated with diabetes.

Nevertheless, little is known about the immunological effects of leptin in pathogenesis of periodontal disease.

1.5 IL-18 and periodontal disease

Cytokines are known to be major participants in acute and chronic inflammation regardless of its location, and there is strong evidence for participation of these mediators in periodontitis (Offenbacher, 1996; Okada and Murakami, 1998; Bjork et al., 2003; Van Dyke and Serhan, 2003; Kinane and Bartold, 2007; Preshaw, 2008). In fact, the pathogenesis of periodontitis is mediated by an unbalanced production of cytokines. production results "Appropriate" cytokine in protective immunity, while "inappropriate" cytokine production can lead to periodontal tissue destruction and disease progression. Cytokines are produced by activated resident gingival cells and infiltrating immune cells. In the periodontitis lesion, high levels of inflammatory mediators/cytokines such as IL-1 β , TNF- α , IFN- γ , IL-6, IL-10, IL-8, MMPs and PGE₂ have been detected (Kornman et al., 1997; Okada and Murakami, 1998). Recent studies reported that there is a positive association between GCF levels of IL-18 and periodontal diseases (Orozco et al., 2006; Figueredo et al., 2008b; Vokurka et al., 2008; Pradeep et al., 2009b; Schallhorn et al., 2010). In addition, IL-18 is elevated in diabetes patients and serum level of IL-18 is correlated with the risk of diabetes development (Aso et al., 2003; Esposito et al., 2004; Hivert et al., 2009). Yet, there is no evidence regarding the role of IL-18 in periodontal disease associated with diabetes. The next part of review will focus on IL-18 literature and its possible role connecting periodontal disease and diabetes.

1.5.1 Structure and gene regulation of IL-18

IL-18 was first discovered in 1995 by Okamura *et al.* (Okamura *et al.*, 1995), and was formerly called INF- γ -inducing factor. IL-18 belongs to IL-1 cytokine family due to its structure, receptor family, signaling pathway, and function (Biet *et al.*, 2002; Muhl and Pfeilschifter, 2004). Despite the moderate sequence identity (17%), the overall 3 dimensional structure of IL-18 shows marked similarity with IL-1 β . Additionally, the β trefoil fold of IL-18 is also similar to those of IL-1 β but the surface residues are totally dissimilar (Kato et al., 2003).

The human IL-18 gene is not within the IL-1 gene cluster on chromosome 2q but is located on chromosome 11q22.2-11q22.3. The transcription factors interferon consensus sequence-binding protein (ICSBP) and PU.1 play a major role in activation of the IL-18 promoter. In addition, IFN- γ stimulation of macrophages has been shown to up-regulate IL-18 gene expression via transcriptional factor AP-1. Similarly, Yamauchi et al. (2008) showed that AP-1 is an important transcriptional regulation of IL-18 expression in gastric epithelial cells. Moreover, the study also demonstrated that NF-kB transcription factor is also involved in IL-18 induction using electrophoretic mobility shift assay and chemical inhibitor experiments (Yamauchi et al., 2008). The synthesized IL-18 protein contains 193 amino acids (Nolan et al., 1998). The 3' untranslated region of human IL-18 mRNA lacks AUUUA destabilization sequences. In addition, the promoter region for IL-18 is TATA-less which is used by a wide range of cell types and IL-18 promoter activity upstream of exon 2 acts constitutively. These observations may explain why IL-18 is expressed not only in human peripheral blood mononuclear cells (PBMCs) but also in some non-immune cells (Tone et al., 1997; Puren et al., 1999; Gracie et al., 2003).

1.5.2 The IL-18 receptor and mechanism of action

IL-18 receptor (IL-18R) is found on many immune cells including T-cells, NK cells, Bcells, and dendritic cells (Sigal, 2005). It belongs to the IL-1R/ TLR superfamily. The IL-18 receptor complex (IL-18RC) is composed of IL-18Ra (also known as IL-1Rrp1, IL-18R1 or IL-1R5) to which IL-18 binds and of IL-18R^β (also termed IL-18RacP, IL-18RII or IL-1R7) which increases the affinity of the receptor and initiates signal transduction (Torigoe et al., 1997; Born et al., 1998). Activation of the IL-18R by IL-18 recruits the IL-1 receptor-activating kinase (IRAK) via the adaptor myeloid differentiation factor MyD88 (Adachi et al., 1998; Kojima et al., 1998). After phosphorylation, IRAK dissociates from the receptor complex and associates with TNF receptor-associated factor 6 (TRAF6), which then leads to sequential activation of NFκB binding kinase (NIK), IKK-1, IKK-2, and NF-κB (Matsumoto et al., 1997; Robinson et al., 1997; Adachi et al., 1998; Dinarello, 1999; Nakanishi et al., 2001a; Reddy, 2004; Alboni et al., 2010). NF- κ B induces gene transcription to produce further inflammatory mediators (such as IFN- γ , TNF- α , IL-6, etc.). In addition, a role for MAPK, p38, JNK and extracellular signal-regulated kinase (ERK), IRAK and STAT3 in IL-18 signalling has been suggested (Tsutsui et al., 1996; Tomura et al., 1998; Kalina et al., 2000; Lee et al., 2004; Netea et al., 2006).

IL-18 binding protein (IL-18BP), a member of the immunoglobulin superfamily, acts as a negative regulator of IL-18 action (Aizawa et al., 1999; Novick et al., 1999).This 38kDa soluble protein displays some sequence homology with IL-18Rα (Novick et al., 1999; Kim et al., 2002). IL-18BP binds selectively and with high affinity to mature IL-18, but not to pro-IL-18, preventing its interaction with IL-18Rα. Four human (18BPad) and two murine (IL-18BPc and d) IL-18BP isoforms have been described (Kim et al., 2000). Of these human IL-18BPb and d lack the structural requirement to inhibit IL-18 action and their role remains to be determined (Boraschi and Dinarello, 2006).

1.5.3 The proIL-18 synthesis and IL-18 processing

IL-18 is synthesized intracellularly as a 24-kDa inactive precursor (pro-IL-18). IL-18 lacks a secretory peptide and requires cleavage by caspase-1 (formerly termed IL-1 β converting enzyme; ICE) to be secreted in an 18-kDa active IL-18 (Ghayur et al., 1997; Gu et al., 1997). Like IL-1 β , there has been proposed that the induction of IL-18 release requires two distinct signals. The first signal is critical for gene expression and synthesis of the IL-18 precursor which are induced by inflammatory signals such as LPS or cytokines. The second signal is required for the activation of the caspase-1 inflammasome to mediate the processing and release of IL-18 (Mehta et al., 2001; Mariathasan and Monack, 2007). However, in monocytes and many other cells, unlike IL-1 β , IL-18 is constitutively produced and released (Puren et al., 1999). Therefore, the research interest has focused attention on the regulation of IL-18 to biological active IL-18.

IL-18 has been identified in a variety of cell types in both haemopoietic and nonhaemopoietic lineages. Thus, IL-18 expression has been reported in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, synovial fibroblasts, oral neutrophils, and epithelial cells (Conti et al., 1997; Matsui et al., 1997; Udagawa et al., 1997; Stoll et al., 1998; Sugawara et al., 2001; Rouabhia et al., 2002; Jablonska et al., 2005). IL-18 is also produced in adipocytes (Skurk et al., 2005), but non-adipocyte cells have been identified as the main source of IL-18 in adipose tissue (Fain et al., 2006). Although IL-18 protein is constitutively released in many cell types, the amount of IL-18 protein can be induced by LPS from Gram-negative bacteria (Seki et al., 2001; Cheng et al., 2005; Foster et al., 2007; Yamauchi et al., 2008). There is increasing evidence demonstrating how pathogens induce IL-18 release by host cells, yet the roles of cytokine or adipokines regulating IL-18 processing and secretion are not fully understood.

1.5.3.1 The inflammasomes

The inflammasome is an intracellular multiprotein complex that mediates the activation of caspase-1. The activation of caspase-1 is central to the processing and secretion of the proinflammatory cytokines IL-1 β and IL-18. The term inflammasome is derived from the word inflammation-to reflect the function of this complex-and the suffix "some" from the Greek soma meaning body. It was named by Tschopp and colleagues (Martinon et al., 2002). The inflammasome acts as a molecular scaffold for caspase-1 activation (Mariathasan and Monack, 2007). The inflammasome classically consists of 3 main components which include NLR proteins, the adaptor ASC and the central effector protein procaspase-1 (Figure 1.1). A critical step in caspase-1 activation is the assembly of the inflammasome. Caspase-1 is synthesized in the cytosol as an inactive 45-kDa zymogen (pro-caspase-1). After stimulation by myriad microbial and endogenous signals, procaspase-1 undergoes auto-catalytic processing to generate p20 and p10 subunits. These two subunits assemble into a heterodimer which in turn forms a tetramer with two independent catalytic sites. ASC (apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain), also known as PYCARD, CARD5 or TMS1, acts as an intracellular adaptor of the inflammasome. ASC encodes a 22-kDa protein that contains an amino (N)-terminal PYD domain and a carboxy (C)-

terminal CARD (Masumoto et al., 1999). As the adaptor protein for the recruitment of other PYD- and CARD- containing proteins, ASC has a central role in the inflammasome. Through homotypic protein-protein interactions with its own CARD and PYD domains, ASC is therefore thought to act as direct bridge between the effector caspase-1 and the protein NLRs. Another significant part of the inflammasome is NLRs which act as a sensor compartment of the inflammasome. To date, three inflammasomes named after the NLR involved (NLRP1, NLRP3 and NLRC4) have been characterized to activate caspase-1 in response to specific stimuli. However, the precise mechanisms controlling the inflammasome activation remains incompletely understood.

1.5.3.2 Pattern recognition receptors (PRRs)

Upon microbial invasion, sensing of pathogenic organisms by the innate immune system is mediated by several classes of germline-encoded pattern recognition receptors (PRRs) that are capable of detecting highly conserved molecular structures that are specific to microbes, called microbe associated molecular patterns (MAMPs) such as LPS, peptidoglycan, flagellin and microbial nucleic acids. The innate immune system identifies not only the presence of microbes, it also contains PRRs that recognize danger signals or danger associated molecular patterns (DAMPs) which are produced by host cells in response to pathogenic conditions. Four main classes of PRRs have been described: TLRs, retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors and the IFI200 family member absent in melanoma 2 (AIM2) (Fitzgerald, 2010; Kanneganti, 2010; Shaw et al., 2010). Whereas TLRs sense MAMPs in the extracellular space and endosomes, NLRs, RLRs and AIM2 function as pathogen sensors in intracellular compartments

(Kanneganti, 2010). Different PRRs recognize and sense specifically different MAMPs. For example, LPS from Gram-negative bacteria is the best-studied ligand for TLR4, whereas Gram-positive bacteria preferentially activate TLR2 through lipoteichoic acid. The different PRRs generate signalling cascades converging on similar effects on host cells, resulting in expression of genes related to the inflammatory response and inflammasome activation (Akira and Takeda, 2004; Latz, 2010).

NLRs are composed of more than 20 family members in mammals. It is characterized by the presence of a central nucleotide-binding and oligomerization NACHT domain, which is commonly flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase-1 recruitment domian (CARD) or pyrin domains (PYD). LRRs are believed to function in ligand sensing and autoregulation, whereas CARD and PYD domains mediate homotypic protein-protein interactions for downstream signalling (Lamkanfi and Dixit, 2009; Fitzgerald, 2010; Kanneganti, 2010; Shaw et al., 2010). The NACHT domain, which is the only domain common to all NLR family members, enables activation of the signaling complex via ATP-dependent oligomerization (Mariathasan et al., 2006). Phylogenetic analysis of NLR family NACHT domains reveals 3 distinct subfamilies within the NLR family: the NODs (NOD1-2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA), the NLRPs (NLRP1-14, also called NALPs) and the IPAF subfamily, consisting of IPAF (NLRC4) and NAIP. Both NOD1 and NOD2 sense bacterial molecules produced during the synthesis, degradation and remodeling of peptidoglycan, a chief component of bacterial cell walls (Lamkanfi and Dixit, 2009). In contrast, several NLRs, including NLRC4 (also called IPAF), NLRP1 and NLRP3 are involved in the assembly of a multiprotein platform called the "inflammasome", which is responsible for caspase-1 activation and the ultimate maturation of cytokines IL-1ß and IL-18 (Martinon et al., 2009). These NLR contain N-

terminal CARDs or PYRIN domains that mediate the assembly of the inflammasome through NOD-mediated oligomerization and interaction with caspase-1 *via* the adaptor ASC. Many of the remaining NLR family members are poorly characterized at present. In following section will discuss the recent studies of the NLRP3 inflammasome, including studies of its regulation and its function in host defense and inflammatory disease.



Figure 1.1 The basic structure of inflammasome

The inflammasome is an intracellular multi-protein scaffold for caspase-1 activation. The inflammasome consists of 3 main components which are NLR proteins, ASC and procaspase-1.

1.5.3.3 The activation of NLRP3 inflammasome

The NLRP3 (also known as NALP3, cryopyrin, CIAS1, PYPAF1, and CLR1.1) inflammasome is currently the best characterized inflammasome, consisting of the NLRP3 scaffold, the ASC (PYCARD) adaptor, and caspase-1. In numerous studies, the NLRP3 has been demonstrated to play a crucial role in caspase-1 activation in response to both microbial and non-microbial stimuli (Ferrari et al., 2006; Martinon et al., 2006; Mariathasan and Monack, 2007; Franchi et al., 2009b; Tschopp and Schroder, 2010). NLRP3 is activated upon exposure to whole pathogens, as well as a number of structurally diverse MAMPs, DAMPs, and environmental irritants (Schroder and Tschopp, 2010). For example, NLRP3 inflammasome can be activated by whole pathogens such as living *Cadida albicans* via Syk signalling (Gross et al., 2009), bacteria-producing pore-forming toxins such as Listeria monocytogenus and Stapphylococcus aureus (Mariathasan et al., 2006), and viruses such as Sendai virus, adenovirus and influenza virus (Kanneganti et al., 2006a; Muruve et al., 2008). A number of crystalline molecules including monosodium urate (MSU), crystalline silica and asbestos have also been demonstrated to activate the NLRP3 inflammasome (Martinon et al., 2006; Cassel et al., 2008; Gasse et al., 2009). In addition, a number of host-derived molecules indicative of injury activate the NLRP3 inflammasome, including extracellular ATP (Mariathasan et al., 2006) and hyaluronan (Yamasaki et al., 2009) that are released by injured cells.

The mechanisms which mediate NLRP3 inflammasome activation are intensely studied. A number of mechanisms have been shown to play a role in activation of the NLRP3 inflammasome; however, the ability of MAMPs to activate the NLRP3 inflammasome has yet to be identified because NLRP3 inflammasome is activated by a large variety of structurally unrelated molecules including TLR ligands and there is no evidence that microbial ligands bind directly to NLRs (Franchi et al., 2010). Recent findings suggest that most or all TLR agonists as well as MDP do not activate the NLRP3 inflammasome directly. Instead, they prime the inflammasome *via* activation of NF- κ B signalling to promote caspase-1 activation (Kahlenberg et al., 2005; Bauernfeind et al., 2009; Franchi et al., 2009b). In addition, a recent study has shown that TNF- α and IL-1 β which result from NF- κ B signalling can promote caspase-1 activation via NLRP3 in response to ATP or silica (Franchi et al., 2009b).

A proposed model of NLRP3 inflammasome activation that is mediated by two signals is shown in Figure 1.2. The first signal 1 is provided by microbial molecules such as TLR ligands or by certain cytokines that induce priming of the inflammasome at least in part by NF-kB and NLRP3 induction (Bauernfeind et al., 2009; Franchi et al., 2009b; Franchi et al., 2010). The second signal directly triggers caspase-1 activation, and can be mediated by at least three separate mechanisms which include ATP-mediated P2X7R signalling, lysosomal membrane rupture with release of cathepsin B and the production of reactive oxygen species (Mariathasan et al., 2006; Gross et al., 2009; Franchi et al., 2010; Schroder and Tschopp, 2010; Tschopp and Schroder, 2010). Extracellular ATP activates the ATP-gated P2X7 receptor (P2X7R), which acts as a cation channel to rapidly induce potent K^+ efflux and a complete collapse of normal ionic gradients (Ferrari et al., 2006). P2X7R activation also recruits pannexin-1 which mediates the formation of a pore that has been implicated in inflammasome activation (Kanneganti et al., 2007). In addition, cathepsin B has been reported to mediate caspase-1 activation in macrophages-induced by silica and amyloid- β but not LPS (Halle et al., 2008). Moreover, either DAMPs or PAMPs, including ATP and particulate/crystalline activators, trigger the generation of reactive oxygen species which could lead to NLRP3 inflammasome complex formation (Martinon, 2010).

Collectively, microbial stimulation and the additional signals may induce different signalling pathways, both of which are required for inflammasome activation. It is interesting to explore the interaction of these different pathways that lead to NLRP3 activation. However, the identification of key mechanisms for NLRP3 activation remains elusive. Future studies are required to determine precise mechanisms required

for NLRP3 activation.



Figure 1.2 The activation of the NLRP3 inflammasome

The activation of NLRP3 inflammasome and subsequent IL-18 processing are mediated by two signals. Microbe and cytokines provide signal 1 for synthesis of pro-IL-1 β and pro-IL-18, as well as priming of the NLRP3 inflammasome. Another important signal is the activation of the NLRP3 inflammasome (signal 2) which could be induced by several stimuli including viruses, bacteria, fungi, danger signal such as ATP, and signalling pathways. The induction of K+ efflux, the generation of mitochondrialderived recative oxygen species (ROS), the lysosomal damage and the resultant release of cathepsin B are also postulated to play a role in NLRP3 inflammasome activation; however, the precise mechanisms controlling NLRP3 inflammasome activation have yet to be fully understood.

1.5.4 Biological roles of IL-18 and clinical relevance

Since its discovery in 1995, IL-18 has been recognized as an immunostimulatory and pro-inflammatory cytokine and has been implicated in several pathologies including rhumatoid arthritis, osteoarthritis, obesity, and inflammatory diseases such as diabetes and periodontitis (Moller et al., 2001; Bresnihan et al., 2002; Esposito et al., 2004; Orozco et al., 2007; Troseid et al., 2010). Indeed, IL-18 is elevated in obesity, metabolic syndrome (Hung et al., 2005), type 2 diabetes (Straczkowski et al., 2007) and periodontal disease (Miranda et al., 2006). IL-18 stimulates either Th1 or Th2 responses, depending on its local cytokine milieu (Nakanishi et al., 2001a). Originally, IL-18 was identified as an IFN- γ inducing factor (IGIF) thus directing the immune system toward Th1 response, in particular when co-stimulation T- and NK cells with IL-12 (Robinson et al., 1997; Okamura et al., 1998). In addition, IL-18 alone has been shown to induce the production of Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 by T- and NK cells, and also PGE2 production by activated macrophages (Kashiwamura et al., 2002). Moreover, IL-18 actions include the induction of the synthesis of TNF- α , IL-1 β , IL-8, intracellular adhesion molecule-1 (ICAM-1), neutrophil activation, and osteoclast activation (Dinarello et al., 1998). Because IL-18 has a variety of immunostimulatory properties, IL-18 may play a role in the pathogenesis of metabolic disease such as diabetes (Troseid et al., 2010) and periodontal disease (Orozco et al., 2007).

1.5.5 IL-18 in diabetes and obesity

Several studies have demonstrated that elevated serum levels of IL-18 is associated with obesity (Hung et al., 2005; Bruun et al., 2007; Evans et al., 2007; Straczkowski et al., 2007), insulin resistance (Bruun et al., 2007) and diabetes (Hivert et al., 2009). In addition, IL-18 has been shown to be elevated in subjects with the metabolic syndrome (Van Guilder et al., 2006). In human type 1 diabetes, elevated serum IL-18 levels are observed in high-risk individuals, before the development of the disease (Nicoletti et al., 2001), whereas in patients there is a correlation between IL-18 levels and autoantibody status (Hanifi-Moghaddam et al., 2003). Cross-sectional studies have also shown that serum levels of IL-18 are higher in patients with type 2 diabetes (Aso et al., 2003; Esposito et al., 2003b; Fischer et al., 2005). In two large studies, elevated IL-18 levels were associated with higher risk of type 2 diabetes, and this association is independent of usual risk factors, including BMI, CRP levels and IL-6 (Hung et al., 2005), as well as other known diabetes risk factors, including dietary aspects (Hivert et al., 2009). Furthermore, circulating levels of IL-18 have been suggested to contribute to complications of diabetes such as microangiopathy and nephropathy in type 2 diabetes (Fujita et al., 2010). Taken together, these data suggest that IL-18 could be a potential biomarker for the development of diabetes.

Polymorphisms in the IL-18 gene at the IL18-BCO2 locus have been shown to be associated with circulating IL-18 levels (He et al., 2010). However, the study had several limitations. For example, the participants included in this study are women of European ancestry, whether this finding applys to men or other ethnicities remains to be determined (He et al., 2010). Interestingly, a study showed that IL-18 gene polymorphism was associated with increased serum levels of IL-18, impaired insulin sensitivity and increased risk of having the metabolic syndrome (Presta et al., 2009), suggesting that IL-18 might be involved in the pathogenesis of the syndrome.

In vivo, the IL-18–knockout (*II18*^{-/-}) and IL-18 receptor–knockout (*II18r1*^{-/-}) mice have a significantly increased body weight compared to wild-type littermates of the same age. IL-18–knockout mice also develop several features characteristic of the metabolic syndrome such as insulin resistance, dyslipidemia, obesity and glucose intolerance (Netea et al., 2006). Furthermore, IL-18 deficiency results in a loss of the circadian regulation of food intake and appetite suppression (Netea et al., 2006). Moreover, IL-18-deficient mice also displayed hyperinsulinemia, consistent with insulin-resistance and hyperglycemia (Boraschi and Dinarello, 2006; Dinarello, 2007).

1.5.6 IL-18 in periodontal diseases

In the periodontitis lesion, high level of pro-inflammatory cytokines and mediators such as IL-1 β , TNF- α , PGE2, IL-8, IL-6 and MMPs have been observed. As there has been demonstrated that IL-18 can induced the release of these key cytokines (Dinarello et al., 1998; Dinarello, 1999; Nakanishi et al., 2001b; Biet et al., 2002). Therefore, IL-18 could possibly be a key factor in the initiation and progression of periodontal diseases. Up-to-date, there is increasing evidence regarding the role of IL-18 in periodontal diseases Table 1.2. The first published data on the levels of IL-18 showed that IL-18 within diseased and healthy human gingiva is positively correlated with deep probing depth (Johnson and Serio, 2005). In agreementt with the findings of Figueredo et al. (2008), levels of GCF IL-18 were higher in patients with chronic periodontitis compared with patients with gingivitis (Figueredo et al., 2008b).

Reference	Study design	Conclusions
(Johnson and Serio,	Cross-sectional	GCF IL-18 is significantly
2005)	Measurement of GCF IL-18 using ELISA	correlated with pocket depth
(Miranda et al., 2005)	Cross-sectional	Serum IL-18 is significantly
	Measurement of serum IL-18 using ELISA	correlated with attachment loss
(Miranda et al., 2006)	Longitudinal study (2 yrs)	No differences were observed
	Measurement of GCF IL-18 using ELISA	for IL-18 levels between
		juvenile idiopathic arthritis
		patients and control subjects
(Orozco et al., 2006)	Cross-sectional	IL-18 levels were higher in
	Measurement of GCF IL-18 using ELISA	GCF from periodontitis
		patients than in that from
		gingivitis patients
(Figueredo et al.,	Cross-sectional	The levels of GCF IL-18 were
2008b)	Measurement of GCF IL-18 using ELISA	higher in periodontitis sites
		than gingivitis sites
(Pradeep et al.,	Cross-sectional	GCF IL-18 levels decreased
2009b)	Measurement of GCF IL-18 using ELISA	after periodontal treatment
(Pradeep et al.,	Cross-sectional	GCF IL-18 level is positively
2009c)	Measurement of GCF IL-18 using ELISA	correlated with the severity of
		periodontal diseases
(Bostanci et al., 2009)	Clinical study: IL-18 mRNA expression in	Positive correlation between
	gingival using real-time PCR	NALP3 and IL-1 β or IL-18
	In vitro study: IL-18 mRNA expression in	expression
	response to P. gingivalis	
(Foster et al., 2007)	In vitro study	P. gingivalis LPS induced IL-
	IL-18 response in monocytes	18 release
(Noack et al., 2008)	Cross-sectional	IL-18 and TLR4 gene
	IL-18 and TLR-4 genotyping	mutations have no major effect
		on aggressive periodontitis
		susceptibility

Table 1.2 The role of IL-18 in periodontal disease

It has been highlighted that the concentration of IL-18 is raised in GCF to the levels above of those IL-1 β (Orozco et al., 2006). In addition, the study examining the relationship between juvenile idiopathic arthritis and periodontitis revealed that serum IL-18 was significantly increased in the subgroup with attachment loss (Miranda et al., 2005; Miranda et al., 2006).Recent studies further comfirmed the positive correlation between IL-18 and the pathogenesis of periodontal diasease (Bostanci et al., 2009; Pradeep et al., 2009b; Pradeep et al., 2009c; Schallhorn et al., 2010). *In vitro*, the secretion of IL-18 and its natural IL-18 binding protein antagonist (IL-18BPa) can be induced by *P. gingivalis* LPS in cultures of the human monocytic cell line (Foster et al.,

2007).

While many of clinical studies have revealed a correlation between IL-18 levels and the clinical parameters of both periodontal disease and diabetes, the knowledge regarding the role of IL-18 that links between these two disorders is unknown. Nevertheless, the biological effects of IL-18 in pathogenesis of periodontal disease remain to be determined. Therefore, the potential role of IL-18 in periodontal diseases and diabetes will be investigated in the present project.

1.6 Aims

- 1. To investigate the concentration of IL-18 in GCF and serum in T2DM and periodontal diseases.
- 2. To investigate the effect of leptin on IL-18 synthesis and secretion in monocytes.
- To analyse whether leptin affects the inflammasome function associated with IL-18 production.
- 4. To investigate the signalling mechanisms that mediate leptin-induced IL-18 release.

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

- 1. Development of IL-18 ELISA and determination of serum and GCF concentrations of IL-18 in T2DM patients with and without periodontal diseases before and after treatment for periodontitis (Chapter 3).
- Stimulation of THP-1 monocytic cell line and primary human monocytes with leptin and LPS from *E.coli* or *P.gingivalis* and investigation of IL-18 mRNA expression and protein secretion. The effect of leptin on production of other cytokines such as IL-1β, IL-6 and IL-8 were also investigated (Chapter 4).
- Stimulation of THP-1 and primary human monocytes with leptin and LPS and investigation of caspase-1 mRNA expression, caspase-1 activation and activity. The inhibitory effect of caspase-1 inhibitor on leptin-induced IL-18 release was also conducted (Chapter 5).
- 4. Analysis of the expression of ObRs genes in THP-1 monocytes and determination of the effect of JAK-2 inhibitor on leptin-induced IL-18 release. Investigation of the effect of ATP on IL-18 release in leptin-treated cells and measurement endogenous ATP production in monocytes. The inhibition of

leptin induced IL-18 release by inhibitor of ATP receptor was also analysed (Chapter 6).

Chapter 2 Materials and Methods

2.1 Cell culture

Unless otherwise stated, all chemical reagents and media were purchased from Sigma-Aldrich (Poole, UK), and all plastic ware from Greiner Bio One (Stonehouse, UK).

2.1.1 THP-1 monocytes

THP-1 is a human monocytic cell line which was originally established from the blood of a one year old boy with acute monocytic leukaemia (Tsuchiya et al., 1980) and is now commercially available. During culture (Figure 2.1), cells are non-adherent and in a pro-monocytic state which exhibit a near-diploid karyotype (Odero et al., 2000). After treatment with vitamin D3 (VitD3), THP-1 cells differentiate into mature monocytes (Schwende et al., 1996), express a range of normal monocytic surface makers, TLRs and other receptors including leptin receptors (Cioffi et al., 1996). Additionally, THP-1 monocytes demonstrate the capability to produce and release a myriad of cytokines in which, therefore, making them a suitable cell model to address the aims of the present study.



Figure 2.1 The morphological characteristics of THP-1 monocyte cell line.

THP-1 monocytes exhibit an even morphology and are large, round, single cells in suspension culture. In addition, THP-1 cells typically do not adhere to the culture plate. After stimulation with VitD3, the cells become more adherent and differentiate into mature monocytes which have similar properties to human primary monocytes.

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. The frozen cells were revived according to the manufacturer's instructions. Briefly, cells were thawed quickly at 37 °C in a water bath by gently agitating vial. The cell suspension was placed in a 75 cm² tissue culture flask. Cell culture medium (RPMI-1640 medium, supplemented with FCS (10 % v/v), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μ g/ml) was added slowly and cells were placed overnight in an incubator set to 37 °C, 5 % CO₂ (Model MCO-17/20 AIC Sanyo, Loughbourgh, UK). 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). The 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

Cultures were examined daily to monitor cell morphology and growth characteristics. Culture of THP-1 monocytes were maintained at a density of $3 - 8 \ge 10^5$ cells/ml in cell culture medium at 37 °C, 5 % CO₂ in an incubator. Medium was changed three times a week. Cells were passaged weekly before they become confluent to assure optimum cell density that allows proper cell growth. 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. Cells in passages from 2-9 were used to set up all experiments.

2.1.1.3 Cell viability

Monitoring of cell viability during routine culture was performed using 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 μ l 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 > 95 % during routine cell culture.

2.1.1.4 Vitamin D3 treatment of THP-1 monocytes

Prior to use in stimulation experiments, THP-1 monocytes (1 x 10^6 cells/ml) were treated with 0.1 μ M vitamin D₃ (1 α , 25-Dihydroxy-Vitamin D₃, Calbiochem, Merck Chemicals, Nottingham, UK) for 48 h. 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 more adherent and have the capability to release mediators like PGE₂ and TNF- α . Also, the expression of the monocyte marker CD14 is increased. The increase of CD14 expression after VitD3 treatment was confirmed in our lab by flow cytometry analysis (Foster et al., 2005).

2.1.1.5 Freezing of THP-1 monocytes

To ensure continuous culture, THP-1 monocytes were frozen in liquid nitrogen. $2 - 4 \times 10^6$ 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 (Model MDF-U30865) over-night. The effect of the propan-2-ol is to allow the cryovials to come to the temperature of the freezer slowly, at about 1°C per minute. The following day, cells were stored in liquid nitrogen storage tank.

2.1.2 Isolation and culture of primary human monocytes

Certain experiments were repeated in human primary monocytes. Primary human monocytes were isolated from buffy coats obtained from the blood of healthy 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). A method which employs anti-CD14 coated magnetic beads was applied to isolate monocytes from buffy coats.

2.1.2.1 Magnetic bead method for isolation of monocytes

Firstly, the peripheral blood mononuclear cells were obtained by differential centrifugation over Histopaque (Sigma-Aldrich). Briefly, blood was transferred to 50 ml polypropylene centrifuge tubes, diluted 1:1 in isolation buffer (PBS/1 mM EDTA, supplemented with 2 % FCS). The diluted blood was overlayed 1:1 on a Histopaque gradient at room temperature. The gradients were then centrifuged at 800 g, 20 °C for 20 min. The buffy coat layer which appeared as a dense white band above the red blood

cell and granulocyte layer was collected and diluted into 50 ml of isolation buffer. After centrifugation for 7 min at 600 g, 4 °C, the cell pellet was resuspended in 50 ml isolation buffer and centrifuged again 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 μ m cell mesh. The mononuclear cells were counted and resuspended to 1 x 10⁸ cells/ml in RoboSep buffer. Subsequently, primary monocytes were purified using a commercial positive selection kit, EasySep® (StemCell Technologies, Grenoble, France) on the fully automated cell separator RoboSep (StemCell Technologies) following the manufacturer's instructions. On the RoboSep, the cell suspension was incubated with monoclonal CD14 antibody 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. As for stimulation experiments, cells were stimulated directly after isolation. For Western blot analysis, 4×10^6 cells were transferred to each well of a 6-well tissue culture plate, incubated overnight at 37 °C, 5 % CO₂ and then used for experiments.

2.1.2.2 Assessing purity of human primary monocytes

To confirm the purification of monocytes yielded by magnetic bead method, CD14 expression on isolated human primary monocytes was analysed with flow cytometry.

4 x 10^6 human primary monocytes were cultured as described in 2.1.2.1. Cells were collected, centrifuged for 5 min at 300 g, 20 °C 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 ml of FACS
buffer were added and cells were centrifuged for 5 min at 300 g, 20 °C. Cells were resuspended in 3 ml of 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 CD14 antibody and isotype control (Serotec, Oxford, UK) as indicated in Table 2.1 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). Acquired data was analysed with WinMDI 2.8 (Joe Trotter, Miscellaneous software).

As stated in manufacturer datasheet, EasySep® CD14 selection kit provides 97.8-99.7% separation performance. Assessing purity by flow cytometry (Figure 2.2) demonstrated that the magnetic bead method yields > 95% pure monocytes compared to isotype control.

In addition, the cytokine response in human primary monocytes was also tested by conducting TNF- α ELISA for cell culture supernatants. TNF- α is one of the first cytokines produced by monocytes in response to pro-inflammatory stimuli (Schindler et al., 1990). As can be seen from Figure 2.3, *E.coli* LPS (100 ng/ml) significantly induced TNF- α production in human primary monocytes compared to unstimulated control indicating an capability to release pro-inflammatory cytokine and a normal response to LPS by the monocytes isolated by magnetic method.

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

Table 2.1 Working concentrations and specifications for flow cytometry antibodies

The table shows the clone, IgG class and the origin of flow cytometry CD14 antibody. Antibody working concentrations were established in titration experiments with the specific antibody and its isotype control.



Figure 2.2 The purity of monocytes isolated by magnetic bead method

After isolation, primary monocytes were analysed for cell surface CD14 expression with flow cytometry. Number presents the percentage of events in the upper left quadrant.



Figure 2.3 TNF- α production in human primary monocytes isolated by magnetic bead method.

Primary monocytes were stimulated *E.coli* LPS (100 ng/ml) for 3 h. Supernatants were collected and analysed with TNF- α ELISA. *E.coli* LPS increased TNF- α secretion in primary monocytes compared with controls. The graph represents mean \pm SD from duplicate cultures of one experiment.

2.2 Reagents

2.2.1 LPS

Ultrapure LPS from *E. coli* 0111.B4 was purchased from Invivogen (via Autogen Bioclear, Wilts, UK) as a TLR4 ligand. Ultrapure LPS from *P. Gingivalis* (#tlrl-pglps) was also purchased from Invivogen as a TLR2 ligand. However, due to the ability of this bacterium to synthesize multiple, structurally different forms of lipid A, recognition of Pg LPS appears to be mediated by either TLR2 or TLR4 (Darveau et al., 2004). LPS working concentrations were prepared in cell culture medium.

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.

2.2.3 Stimulation of monocytes with LPS or leptin

Unless otherwise stated, THP-1 monocytes or primary monocytes of 5 x 10^5 cells /well in 24-well plate were used for all stimulation experiments. For mRNA expression experiments, large scale experiments were set up with 4 x 10^6 cells/well in 6-well plate. Cells were stimulated with either of ultrapure LPS from *E. coli* 0111:B4 (see 2.2.1) or recombinant human leptin (see 2.2.2) at different timepoints (see 4.2.1). Our laboratory has shown that 100 ng/ml *E.coli* LPS is an optimal concentration used in stimulation experiments (Foster *et al.*, 2005) and 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). In addition, the results in dose response experiments showed that 1000 ng/ml leptin stimulated a significant release of IL-18 in THP-1 monocytes (see chapter 4). These LPS and leptin concentrations were used in all subsequent experiments. Cells treated with LPS or leptin were compared with unstimulated cells cultured for the same time period. At any relevant timepoints, supernatants were collected and stored at -80 °C. Also, cells were harvested and after lysation stored at -80 °C for future experiment.

2.2.4 Testing for endotoxin contamination

Using recombinant proteins produced in bacteria such as *E. coli* always involves a risk of contamination with LPS. To exclude the false positive effect of leptin on cytokine production, the endotoxin assay was performed in our laboratory (Jaedicke, 2010).

Recombinant human leptin was tested by Limulus amebocyte lysate (LAL) assay at a concentration of 100 μ g/ml and was found to contain 0.053 ng/ml LPS. The highest leptin concentration used for stimulation experiments in the present study was 1 μ g/ml. This leptin concentration would contain 0.0005 ng/ml LPS which is a significantly low concentration and has no effect on TNF- α production (Jaedicke, 2010). Moreover, leptin at 1 μ g/ml also had no effect on IL-1 β and IL-6 production in THP-1 monocytes (see 4.2.4). Any observed effect of leptin is therefore unlikely due to LPS contamination.

2.2.4.1 Evaluation of mitogenic or cytotoxic effects of reagents

The potential mitogenic or cytotoxic effect of leptin and LPS in THP-1 monocyte cultures were evaluated with the Cell Titer 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK). In this assay, NADH and NADPH, which are

secreted in by living cells, convert a chemical compound (Owen's reagent) to a coloured product. The conversion directly corresponds to cellular activity, providing readout for changes in cell numbers.

The assay was carried out according to the manufacturer's instructions. An 8-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 1 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 30 min of incubation at 37 °C, 5 % CO₂, 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, Preston, UK) 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 2.4.



Figure 2.4 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 8-point standard curve for the Cell Titer 96 cell proliferation assay. Δ OD: OD 460 nm – OD 645 nm.

2.2.4.2 The mitogenic and cytotoxic effect of leptin and LPS

To test the mitogenic or cytotoxic effect of leptin and LPS, THP-1 monocytes (5 x 10^5) were stimulated with 1000 ng/ml leptin or 100 ng/ml *E. coli* LPS for 6 h in triplicate cell cultures. Unstimulated cells served as controls. Cells were collected and processed in the Cell Titer 96 cell proliferation assay as described in 2.2.2.2.

After 6 h of leptin stimulation, no significant change in the cell number of THP-1 monocytes was detected in comparison to the control (p > 0.05, Figure 2.5). The mean cell number was 6.38 x 10^6 cells/ml for leptin stimulated THP-1 monocytes and 7.20 x 10^6 cells/ml for the control. These data show that after 6 h, human recombinant leptin neither has a mitogenic nor a cytotoxic effect on THP-1 monocytes. In contrast, cell numbers of THP-1 monocyte stimulated with *E.coli* LPS were significantly lower (p < p0.05) in comparison to control indicating that E. coli LPS has a cytotoxic effect on the THP-1 monocytes at 6 h. 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 comparable to starting cell number (5 x 10^5) of the experimental setup (Jaedicke, 2010). In summary, results of the Cell Titer 96 cell proliferation assay show that leptin has no proliferative or cytotoxic effects on THP-1 monocytes. Any leptin-induced changes in cytokine response are therefore not due to changes in cell numbers. In addition, although cell numbers are lower after 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 2.5 The effect of leptin on cell proliferation in THP-1 monocytes after 6 h

THP-1 monocytes were stimulated with leptin (1000 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 6 h. The graph represents median \pm SD from one experiment with three independent cultures. Statistics: Mann-Whitney *U* test: * *p*< 0.05.

2.2.5 ATP and inhibitors

Adenosine 5-triphosphate (ATP) A26209 was purchased from Sigma-Aldrich. ATP was freshly prepared and reconstituted to a stock solution with 200 mM Trisbase for each experiment. In addition, ATP concentration used in experiments was chosen based on previous studies applying a comparable experimental setup (Perregaux et al., 2000; Mehta et al., 2001).

To investigate the role of leptin in post-translational processing of IL-18 production, a variety of chemical inhibitors were applied in various experiments. A caspase-1 inhibitor Ac-YVAD, Cat# 400012 was purchased from Calbiochem, Merck Chemicals (Nottingham, UK) and reconstituted to a stock solution of 5 mg/ml with DMSO according to the manufacturer's instructions. Working dilutions were prepared in cell culture medium. Reconstituted caspase-1 inhibitor Ac-YVAD was stored at -20 °C.

Also, JAK-2 inhibitor AG490, Cat# 658411 was purchased from Calbiochem, Merck Chemicals as a liquid solution of 100 mM (5 mg/170 μ l) in DMSO. The inhibitor was aliquoted and stored in -80 °C.

Furthermore, P2X7 receptor inhibitor, pyridoxal phosphate-6-azo (benzene-2, 4disulfonic acid) tetrasodium salt hydrate (PPADS), Cat# P178 was purchased from Sigma-Aldrich (Poole, UK). The inhibitor was reconstituted to a stock solution of 10 mM with H_2O according to the manufacturer's instructions. Reconstituted PPADS was aliquoted and stored at -20 °C.

All the inhibitors used in this study were tested for mitogenic or cytotoxic effect by the Cell Titer 96 cell proliferation assay. As shown in Figure 2.6, after 6 h of caspase-1 inhibitor Ac-YVAD stimulation, no significant change in the mean cell number of THP-1 monocytes was detected in comparison to the control (p > 0.05). The cell number was

7.20 x 10^5 cells/ml for the control cultures and 7.45 x 10^5 cells/ml for Ac-YVAD stimulated THP-1 monocytes, respectively. The same number was also observed in DMSO solvent control. Also, as shown in Figure 2.7, there is no significant change in the cell number of THP-1 monocytes after treated with AG 490 and PPADS in comparison to the control (p > 0.05). The cell number was 1.13 x 10^6 cells/ml, 1.17 x 10^6 cells/ml and 1.18 x 10^6 cells/ml for the control, AG 490 and PPADS, respectively. These data show that caspase-1 inhibitor Ac-YVAD, JAK-2 inhibitor AG490 and PPADS neither had a mitogenic nor a cytotoxic effect on THP-1 monocytes.



Figure 2.6 The cytotoxicity of caspase-1 inhibitor Ac-YVAD on THP-1 monocytes after 6 h

THP-1 monocytes were stimulated with leptin (1000 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 6 h. The graph represents median \pm SD from one experiment with three independent cultures. Statistics: Mann-Whitney *U* test: * *p*< 0.05.



Figure 2.7 The cytotoxicity of JAK-2 inhibitor (AG490) and P2X7 receptor inhibitor PPADS on THP-1 monocytes after 3 h

THP-1 monocytes were stimulated with AG 490 and PPADS for 3h. Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay. The graph represents median \pm SD from one experiment with three independent cultures. Statistics: Mann-Whitney *U* test: * *p*<0.05.

2.3 Enzyme-linked immunosorbent assays (ELISA)

2.3.1 Development of IL-18 ELISA

Sandwich ELISAs were developed in our laboratory to detect IL-18 as a protocol adapted from Dr. Xiaoqing Wei (Cardiff University, UK). Briefly, 96-well microplates with high protein binding properties (Greiner Bio One) were coated overnight with monoclonal anti-human IL-18 antibody (Cat No. DO44-3, R&D Systems, Abingdon, UK) in 0.1 M NaHCO₂ pH 8.5. After washing with PBS/0.05% Tween-20 and blocking with 10% FCS PBS for 2 h, plates were again washed, and samples of IL-18 standard (rhIL-18 (Cat No. B001-5, MBL, R&D Systems) or unknowns were added. Samples were incubated for 2 h and then washed in PBS/0.05% Tween-20 and the detection Ab (biotinated anti-hIL-18 antibody, Cat No. DO45-6, R&D Systems) added at a 1:1000 dilution in 10% FCS PBS and incubated for 2 h. After washing, the bound Ab was detected with 1/200 diluted Avidin-HRP (Sigma-Aldrich) that was incubated for 30 min. A final wash was then performed. Tetramethylbenzidine (TMB) and H_2O_2 substrate solution was added to each well and incubated for 30 min. Color development was stopped with 2 N H_2SO_4 , and the resulting absorbances were read at 450 nm on a FL600 Microplate fluorescence reader (BioTek). To correct for the background absorbance of the plate, a second reading at 550 nm was subtracted. Concentrations were determined by 4-parameter standard curve-fitting procedures using KC4 Kineticalc 2.7 for windows.

2.3.2 IL-8, IL-6, IL-1β and TNF-α ELISA

IL-8 IL-6, IL-1 β and TNF- α concentrations in cell culture supernatants (retained from cell collections for other experiments and frozen at -80 °C until further processing) were also 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 2.2.

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 block buffer (1 % BSA in PBS, w/v for the IL-6, IL-1 β and TNF- α ELISA; 1 % BSA in PBS, w/v with 0.05% NaN₃ for the IL-8 ELISA) for one hour. After repeating the washing step, $100 \,\mu$ l standards (in triplicates, serial dilution prepared in reagent diluent (RD)) or sample (in duplicates) were applied. A triplicate RD sample (1 % BSA in PBS, w/v for IL-6, IL-1 β and TNF- α ELISA; 0.1 % BSA, 0.05% Tween 20 in Tris-buffered Saline, w/v for IL-8 ELISA) 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 plate was incubated with 100 µl horse radish peroxidase (diluted in RD) for 20 minutes and kept away from direct light by covering with aluminium foil. The plate was washed again and 100 µl of a 1:1 dilution of TMB and H₂O₂ (Substrate reagent kit, R&D Systems) was applied. The plate was again incubated with a foil covering and the reaction was stopped with 50 μ l 2 N H₂SO₄ 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 curve fit.

Examples of a standard curve for each ELISA are shown in Figure 2.8 - Figure 2.11.

Table 2.2 Antibody	working	concentrations	and	standard	curve	detection	ranges
for ELISAs							

ELISA	Capture antibody	Detection antibody	Standard curve range [pg/ml]
IL-8	4 µg/ml	20 ng/ml	2000 - 31.25
IL-6	2 µg/ml	200 ng/ml	600 - 9.375
IL-1β	4 μg/ml	300 ng/ml	250 - 3.9
TNF-α	4 µg/ml	250 ng/ml	1000 - 15.625

The table lists the capture and detection antibody working concentrations and the ranges of the standard curves for the IL-8, IL-6, IL-1 β and TNF- α ELISA.



Figure 2.8 Standard curve of the IL-8 ELISA

The Δ OD of the different IL-8 concentrations was plotted against the IL-8 concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the IL-8 ELISA. Delta OD: OD 450 nm – OD 550 nm



Figure 2.9 Standard curve of the IL-1ß ELISA

The Δ OD of the different IL-1 β concentrations were plotted against the IL-1 β concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the IL-1 β ELISA. Delta OD: OD 450 nm – OD 550 nm.



Figure 2.10 Standard curve of the IL-6 ELISA

The Δ OD of the different IL-6 concentrations was plotted against the IL-6 concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the IL-6 ELISA. Delta OD: OD 450 nm – OD 550 nm.



Figure 2.11 Standard curve of the TNF-α ELISA

The delta OD of the different TNF- α concentration 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. Delta OD: OD 450 nm – OD 550 nm.

2.4 mRNA analysis

2.4.1 Analysis of mRNA expression

The investigation of mRNA expression in THP-1 cells was performed using conventional RT-PCR and Real-time RT-PCR.

2.4.2 RNA extraction

Total RNA was isolated from culture cells (4 x 10^{6} cells/well) using a commercial RNA extraction kit (GenEluteTM, Sigma-Aldrich). The procedure was performed according to the manufacturer's instructions. Cells 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, Buckinghamshire, UK). 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 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.

2.4.3 The cDNA synthesis by reverse transcription

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

2 µl 10X RT buffer

0.8 µl 10X Random Primers

2 µl 25X dNTP mix

1 µl MultiScribe Reverse Transcriptase

1 µl RNase Inhibitor

 $3.2 \,\mu l$ Nuclease-free H₂O

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, Warrington, UK) with the following settings:

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

cDNA samples were then stored at 4 °C until further processing.

2.4.4 The conventional RT-PCR

The mRNA expression of IL-18 and other related genes was analysed using a conventional PCR method. The primer sets specific for each gene and the PCR conditions are summarized in the Table 2.3. The PCR was performed using Biomix Red DNA polymerase (Bioline, London, UK) with 2.5 μ l of cDNA sample. PCR amplification was conducted for 35 cycles and the products of the PCR were then separated by 3 % agarose gel electrophoresis visualized with ethidium bromide staining, and viewed under UV light.

		Annealing	Product
Gene	Primer sequence (5'-3')	Temp. (° C)	size (bp)
IL-18	FGCT TGA ATC TAA ATT ATC AGT C	50	335
(Akita et al., 1997)	RCAA ATT GCA TCT TAT TAT CAT G		
IL-1β	FGGA TAT GGA GCA ACA AGT GG	60	264
(Netea et al., 2009)	RATG TAC CAG TTG GGG AAC TG		
Ob-Ra	FCCC ATT GAG AAG TAC CAG TTC AGT C	60	297
(Tsiotra et al., 2000)	RCAT GAT TAG ACT TCA AAG AAT GTC CG		
Ob-Rb	FGAA GAT GTT CCG AAC CCC AAG AAT TG	60	428
(Bennett et al., 1996)	RCTA GAG AAG CAC TTG GTG ACT GAA C		
Cas-1	FATC CGT TCC ATG GGT GAA GGT ACA	60	616
(Tardif et al., 2004)	RCAA ATG CCT CCA GCT CTC TAA TCA		
ASC	FGGA CGC CTT GGC CCT CAC CG	65	150
(Das et al., 2006)	RGGC GCG GCT CCA GAG CCC TG		
NALP-3	FCGA GGG GTC AGA CAG AGA AG	58	206
(Ustek et al., 2007)	RTTC CTG GCA TAT CAC AGT GG		
β2Μ	F ACC CCC ACT GAA AAA GAT GA	60	120
	RCTT ATG CAC GCT TAA CTA TC		

Table 2.3 List of primers used in the RT-PCR analyses

2.4.5 Real-Time RT-PCR

The quantification of IL-18 and other gene of interest mRNA levels were further analysed using Real-Time RT-PCR. Real-Time PCR, also called quantitative polymerase chain reaction (qPCR), is one of the most powerful and sensitive gene analysis techniques available. A basic PCR run can be divided into three phases: exponential, linear and plateau phase (Figure 2.12). The reaction at exponential phase is very specific and precise. Exponential amplification occurs because all of the reagents are fresh and available, the kinetics of the reaction push the reaction to favor doubling of amplicon. As the reaction progresses to linear phase, some of the reagents are being consumed as a result of amplification. The reactions start to slow down and the PCR product is no longer being doubled at each cycle. Eventually, when the reaction reaches the plateau phase, the reaction has stopped. The PCR products will begin to degrade. Each reaction will plateau at a different point, due to the different reaction kinetics for each sample. These differences can be seen in the plateau phase. The plateau phase is where conventional PCR takes its measurement which only gives 'semi-quantitative' results. However, in Real-Time PCR, data is collected during the exponential phase of PCR when the quantity of the PCR product is directly proportional to the amount of template nucleic acid. Therefore, Real-Time PCR provides the most precise and accurate data for quantitation.



Figure 2.12 The basic principle of PCR analysis

The conventional PCR measures the amplification at the plateau phase, giving variable results. However, Real-Time PCR measures the product at the exponential phase, obtaining more accurate quantitative data. The PCR cycle at which the sample reaches a fluorescent intensity above background is the Cycle threshold or Ct.

85

Within the exponential phase, the Real-Time PCR machine calculates two values (Figure 2.12). The Threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the sample reaches this level is called the cycle threshold (Ct). The lower the Ct value, the more DNA of the gene of interest was in the sample and therefore the threshold is reached earlier. 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 (NFQ) at the 3' end of the probe, which suppresses the reporter dye fluorescence unless activated. During the reaction, cleavage of the probe by DNA polymerase separates the reporter dye and the quencher dye. This separation results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any nonspecific amplification is not detected. 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. $\delta Ct = Ct_{(target gene)} Ct_{(reference gene)}$
- 2. $\delta\delta Ct = \delta Ct$ (stimulation) δCt (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 DNase/RNase-free water was added.

A master mix of SensiMix with either the TaqMan Probe (IL-18: Hs99999040_m1; NLRP-3: Hs00918082_m1; IL-1β: Hs01555410_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 H₂O

2.5 μ l 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 for 2 min – 95 °C for 10 min – 40 X 95 °C for 15 s – 60 °C for 1 min

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

2.5 Determination of caspase-1 activity

The activity of caspase-1 was measured using a caspase-1 colorimetric assay (R&D Systems). In this assay, caspase-1 cleaves a specific peptide (WEHD) conjugated to the color reporter molecule p-nitroanaline (pNA) producing a color which can be quantified spectrophotometrically. The assay was carried out according to the manufacturer's instructions. Briefly, 4×10^6 cells were lysed in 150 µl lysis buffer on ice for 10 min and then centrifuged at 10,000g for 1 min. The lysates were collected and assayed for caspase-1 activity. The protein content in cell lysates was determined using a Bradford protein assay kit (Pierce Chemicals, Fischer Scientific, Leicestershire, UK). An 8-point standard curve of 2 units recombinant caspase-1 (Sigma-Aldrich) as the highest standard was produced using a 2-fold dilution series in dilution buffer. Dilution buffer alone was used as a zero standard. In a 96 well flat bottom microplate, 50 µl of standards or lysates were added into each well. Following this, 50 µl of reaction buffer was added in to each standards and samples. Finally, the caspase-1 colorimetric substrate (WEHD-pNA) was added into each reaction and then incubated at 37 °C for 2 h. The plate was read at 405 nm wavelength on microplate reader. The caspase-1 activities were calculated by linear standard curve fitting (Figure 2.13) and expressed as a specific activity (pmol/min/mg protein). One unit was defined as an activity that releases 1 pmol WEHD-pNA per minute.



Figure 2.13 The standard curve of caspase-1 assay

A seven point standard curve was created using 2-fold serial dilutions with 2 units of recombinant caspase-1 as a highest standard. The standard curve was calculated using linear curve fit. Each value is a mean of duplicate standards.

2.6 Immunoprecipitation and Western blot

2.6.1 Lysis of cells

THP-1 monocytes or primary monocytes (4 x 10⁶) were collected and centrifuged for 5 min at 150 g, 20 °C. The cell pellet was resuspended in 100 μ l (500 μ l for immunoprecipitation (IP) see 2.6.3) cell lysis buffer (150 mM NaCl; 1% NP40; 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8) and incubated on ice for 15 min. The cell lysate was centrifuged for 10 min at 12000 g, 4 °C. 20 μ l of the supernatants were retained for total protein analysis as described in 2.6.2. 80 μ l were diluted 1:1 with sample loading buffer (Sample loading buffer 1; 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, 0.1M DTT, w/v: Sample loading buffer 2; 0.01M NaPP, / 60mM Tris, 1% SDS, 8M Urea, 0.1% Triton X100, 0.01mM β -mercaptoethanol, 0.15% Bromophenol blue). The sample lysates were processed immediately for Western blot.

2.6.2 Bradford protein determination assay

Bradford protein assay (Pierce Chemicals) was performed to determine the protein concentration in cell lysates for western blot and caspase-1 activity experiments. In this assay, proteins form a complex with the Coomassie G-250 dye inducing a colour change from brown to blue. The intensity of the colour change correlates directly with the amount of protein in a sample. The assay was carried out according to the manufacturer's instructions. Briefly, a twelve-point standard curve of BSA in lysis buffer was produced with the following concentrations: 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250, 125 and 25 μ g/ml. Lysis buffer was included as a negative control. In a 96-well tissue culture plate, 5 μ l of standards and samples were added into

each well in triplicates. The Coomassie reagent (250 μ l) was added to each well. The assay was placed on a plate shaker for 30 s and incubated for 10 min at room temperature. The plate was read on a spectrophotometer (FL 600 Microplate Fluorescence Reader, BioTek,) at 562 nm (Figure 2.14).



Figure 2.14 The standard curve of Bradford protein determination assay.

A twelve- point standard curve was generated using a series dilution of BSA with highest concentration at 4000 μ g/ml. The standard curve was calculated using 4-parameters curve fit. Each value is a mean of triplicate standards.

2.6.3 Immunoprecipitation

The immunoprecipitation of caspase-1 p10 was performed using Sigma Protein G Immunoprecipitation kit, following the manufacturer's instructions. Cells were collected and lysates prepared as described above (2.6.1). 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 anti human caspase-1 p10, sc-515 (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 μ l 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.

2.6.4 Western blot analysis

Protein concentration of total cell lysates and immunoprecipitates were analysed with Western blot using XCell SureLock[™] Mini-Cell electrophoresis system (Invitrogen®, Paisley, UK). Samples were heat denatured for 5 min, at 95 °C on a dry heat block and
20 µl of samples were loaded on each lane of the 10-20% Novex® pre-cast Tris-glycine gel (Invitrogen, Paisley, UK) in running buffer (192 mM Glycine, 25 mM Tris Base, 0.1 % SDS, w/v, pH 8.3). 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 90 min at 125 V constant, 30-40 mA (Consort, Flowgen Instruments Ltd., Kent, UK).

Proteins were blotting to a Hybord P PVDF Membrane (GE Healthcare, Amersham, Buckinghamshire, UK) using the XCell IITM Blot Module (Invitrogen ®, Paisley, UK) in transfer buffer (96 mM Glycine, 12 mM Tris Base, 20% methanol, w/v, pH 8.3), 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 90 min at 25 V constant, 100 mA (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 H2O, pH 7.5) until clear. The membrane was blocked for 1 h at RT 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 rabbit anticaspase-1 p10 (Santa Cruz, Biotechnology, product sc-515) (1:100, in blocking solution) or rabbit-anti human IL-18 (Santa Cruz, Biotechnology, product sc-7954) (1:200, in blocking solution) or goat-anti human caspase-1 p20 (Santa Cruz, Biotechnology, product sc-1780) (1:100, in blocking solution) at 4 °C. Next day, the membrane was washed 3 times with TBS-T and incubated with the secondary HRPlinked polyclonal antibody donkey-anti goat (Santa Cruz, Biotechnology, product sc-2020, Wembley, UK) 1:20000, or donkey-anti rabbit (GE Healthcare, Amersham) 1:20000, in blocking solution) for 1 h at RT. The membrane was washed 2 times for 5 min in TBS-T. 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).

2.7 ATP determination assay

The quantitative determination of ATP was performed using ATP determination kit (Molecular ProbesTM (A22066), Invitrogen, Paisley, UK). The assay is based on the requirement for ATP in order to produce light from the reaction between luciferase and its substrate D-luciferin. Supernatants were collected and assayed freshly because of the instability of ATP in the samples. Briefly, standard reaction solution was prepared following the manufacturer's instructions. A seven-point standard curve of ATP standard solution was generated with the following concentrations: 1000, 500, 250, 125, 62.5, 31.25 and 15.625 nM. For standard curve and sample analysis, 90 μ l of the standard reaction solution was placed in 96- well plate and measured the background luminescence at 560 nm in the luminometer (Microlumat Plus LB 96V, Berthold Technology, Hertfordshire, UK). The reaction was started by adding 10 μ l of each standard or sample solutions. Protect the plate from light. After 15 min incubation, the reaction luminescence was read. The background luminescence was subtracted and a standard curve generated using linear curve fit (Figure 2.15). The ATP concentration in the experimental samples was then calculated from the standard curve.



Figure 2.15 The standard curve of ATP determination assay.

A seven- point standard curve was generated using a series dilution of ATP solution with highest concentration at 1000 nM. The standard curve was calculated using linear curve fit. Each value is a mean of duplicate standards.

2.8 Subject population

The measurement of IL-18 levels in clinical samples from T2DM and non-diabetic control subjects was investigated. The study was approved by the Sunderland Research Ethics Committee (ref 06/Q0904/8). Written informed consent was obtained from all subjects before inclusion in the study. The recruitment of subjects including screening, clinical data and sample collection and treatment of patients were conducted with the help of a clinical team (Prof Philip Preshaw, Rebecca Wassall, Susan Bissett, Hannah Fraser, Kerry Stone, School of Dental Sciences, Newcastle University). Diabetic patients were enrolled from the GP practices and secondary care diabetes clinics (Newcastle). The non-diabetic controls were invited from consultant clinics at Newcastle Dental Hospital or from staff of the dental School, Newcastle University. T2DM and non-diabetic patients with and without periodontal disease were examined in a periodontitis/diabetic clinic at School of Dental Sciences, Newcastle University.

A total of 101 T2DM and 83 non-diabetic subjects were recruited in this study. Subjects had a minimum of 20 natural teeth and all subjects had to be in good general health. Individuals were excluded from the study if they had any bleeding disorder, were pregnant, had a disease requiring prophylactic antibiotics before dental management, were taking drugs that induce gingival overgrowth, were taking immunosuppressant drugs or if the subject had scaling and root planing in the previous 6 months. An overview of demographics of study population was shown in Table 2.4. The patients were aged from 30-55 years old. Diabetic patients had a mean disease duration of 7 ± 5 years. The HbA1c levels (mean±SD) in diabetic patients and non-diabetic patients were 7.61±0.16 and 5.54±0.3, respectively.

Parameters	Non-diabetic control (n=83)	T2DM (n=101)	p value
Age (years)	48±7	48±6	0.7
Gender (male/female)	50/33	67/34	0.5/0.7
Years with T2DM	-	7±5	-
HbA1c	5.54±0.3	7.61±0.16	0.00
Race (n)	83	96	0.3
Caucasian	-	1	-
Black	-	4	-
Asian			
Smoking status (n)			
Current	9	10	0.8
Ex	25	37	0.1
Non	49	54	0.6

Table 2.4 Demographics of the study population

The table shows characteristics of the study population according to diabetic status. Age, years with T2DM and HbA1c levels are given as mean \pm SD. Chi-square (χ^2) showed no statistical differences in number, ethnic background and smoking status between diabetic individuals and the control group (p > 0.05). Student's *t*-tests showed no statistical differences in age between diabetic individuals and the control group (p > 0.05). Student's *t*-tests showed significantly higher than of those in non-diabetic subjects (p < 0.05). The subjects were matched for age and smoking status. The differences in age, gender, race and smoking status at baseline were not statistically significant (p > 0.05).

2.8.1 Clinical assessments and periodontal treatment

After confirmation of enrolment, clinical data were recorded including patient characteristics, age, gender, race, smoking status, body mass index (BMI), blood pressure, and results of laboratory tests including triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, serum high sensitive C-reactive protein (hsCRP) and haemoglobin A1c (HbA1c). Full-mouth periodontal examinations were carried out on each patient for baseline information (month 0) including plaque index, probing depths, clinical attachment loss, bleeding on probing and radiographic examination. The subjects were then classified into three subgroups as follows; periodontal health, gingivitis or periodontitis group according to the diagnostic criteria that were formulated after consideration of the 2005 European Workshop of Periodontology and the 2007 CDC-AAP collaboration (Tonetti and Claffey, 2005; Page and Eke, 2007) which were as follows:

Periodontal health was defined as ≤ 15% bleeding on probing, no attachment loss, no bone loss and probing depth ≤ 3 mm. The subjects with gingivitis had bleeding on probing > 15%, no attachment loss, no bone loss and probing depth ≤ 4 mm. The subjects with chronic periodontitis had ≥ 6 sites with and probing depth ≥ 5 mm with loss of attachment and/or bone loss.

Along with the clinical examination GCF and blood samples were obtained from the each participant. The patients received non-surgical periodontal treatment comprising oral hygiene instruction and a full mouth instrumentation approach (Quirynen et al., 2000). The periodontitis subjects were followed up after three (month 3) and six (month

6) months. GCF and blood samples were collected again and further periodontal treatment was provided as necessary. Subjects with gingivitis were given oral hygiene instructions and a full mouth instrumentation at the baseline but were not followed up any further. A summary of study flow chart was demonstrated in Figure 2.16.

2.8.2 Sample collection

Blood and GCF samples were taken from all groups at baseline. For periodontitis patients, samples were collected again at month 3 and month 6 after periodontal treatment (Figure 2.16) by the clinical team. Blood samples were obtained by venipuncture 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 cytokine 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 150 µl PBS. Four GCF samples were taken from four different teeth in each subject. 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 2.16 A summary of study flow chart

After screening and confirmation of enrolment, a full- mouth periodontal baseline data were recorded (month 0). In addition, blood and GCF samples were taken from all study subjects and non-surgical periodontal treatment was provided including oral hygiene instruction and a full-mouth instrumentation. For subjects with periodontitis, treatment was continued and blood and GCF samples were taken again at month 3 and 6.

2.9 Statistical analyses

Statistical analysis was performed in SPSS 15.0. Graphs were created in Sigmaplot 11.0. or Microsoft Excel 2007. Box and dot plots were created in SPSS 15.0.

2.9.1 Analysis of cell culture data

Unless otherwise stated, data are presented as the means \pm S.D. of the results from three independent experiments. Parametric data was analysed with ANOVA. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. Non-parametric data was analysed with Kruskal-Wallis. Student's *t*-test or Mann-Whitney *U* test was applied for *post hoc* analysis 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. Statistical analysis of Realtime RT-PCR data was performed on δ Ct values as described by Yuan and colleagues (Yuan et al., 2006).

2.9.2 Analysis of clinical data

Standard transformations such as *square root*, *common log* or *inverse* were used to achieve normal distribution and equality of variance. Data were presented as a box plot, dot plot or table. Parametric data was analysed with ANOVA. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. Non-parametric data was analysed with Kruskal-Wallis. Student's *t*-test or Mann-Whitney *U* test was 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. Non-

parametric data were analysed with Friedman test and Wilcoxon Mann-Whitney test. *P*-values were corrected for multiple comparisons with Bonferroni-Holm. A *p*-value of < 0.05 was considered significant. Spearman correlation analysis was used to determine possible associations between two parameters. Spearman ρ values were considered to be significant when $\rho < 0.05$.

Chapter 3 Analysis of IL-18 concentrations in type 2 diabetes and periodontal disease

3.1 Introduction

Periodontal disease is considered the sixth complication of diabetes (Southerland et al., 2006). A meta-analysis revealed that the prevalence, progression and severity of periodontal diseases are higher in diabetic subjects when compared with non-diabetic ones (Taylor, 2001; Taylor and Borgnakke, 2008; Chavarry et al., 2009), supporting diabetes as a risk factor for periodontitis (Kinane and Bouchard, 2008). Clinical studies have consistently demonstrated a positive association between poor glycaemic control and the severity of periodontal diseases (Seppala and Ainamo, 1994; Tsai et al., 2002; Lim et al., 2007; Bandyopadhyay et al., 2010; Chen et al., 2010). For example, Seppala and Ainamo (1994) reported that the mean percent of sites with loss of proximal alveolar bone was greater in poorly controlled diabetic subjects than in the wellcontrolled diabetic subjects after 2-year follow up. In addition, subjects with acceptable glycaemic control (HbA1c < 8%) showed a lower percentage of sites with bleeding on probing and probing depths \geq 5 mm when compared with those having inadequate glycaemic control (Lim et al., 2007). Furthermore, a recent study demonstrated that the mean probing depth in periodontitis subjects with type 2 diabetes was positively correlated with the glycaemic control status (Chen et al., 2010). These findings are confirmed by a large epidemiological study showing that individuals with poorly controlled diabetes have a significantly higher prevalence of severe periodontitis than those with well-controlled diabetes (odds ratio = 2.90), after controlling for age, education, smoking status, and calculus (Tsai et al., 2002). However, initial analysis in the same study did not demonstrate a significant difference in severe periodontitis prevalence between those with and those without diabetes. A significant association was identified only when glycaemic control status was considered.

Several biological mechanisms have been proposed to explain the inter-relationship between periodontal disease and diabetes including microangiopathy, alterations in collagen metabolism, altered host response, altered subgingival microflora, the AGE-RAGE interaction and hereditary predisposition (Salvi et al., 1997b; Mealey and Ocampo, 2007; Nishimura et al., 2007; Preshaw, 2009). However, the precise mechanistic link between diabetes and periodontal disease is still not completely understood.

Cytokines play a central role in the pathogenesis of periodontal disease (Seymour and Taylor, 2004) and also in the development and progression of diabetes (King, 2008). Elevated serum levels of pro-inflammatory cytokines are detected in the periodontal disease lesion, whereas patients with diabetes have hyper-responsive immune cells that can potentiate the elevated production of pro-inflammatory cytokines (King, 2008; Preshaw, 2009). An increasing number of studies have reported that alterations in inflammatory cytokine levels may mediate the effect of diabetes on periodontal health (Engebretson et al., 2004; Engebretson et al., 2006; Duarte et al., 2007; Santos et al., 2010; Venza et al., 2010). It has been suggested that elevated secretion of PGE₂, IL-1 β , and TNF- α by monocyte, as well as increased levels of PGE₂ and IL-1 β in gingival exudates, are associated with increased severity of periodontal disease in patients with diabetes (Salvi et al., 1997a; Salvi et al., 1997c; Salvi et al., 1998). In addition, analysis of cytokine mRNA expression in gingival biopsies revealed that IL-1 β , IL-6, IFN- γ and RANKL mRNA levels were higher in the diabetic group when compared with the healthy control subjects (Duarte et al., 2007). In contrast, chronic periodontitis patients with type 2 diabetes had significantly lower levels of IL-8 than those without type 2

diabetes and this may be due to an inadequate local response of PMNs which are a major source of IL-8 (Engebretson et al., 2006). Furthermore, Engebretson et al. (2007) reported that the plasma levels of TNF- α were positively correlated with attachment loss and GCF IL-1 β in chronic periodontitis subjects with type 2 diabetes but this study failed to show a correlation between plasma TNF- α and serum glucose or HbA1c which are a strong predictor of diabetes. In addition, no change of TNF- α level was observed in the gingival tissue of diabetic rats (Doxey et al., 1998) and diabetic subjects (Duarte et al., 2007). Thus, it remains unclear whether TNF- α levels influence periodontitis severity or indeed circulating TNF- α influences diabetes progression.

IL-18 is a pleiotropic cytokine with regulatory roles in both innate and adaptive immune responses. Elevated systemic IL-18 levels were reported to be positively associated with several chronic diseases including inflammatory bowel disease, Sjogren's syndrome, rheumatoid arthritis, and diabetes (Mosaad et al., 2003; Manoussakis et al., 2007; Sanchez-Munoz et al., 2008; Hivert et al., 2009). In addition, there is increasing evidence supporting a role for IL-18 in periodontal disease (Foster et al., 2007; Orozco et al., 2007; Figueredo et al., 2008b; Bostanci et al., 2009; Pradeep et al., 2009a). The concentration of IL-18 was investigated in gingival biopsies from periodontitis subjects, and this cytokine was significantly increased adjacent to deep sites (probing depth >6 mm) compared to healthy sites (Johnson and Serio, 2005). It was suggested that periodontal inflammation may be not successfully resolved due to the accumulation of IL-18 within the diseased tissues. Interestingly, the investigation of serum IL-18 levels in juvenile idiopathic arthritis patients with the presence or absence of attachment loss found that there was a markedly increased of serum IL-18 in juvenile idiopathic arthritis patients with the presence of attachment loss.

subgingival microbiota (Miranda et al., 2005). The findings from this study suggested a potential role of IL-18 in periodontal destruction. The level of IL-18 in GCF has also been reported in subsequent studies. Consistently, GCF IL-18 was higher in patients with periodontitis compared with of those with gingivitis (Orozco et al., 2006; Figueredo et al., 2008b; Pradeep et al., 2009b). *In vitro*, Foster et al. (2007) demonstrated that LPS from *P. gingivalis* induced a production of IL-18 and IL-18BPa by THP-1 monocytes suggesting an interaction between periodontal pathogen and IL-18 in immune responses. Oral epithelial cells, which are the primary host-pathogen interface, have also been reported to produce IL-18 in response to LPS stimulation (Sugawara et al., 2001).

In diabetes, the levels of serum IL-18 were found to be elevated in type1 diabetes (Nicoletti et al., 2001; Altinova et al., 2008) and type 2 diabetes patients (Aso et al., 2003; Esposito et al., 2003b; Moriwaki et al., 2003) compared to subjects without diabetes. In addition, circulating IL-18 levels were positively correlated with glycaemic control since serum IL-18 levels increased when patients had higher levels of HbA1c or fasting plasma glucose (Moriwaki et al., 2003). Multivariate regression analysis revealed that plasma IL-18 is closely associated with insulin resistance in both type 2 diabetes subjects and non-diabetic controls, even after adjusting for age, gender, BMI and smoking status (Fischer et al., 2005). In cohort studies, elevated serum IL-18 concentrations were strongly associated with an increased risk of type 2 diabetes (Thorand et al., 2005; Hivert et al., 2009). The association between IL-18 and type 2 diabetes is independent of other known risk factor for diabetes such as age, sex, BMI and parental history of diabetes (Thorand et al., 2005), specific dietary information and inflammatory biomarkers (Hivert et al., 2009).

Although many studies have demonstrated that the biologic activity of a variety of cytokines may be directly relevant to periodontal destruction in diabetic individuals, the mechanisms are not fully understood and still remain controversial. In addition, to my knowledge, there are no detailed studies which have investigated the serum levels of IL-18 in type 2 diabetes subjects with periodontal disease. Therefore, the aim of this study was to investigate the role of serum IL-18 as a potential biomarker in periodontal disease subjects with and without type 2 diabetes. In addition, the serum IL-18 levels were further evaluated 3 months and 6 months after non-surgical periodontal treatment. Furthermore, the possible relationships between serum IL-18 levels and clinical parameters of diabetes such as HbA1c, hsCRP and BMI were also examined. Finally, in order to determine the serum concentrations of IL-18, a sensitive sandwich IL-18 ELISA was initially developed in our laboratory, as the available commercial IL-18 ELISA kits are relatively expensive.

3.2 Results

3.2.1 IL-18 ELISA Development

Development of an IL-18 ELISA requires the optimum working concentrations of capture antibody (CAb), detection antibody (DAb), and the series of IL-18 standard (Wreghitt and Morgan-Capner, 1990). According to a protocol from Dr. Xiaoqing Wei, a pair antibody concentration of 2 μ g/ml of CAb and 0.5 μ g/ml of DAb was used. The ten series of 2-fold serial dilution of IL-18 standard starting from 5000 to 9.77 pg/ml were performed. The standard curve of IL-18 ELISA was shown in Figure 3.1. The R² value of standard curve was close to 1 showing the regression line has a close fit to the data (Schraw and Whiteheart, 2005). The sensitivity of the ELISA was considered as the concentration corresponding to a response at 2.5 standard deviation away from the

mean value of the zero standards (Wreghitt and Morgan-Capner, 1990). The "solve" function in SigmaPlot programme was used to calculate the sensitivity value applying on the 4 parameter curve fit equation. The sensitivity of first IL-18 ELISA using 2 μ g/ml of CAb and 0.5 μ g/ml of Dab was 25.89 pg/ml.



Figure 3.1 The standard curve of IL-18 ELISA

IL-18 ELISA was produced by an adapted protocol from Dr. Xiaoqing Wei. Each value is a mean of triplicate standards.

3.2.1.1 Developing the ELISA to increase sensitivity

Since the levels of IL-18 found in GCF were between 20.6 to 103.3 pg/ml (Miranda et al., 2006; Figueredo et al., 2008a), development of an assay sensitive at low IL-18 concentrations was considered important. Improving of assay sensitivity can be achieved by a variety of adaptations to the ELISA protocol including increasing CAb and DAb concentrations, increasing the incubation times, and appropriate incubation temperature (Kemeny and Challacombe, 1988). The determination whether increasing CAb and/or DAb improved the assay sensitivity was performed. As shown in Figure 3.2, the use of higher concentration of CAb and DAb appeared not to significantly increase assay sensitivity as lower readings and less R^2 value were achieved. In fact, the previous pair antibody concentration (CAb 2 µg/ml vs DAb 0.5 µg/ml; Figure 3.2A) showed a stronger reading than the other three pairs of antibody concentration. Therefore, the CAb 2 µg/ml paired with the DAb 0.5 µg/ml was used in the subsequent assays to determine the IL-18 level of samples.





(A) CAb 2 μ g/ml vs DAb 0.5 μ g/ml (B) CAb 2 μ g/ml vs DAb 1.0 μ g/ml (C) CAb 4 μ g/ml vs DAb 0.5 μ g/ml (D) CAb 4 μ g/ml vs DAb 1.0 μ g/ml

The sensitivity of ELISA is influenced by a variety of factors such as room temperature, incubation time and antibody and standard storage. Either incubation of ELISA plate in an accurate 37 °C incubator or prolonging the incubation time increased the background reading to some extent. However, it was noted that antibody and standard storage are essential in development of sensitive ELISAs. Discrepancies in output reading such as low readings in high concentration standards or high standard deviations between replicates were observed when using old antibodies or inappropriate storage of antibody or standard. Thus, use of all reagents was strictly according to the manufacturer's instructions. In all subsequent ELISAs, an 8-point standard curve of 2500 pg/ml IL-18 as the highest standard was produced using a 2-fold dilution series in reagent diluent. Reagent diluent alone was used as a zero standard. A representative standard curve of IL-18 ELISA is shown in Figure 3.3. The higher reading of the highest standard and the lower reading of zero standards were observed. In addition, a stable absorbance reading was achieved throughout the whole study and the R^2 values were all close to 1. The sensitivity of the IL-18 ELISA was improved, showing the average sensitivity from three independent ELISAs was 5.94 pg/ml (Table 3.1). In addition, the sensitivity of 5.94 pg/ml was generally maintained in all subsequent ELISAs allowing confidence in detection down to this concentration.



Figure 3.3 A representative standard curve of IL-18 ELISA

The Δ OD of the different IL-18 concentrations was plotted against the IL-18 concentration. A 4-parameter curve fit was created to produce the 7-point standard curve for the IL-18 ELISA. Delta OD: OD 450 nm – OD 550 nm

IL-18 ELISA	ELISA 1	ELISA 2	ELISA 3	Mean (n=3)
Mean delta OD of zero standards	0.007	0.012	0.004	0.008
Sensitivity (pg/ml)	5.17	6.82	5.83	5.94

Table 3.1 The sensitivity of IL-18 ELISA from three independent ELISAs

The sensitivity of IL-18 ELISA was calculated using the "solve" function in SigmaPlot programme. The mean OD of zero standards added by 2.5 SD was applied on 4-parameter equation obtained from three independent ELISAs.

3.2.1.2 Intra-and inter-assay variation of IL-18 ELISA

Intra-assay variation for the IL-18 ELISA was determined in 3 different samples with varying IL-18 concentrations. Each sample was assayed for IL-18 levels in 4 replicates in one assay. Inter-assay variation for the IL-18 ELISA was determined in 3 independent assays for 3 different samples. Results are shown in Table 3.2 and Table 3.3. The percentage coefficient of intra-and inter-assay variation was calculated as

follows:
$$\frac{SD}{\text{average}} \ge 100$$

The assay variations were considered the acceptable variation below 10 per cent (Kemeny, 1991). As shown in Table 3.2, the coefficients of intra-assay variation of IL-18 ELISA ranged from 2.17 to 7.58 %. In addition, Table 3.3 shows inter-assay variations were ranged from 1.65 to 6.78 %. Taken together, these indicate a high reproducibility for the established IL-18 ELISA and increase the confidence in this ELISA for testing samples.

Sample	sample 1	sample 2	sample 3
Number of determinations	4	4	4
Mean (pg/ml)	189.09	426.94	1205.08
C.V. (%)	2.17	7.58	4.67

 Table 3.2 Intra-assay variation of IL-18 ELISA

Three different samples were analysed in 4 replicates for IL-18 in one assay for each sample and the intra-assay variation was calculated. The intra-assay variation of IL-18 ELISA expressed as the percentage coefficient of variation (C.V.).

Sample	sample 1	sample 2	sample 3
Number of assays	3	3	3
Mean (pg/ml)	231.24	279.20	793.05
C.V. (%)	6.09	6.78	1.65

Table 3.3 Inter-assay variation IL-18 ELISA

Three different samples were analysed for IL-18 in three independent assays and the inter-assay variation was calculated. The inter-assay variation of IL-18 ELISA expressed as the percentage coefficient of variation (C.V.).

3.2.1.3 Validation of IL-18 ELISA for serum 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, in order to use the IL-18 ELISA for analysing human samples such as serum or GCF samples, validation experiments for the IL-18 ELISA were performed, including spike/recovery and linearity experiments. 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 with 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.

3.2.1.4 Spike/recovery assay

From different studies, serum IL-18 concentrations were found up to 759.2 pg/ml (Moriwaki et al., 2003; Orozco et al., 2006; Dong et al., 2007; Altinova et al., 2008; Hivert et al., 2009) which are still in the range of standard curve of the established IL-18 ELISA. Therefore, neat serum samples were used in spike/recovery experiments. Neat human serum samples were spiked with 1000 pg/ml human recombinant IL-18 (R&D). Reagent diluent (RD) was spiked with the same amount of recombinant IL-18 to create a spiked control. The spiked sample, the neat sample and the spiked control were then diluted 1:2 and 1:4 in RD in a 2-fold dilution series. IL-18 concentrations of all samples were determined with ELISA. Recovery of the spiked sample, the control spike and the serial dilutions was calculated as follows:

spiked sample % recovery: $\frac{\text{spiked sample-neat sample}}{\text{amount spiked}} \ge 100$

spiked control % recovery: $\frac{\text{spiked control}}{\text{amount spiked}} \times 100$

example calculation for % recovery of 1:2 dilutions:

Spiked sample value was used as the expected value if testing linearity of the spiked sample. The neat sample value was used as the expected value if testing linearity of the unspiked sample.

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 and testing sample linearity for IL-18 ELISA are shown in Table 3.4.

The recovery of spiked sample was 81.55 % indicating that there is no significant interfering substances in tested serum samples on the established IL-18 ELISA. In addition, the recovery of the spiked control was 91.87 % indicating that there was no problem in preparing spiked controls. Results from testing of sample linearity were in 80-120 % range except only the 1:4 dilution of spiked sample was 121.37 %. These results indicate the linear relationship between sample dilutions and give confidence that the values of sample dilutions are accurate. Additionally, this will allow comparison of sample values generated from samples run at different dilutions. In conclusion, the validation of the IL-18 ELISAs successfully established the use of these ELISAs for analysis of human serum samples.

Samples	IL-18 (pg/ml)	% Recovery	Linearity (% recovery)
Spiked sample (SS)	927.70	81.56	-
SS 1:2	492.24	-	106.12
SS 1:4	281.49	-	121.37
Neat sample (NS)	112.13	-	-
NS 1:2	49.45	-	88.20
NS 1:4	32.87	-	117.26
Spiked control (SC)	918.74	91.87	-
SC 1:2	486.89	-	105.99
SC 1:4	228.67	-	99.56

Table 3.4 Spike/recovery and linearity of IL-18 ELISA for human serum samples

Human serum samples were spiked with 1000 pg/ml recombinant IL-18. RD was spiked with the same amount (spiked control). 2-fold serial dilutions were prepared and spiked samples, neat samples and spiked controls were assayed for IL-18 concentrations. Results show the obtained IL-18 concentrations, the % recovery and the testing of sample linearity.

3.2.2 IL-18 in serum and GCF samples from T2DM and periodontal disease

A total of 101 T2DM and 83 non-diabetic subjects were screened and recruited in the study as described in section 2.8. Blood and CGF sample were collected and analysed for IL-18 concentrations using the IL-18 ELISA. 98 T2DM and 83 non-diabetic serum samples were analysed for serum IL-18 levels at the baseline of the study. As also shown in section 2.8, demographic analysis demonstrated that there were no significant differences in age, gender, race and smoking status between the T2DM group and the non-diabetic control group.

3.2.2.1 Analysis of serum and GCF IL-18 concentrations in T2DM subjects and non-diabetic controls with and without periodontal disease before periodontal treatment

As shown in Figure 3.4, the serum IL-18 concentrations (median \pm IQR) in the nondiabetic control group and the T2DM group were 66.67 \pm 70.90 pg/ml and 114.54 \pm 89.28 pg/ml, respectively. The statistical analysis demonstrated that serum IL-18 levels were significantly higher in the T2DM group than of those in the non-diabetic control group (p < 0.01). IL-18 concentrations in T2DM subjects were compared according to periodontal status, and the data are shown in Figure 3.5. In the T2DM group, the levels of IL-18 (median \pm IQR) in the healthy, gingivitis and periodontitis subgroups were 82.16 \pm 47.93 pg/ml, 114.31 \pm 144.81 pg/ml and 122.48 \pm 96.96 pg/ml, respectively. In addition, statistical analysis showed that the T2DM with periodontitis subgroup had significantly higher levels of IL-18 than the T2DM with periodontally healthy subgroub (p < 0.05). However, there was no significant differences in serum IL-18 levels between the T2DM with gingivitis patients and the T2DM with periodontitis patients (p > 0.05). The same pattern of IL-18 levels in the non-diabetic control group with different periodontal status was observed. The serum IL-18 concentrations of the non-diabetic control subjects in the periodontally healthy, gingivitis and periodontitis groups were 47.38 \pm 44.77 pg/ml, 65.75 \pm 42.79 pg/ml and 89.97 \pm 90.95 pg/ml, respectively. Interestingly, the concentrations of IL-18 in the non-diabetic controls with periodontitis were significantly higher than those in the non-diabetic controls with gingivitis (p < 0.05), and also those in the non-diabetic controls who were periodontally healthy (p < 0.01). Furthermore, the concentrations of IL-18 between the T2DM group and the non-diabetic control group with the same periodontal status were compared. The T2DM with periodontitis subgroup had significantly higher levels of IL-18 than the non-diabetic controls with periodontitis (p < 0.05). Similar results were obtained when comparing the gingivitis subgroup of T2DM patients to the gingivitis subgroup of non-diabetic controls (p < 0.05) and also when comparing the periodontally healthy subgroup of T2DM patients to the periodontally healthy subgroup of the non-diabetic controls (p < 0.05) and also when comparing the periodontally healthy subgroup of T2DM patients to the periodontally healthy subgroup of the non-diabetic controls (p < 0.05) and also when comparing the periodontally healthy subgroup of T2DM patients to the periodontally healthy subgroup of the non-diabetic controls (p < 0.05).

IL-18 concentrations were also determined in GCF samples. Only one spare GCF sample from each patient was analysed for IL-18 concentrations. GCF volumes were not taken into account to calculate the final concentrations. GCF IL-18 levels are shown in Table 3.5. The concentrations of GCF IL-18 in the periodontally healthy non-diabetic controls and the T2DM patients with periodontitis were 0.58 pg/ml and 1.78 pg/ml, respectively. However, these concentrations were all below detection limit of the IL-18 ELISA. Additionally, IL-18 concentrations in GCF samples of other subgroups were below detection limit of ELISA.



Figure 3.4 Serum IL-18 concentrations between non-diabetic subjects and T2DM subjects

Box plots of serum IL-18 concentrations in non-diabetic control subjects (n = 83) and T2DM patients (n = 98) at the baseline of the study. Statistics: Mann-Whitney U test, ** p < 0.01. • outlier more than 1.5 but less than 3 times of the IQR.



Diabetes status

Figure 3.5 Serum IL-18 concentrations according to periodontal status in nondiabetic control subjects and T2DM subjects

Box plots of serum IL-18 concentrations in non-diabetic control subjects (n = 83) and T2DM patients (n = 98) according to periodontal status at the baseline of the study. Both the non-diabetic and T2DM groups were divided into 3 subgroups; healthy periodontium (control/T2DM; n = 15/14), gingivitis (control/T2DM; n = 20/39) and periodontitis (control/T2DM; n = 48/45). Statistics: Kruskal Wallis test and Mann-Whitney U test. *p*-values were corrected for multiple comparisons with Bonferroni-Holm, * p < 0.05. ** p < 0.01 (according to periodontal status within diabetes or control group); § p < 0.05 (comparison between non-diabetic control and T2DM with the same periodontal status).

Subjects	GCF IL-18 (pg/ml)		
Non-diabetic controls			
healthy periodontium (n=2)	0.58		
Gingivitis (n=1)	n.d.		
Periodontitis (n=4)	n.d.		
T2DM			
healthy periodontium (n=2)	n.d.		
Gingivitis (n=1)	n.d.		
Periodontitis (n=3)	1.78		

Table 3.5 The GCF IL-18 concentrations in T2DM patients and non-diabetic

controls with different periodontal status

The table shows the preliminary data of GCF IL-18 concentrations in different subgroups of study population. GCF IL-18 was measured using ELISA. n.d., non detected

3.2.2.2 Analysis of serum IL-18 concentrations in T2DM subjects and nondiabetic controls with periodontitis after periodontal treatment

For the periodontitis subgroups within both the T2DM patients and the non-diabetic controls, patients were recalled at month 3 and month 6 after initial periodontal treatment and clinical periodontal status was re-evaluated. Blood samples were taken and assayed for IL-18 concentrations using ELISA.

Figure 3.6 shows the levels of serum IL-18 in periodontitis subgroups of the T2DM patients and the non-diabetic controls at baseline of the study and at month 3 and month 6 after periodontal treatment. In the non-diabetic control subjects, the serum IL-18 concentrations (median \pm IQR) at baseline, month 3 and month 6 of the study were 89.97 \pm 90.52 pg/ml, 108.39 \pm 76.77 pg/ml and 103.72 \pm 60.05 pg/ml, respectively. The serum IL-18 levels significantly increased 3 months after periodontal treatment compared to baseline levels in non-diabetic control subjects (p < 0.05). At month 6 after periodontal treatment, serum IL-18 concentrations significantly decreased compared to month 3 concentrations (p < 0.05) but were not significantly difference to the baseline IL-18 concentrations.

Periodontal treatment had no effect on serum IL-18 concentrations in T2DM patients associated with periodontitis. In these patients, the serum IL-18 concentrations at baseline, month 3 and month 6 of the study were 122.48 ± 96.96 pg/ml, 160.49 ± 78.18 pg/ml and 142.04 ± 46.60 pg/ml, respectively. The serum IL-18 concentrations appeared to increase at month 3 after periodontal treatment. However, there were no significant differences in serum IL-18 concentrations after periodontal treatment at month 3 and month 6 compared to pre-treatment values (p > 0.05).



Diabetes status

Figure 3.6 The effect of periodontal treatment on serum IL-18 concentrations in non-diabetic control subjects and T2DM subjects.

Box plots of serum IL-18 concentrations in non-diabetic subjects with periodontitis and T2DM patients with periodontitis. Periodontitis subgroubs in both non-diabetic control subjects and T2DM patients were followed up at month 3 (control/T2DM; n = 40/33) and month 6 (control/T2DM; n = 33/34) after non-surgical periodontal treatment. Statistics: Freidman test and Wilcoxon signed ranks test. * p < 0.05. • outlier more than 1.5 but less than 3 times of the IQR.

3.2.3 The relationship between serum IL-18 and clinical parameters of T2DM

To clarify whether the serum IL-18 levels are associated with glycaemic control, systemic inflammation or risk factors for diabetes such as BMI, the relationships between serum IL-18 concentrations and HbA1c, hsCRP or BMI were analysed using the Spearman correlation test. Blood samples taken from either T2DM patients or non-diabetic control subjects were sent to the Heamatology and Clinical Biochemistry labs of the Royal Victoria Infirmary (Newcastle) to analyse for HbA1c and hsCRP (see 2.8.2). Analysis of correlations between serum IL-18 levels and BMI, HbA1c and hsCRP levels was performed for the whole study population (n= 181) because the same results were obtained when splitting data into different groups according to diabetic or periodontal status.

Spearman correlations between serum IL-18 levels and HbAlc, hsCRP or BMI are shown in Figure 3.7, Figure 3.8, and Figure 3.9, respectively. Levels of serum IL-18 were significantly and positively correlated with HbA1c and BMI (both p < 0.001). In addition, serum IL-18 levels showed a stronger correlation with HbA1c (spearman's $\rho = 0.353$) than serum IL-18 levels with BMI (spearman's $\rho = 0.253$). However, there was no significant correlation between serum IL-18 levels and hsCRP (spearman's $\rho = 0.096$).


Figure 3.7 The relationship of serum IL-18 with HbA1c

The serum IL-18 levels from a total number of 98 T2DM and 83 non-diabetic control subjects were included to determine the relationship between serum IL-18 concentrations and percentage of HbA1c. A dot plot was created to illustrate the association between serun IL-18 levels and HbA1c. Statistics: Spearman correlation analysis.



Figure 3.8 The relationship of serum IL-18 with hsCRP

The serum IL-18 levels from a total number of 98 T2DM and 83 non-diabetic control subjects were included to determine the relationship between serum IL-18 concentrations and serum concentrations of hsCRP. A dot plot was created to illustrate the association between serun IL-18 levels and hsCRP concentrations. Statistics: Spearman correlation analysis.



Figure 3.9 The relationship of serum IL-18 with BMI

The serum IL-18 levels from a total number of 98 T2DM and 83 non-diabetic control subjects were included to determine the relationship between serum IL-18 concentrations and BMI. A dot plot was created to illustrate the association between serun IL-18 levels and BMI. Statistics: Spearman correlation analysis.

3.3 Discussion

It has been suggested that an imbalance of cytokine responses against periodontal pathogens is critical in the initiation and progression of periodontal disease (Okada and Murakami, 1998; Liu et al., 2010). A more thorough understanding of cytokine networks may lead to the development of novel therapies by means of host response modulation for chronic inflammatory diseases such as rheumatoid arthritis and periodontal disease (Preshaw, 2008; Deo and Bhongade, 2010; Feldmann and Maini, 2010). With increased knowledge of the IL-1 family, therapies have now been developed using a recombinant IL-1Ra called anakinra which blocks the IL-1 signaling pathway (Dinarello, 2010). Evidence of IL-18 augmentation of inflammatory response has lead to the development of therapies currently in clinical trials (Anderson et al., 2006; Lu et al., 2008; Wang et al., 2008; Troseid et al., 2010).

A number of studies support the hypothesis that diabetes is a risk factor for periodontitis (Taylor and Borgnakke, 2008; Chavarry et al., 2009). Diabetes manifests a proinflammatory state that exacerbates the host response to periodontal bacteria in periodontitis patients (Southerland et al., 2006; Nishimura et al., 2007; King, 2008). In fact, it has been reported that pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-18 are elevated in diabetic individuals and this could lead to development of diabetic complications such as nephropathy (Moriwaki et al., 2003), atherosclerosis (Aso et al., 2003) and periodontitis (Salvi et al., 1998; Ryan et al., 2003; Nishimura et al., 2007).

The present study demonstrated through the development of an ELISA specific for IL-18, that serum IL-18 concentrations in T2DM patients are significantly higher than nondiabetic controls. In particular, this study showed for the first time that serum IL-18 levels are elevated in T2DM patients with periodontal disease as compared to periodontally healthy non-diabetic controls. Moreover, significant differences in serum IL-18 concentrations in patients with periodontitis, gingivitis and periodontal health were observed irrespective of diabetic status. Subjects with periodontitis have significantly higher serum IL-18 concentrations than subjects with gingivitis or periodontal health. However, the serum IL-18 levels in T2DM patients remained unchanged either after 3-month or after 6-month of periodontal treatment. Finally, the present study revealed that the levels of serum IL-18 are positively correlated with HbA1c and BMI but not with hsCRP levels.

The IL-18 ELISA produced in the present study is able to specifically detect the presence of IL-18 in samples demonstrated by the positive and negative controls. The IL-18 ELISA is sensitive to a concentration of 5.94 pg.ml, equivalent to commercial ELISA cytokine kits by R&D systems. The standard curve showed a close fit with 4parameter regression analysis as consistently strong R^2 values were obtained throughout the whole study. In addition, the ELISA has a high reproducibility as clearly shown by a good intra and inter-assay variation, a validated recovery performance and optimum sample linearity which allow confidence in detection of IL-18 concentrations in clinical samples. However, the present study did not obtain a detectable amount of IL-18 in GCF samples. Unlike previous studies, the levels of GCF IL-18 were found to be elevated in periodontitis and associated with the severity of periodontal disease (Orozco et al., 2006; Figueredo et al., 2008b; Pradeep et al., 2009b). The cause of these discrepancies may be due to differences in GCF collection method and data analysis. In Figueredo et al. (2008b) study, five samples were collected and were pooled together before elution and assay for IL-18 concentration. GCF volumes were also taken into account to calculate the actual concentration. Orozco et al. (2006) also used a different technique to collect GCF sample as the sample was collected from two sites and the periopaper strip was left in the gingival sulcus for 30 s for three times per site. In contrast, the present study only analysed GCF IL-18 concentration from one sample for each individual. Therefore, it is likely that the GCF IL-18 concentration may be below the detection limit of the established ELISA.

In agreement with previous studies (Aso et al., 2003; Esposito et al., 2003b; Moriwaki et al., 2003; Fischer et al., 2005; Hivert et al., 2009), the results of the present study have shown that higher serum IL-18 levels were found in T2DM patients when compared with age-, sex-, and smoking status–matched non-diabetic control patients.

The mechanisms responsible for elevation of serum IL-18 in diabetic patients remain unclear. Although hyperglycemia appears likely to play a role, conflicting findings were previously reported regarding the relationship between serum IL-18 levels and HbA1c or plasma glucose levels (Esposito et al., 2002; Aso et al., 2003; Esposito et al., 2003b; Moriwaki et al., 2003). A hyperglycemic glucose clamp study has shown that plasma IL-18 increases within 2 h of clamping in both control subjects and subjects with impaired glucose tolerance (Esposito et al., 2002). In addition, Aso et al. (2003) showed that there is a significant positive correlation between plasma IL-18 levels and fasting plasma glucose. However, this study failed to show a significant correlation between plasma IL-18 levels and HbA1c suggesting a differential effect of the hyperglycemic state (acute vs chronic) on plasma IL-18 concentrations. In contrast, other studies found that serum IL-18 levels are correlated with both HbA1c (Moriwaki et al., 2003) and fasting blood glucose (Esposito et al., 2003b; Moriwaki et al., 2003). The results of the present study are in accordance with the findings of the latter studies. A significant relationship between serum IL-18 levels and HbA1c was detected. Although statistical analysis revealed a significant correlation, it is important to note that the correlation between serum levels of IL-18 and HbA1c was relatively weak (spearman's $\rho = 0.353$). Therefore, the biological importance of the correlation is questionable.

CRP is a member of the class of acute-phase reactants. It is believed to play a role in innate immunity for it is able to promote the function of complement binding to foreign bodies and damaged cells and enhance phagocytosis by macrophages (Marnell et al., 2005; Peisajovich et al., 2008). CRP is considered as an important biomarker of the systemic inflammatory response because the serum levels of CRP are elevated in response to inflammation (Peisajovich et al., 2008). Substantial evidence has demonstrated that increased CRP levels represent a high inflammatory state in several disease pathologies such as rheumatoid arthritis, cardiovascular disease as well as diabetes (Jialal et al., 2004; Emery et al., 2007; Mugabo et al., 2010). Monitoring CRP values is also useful to determine disease progression or the effectiveness of treatment (Emery et al., 2007; Sun et al., 2010). In the present study, although serum levels of IL-18 were increased in T2DM patients, the concentrations of this cytokine did not reflect systemic inflammation, as indicated by the data which showed no significant relationship with hsCRP levels. Similar findings were reported in the previous study (Moriwaki et al., 2003). This could be due to the small sample size of the present study. Further studies in larger patient cohorts will be required to clarify this issue.

BMI is considered a risk indicator for the development of diabetes (Hivert et al., 2009). It has been suggested that IL-18 could be one link through which obesity influences the risk for type 2 diabetes and insulin resistance. The results of present study demonstrated that serum IL-18 levels are significantly correlated with BMI. The relationship between serum IL-18 levels and BMI was previously reported (Hivert et al., 2009). Elevated levels of circulating IL-18 were also found to be correlated with BMI (Esposito et al., 2004; Bruun et al., 2007). In a large case-cohort study, adjustment for BMI attenuated

the associations between IL-18 and incident diabetes (Thorand et al., 2005). These findings are supported by a randomized single-blind trial (Esposito et al., 2003a) which reported that the subject group who successfully underwent a weight loss programme had significantly lower concentrations of serum IL-18 compared with the control group. Additionally, it has been shown that adipocytes are also a source of IL-18, *in vitro* (Skurk et al., 2005).

Taking these findings together, serum IL-18 concentrations are elevated in T2DM patients. The elevation of serum IL-18 is associated with HbA1c and BMI. These data emphasize the systemic pro-inflammatory state in T2DM patients. However, the role of systemic IL-18 in the progression of periodontitis in non-diabetic subjects remains inconclusive. The mean BMI scores of periodontitis subjects were higher than of those periodontally healthy groups in non-diabetic subjects, although the difference was observed as a statistical trend, only (p > 0.05). These data likely suggest that serum IL-18 may not function as a sole mediator but it is mediated by the negative effect of diabetes status and other related risk factor such as obesity. In fact, all diabetic patients in the present study had a significant higher BMI and HbA1c than the non-diabetic subjects. Further studies recruiting BMI-matched subjects may be useful to determine the systemic role of IL-18 in pathogenesis of periodontal disease.

A number of studies have demonstrated that serum IL-18 levels are elevated in various immune diseases (Mosaad et al., 2003; Sakai et al., 2008; Sanchez-Munoz et al., 2008) including diabetes mellitus (Aso et al., 2003; Esposito et al., 2003b; Fischer et al., 2005; Altinova et al., 2008). However, very little is known about the role of IL-18 in T2DM associated with development of periodontal disease. To the best of my knowledge, this is the first study to demonstrate that serum IL-18 concentrations are elevated in T2DM patients with periodontitis. In fact, the levels of serum IL-18 were highest in T2DM

patients with periodontitis compared with either non-diabetic subjects or T2DM subjects with gingivitis or healthy periodontium.

Several studies have examined IL-18 levels in periodontal disease and demonstrated that IL-18 concentrations are higher in GCF and periodontal tissues from diseased sites compared with healthy sites (Johnson and Serio, 2005; Orozco et al., 2006; Figueredo et al., 2008b; Pradeep et al., 2009b). In the present study, serum IL-18 levels were found to be higher in the periodontitis subgroup compared with gingivitis and healthy periodontium subgroups of the non-diabetic subjects. It is more interesting that in T2DM subjects, the same pattern of serum IL-18 concentrations was observed in relation to periodontal status, but the levels found in this group were significantly higher than in the non-diabetic controls. To date, only two studies have reported the serum IL-18 levels in periodontal disease (Miranda et al., 2005; Orozco et al., 2006). Miranda et al. (2005) found that elevated serum IL-18 concentrations in juvenile idiopathic arthritis patients are associated with periodontal attachment loss. This study suggested that IL-18 might be involved in tissue destruction process in the periodontium. However, Orozco et al. (2006) reported that there was no or very little IL-18 detected in the serum samples of both gingivitis and periodontitis patients. One possible explanation for these conflicting data may be differences in the serum collection method. In addition, a different ELISA was used in the latter study and the authors did not report the validation of the ELISA for human serum samples. There is a possibility that certain antibodies used in ELISAs could cross-react with the components in human serum.

The results of the present study showed that the serum levels of IL-18 were higher in T2DM subjects, when compared with non-diabetic controls indicating an alteration in the systemic inflammatory response in T2DM patients. This type of response might make these patients more prone to periodontal disease. However, we should be careful

not to draw premature conclusions regarding the role of IL-18 in the link between T2DM and periodontal disease. Serum cytokine levels often reflect their local concentration in GCF or periodontal tissues in response to periodontal pathogens (Griffiths, 2003). Although, the levels of IL-18 in periodontal tissues and GCF from healthy and diseased conditions were addressed in previous studies (Johnson and Serio, 2005; Orozco et al., 2006; Figueredo et al., 2008b; Pradeep et al., 2009b), the present study failed to detect significant amounts of GCF IL-18 due to study limitations discussed previously. Therefore, further study with a robust GCF collection method and higher sensitivity ELISA is needed to clarify the relationship between serum IL-18 and GCF IL-18 levels in T2DM patients.

Serum IL-18 levels for both T2DM and non-diabetic groups did not change significantly following the non-surgical periodontal therapy. However, a significant increase in the concentration of serum IL-18 was observed at 3 months for non-diabetic control subjects. Only two studies have evaluated the effect of periodontal therapy on GCF levels of IL-18 in diabetic subjects and conflicting results have been shown (Correa et al., 2008; Pradeep et al., 2009b). Correa et al. (2008) found no significant difference in GCF IL-18 levels at 90 days after non-surgical periodontal treatment. In contrast, Pradeep et al. (2009b) demonstrated that GCF IL-18 concentrations are correlated with the severity of periodontal disease and significantly decreased at 6 to 8 weeks after periodontal treatment. Differences in experimental designs among studies, including the GCF collection method, periodontitis severity and ELISA sensitivity play a crucial role in the different findings between the studies.

An increasing number of studies demonstrated that periodontal disease can influence the levels of systemic pro-inflammatory cytokines in T2DM patients (Shimada et al., 2010; Sun et al., 2010). However, the impact of periodontal disease on systemic inflammation

associated with diabetes remains controversial. In individuals with diabetes, clinical intervention trials showed a significant reduction of acute-phase proteins levels such as CRP following periodontal therapy (Lalla et al., 2007b; Sun et al., 2010). Studies have shown that periodontal treatment reduced circulating TNF- α in T2DM subjects with a subsequent reduction in the insulin concentration and HbA1c levels (Iwamoto et al., 2001; Nishimura et al., 2003; Correa et al., 2010). On the other hand, some studies have demonstrated that circulating TNF- α is not reduced following periodontal treatment in subjects with diabetes (Talbert et al., 2006; Lalla et al., 2007b; O'Connell et al., 2008). To the best of my knowledge, there are no published data regarding the effect of periodontal treatment on serum IL-18 levels in T2DM patients. The present study found no significant change in serum IL-18 concentrations in either T2DM or non-diabetic subjects 6 months after periodontal treatment. The serum IL-18 levels are positively correlated with HbA1c as shown by a previous study (Moriwaki et al., 2003), and as shown in the present study. However, one important additional finding of this study is that the levels of HbA1c did not change significantly at 3 and 6 months following nonsurgical periodontal therapy. When considering the limitations of the present study, it should be noted that the role of other variables on glycaemic condition such as diet, physical activity and compliance to medications and changes in hypoglycaemia medications during study period was not determined. Additionally, samples of the present study were obtained under non-fasting conditions, which may somewhat influence the detected serum IL-18 concentrations.

In conclusion, the present study demonstrated for the first time that serum IL-18 levels were elevated in T2DM patients with periodontitis. The levels of serum IL-18 were positively correlated with HbA1c and BMI. These findings suggest that diabetes may be associated with the upregulation of specific proinflammatory cytokines as reflected by

140

elevated levels of serum IL-18, which, in turn, may play a crucial role in the pathogenesis of periodontal disease in diabetic subjects. However, within the limitations of the study, the non-surgical periodontal treatment had no effect on serum IL-18 concentrations. Therefore, further studies with detailed assessment of potential confounding factors are required to determine the role of IL-18 in the bi-directional relationship between periodontal disease and T2DM.

Chapter 4 An investigation into the effect of leptin on IL-18 expression and secretion in monocytes

4.1 Introduction

Leptin is a 16-kDa non-glycosylated peptide hormone which is mainly produced by adipocytes (Maffei et al., 1995). Besides its actions on the regulation of appetite and energy homeostasis (Halaas et al., 1995), leptin has also been implicated in regulating host response to infection and inflammation (Fernandez-Riejos et al., 2010). The leptin receptor is expressed in a number of immune cells such as neutrophils, dendritic cells, lymphocytes and monocytes (Tsiotra et al., 2000; Zarkesh-Esfahani et al., 2001; Mattioli et al., 2005; Schroeter et al., 2007). Several studies have reported that leptin has a range of effects on immune cells (Loffreda et al., 1998; Tilg and Moschen, 2006; Lago et al., 2007a; Kanda and Watanabe, 2008; Kiguchi et al., 2009). For example, it has been shown that leptin can increase the number of macrophage and granulocyte colonies (Mikhail et al., 1997). In addition, Loffreda et al. (1998) demonstrated that peritoneal macrophages from leptin (ob/ob) knock-out mice and leptin receptor (db/db) knock-out mice have an impaired ability to phagocytose and kill *Candida parasilopsis*. In peripheral human monocytes, leptin stimulates proliferation in a dose-dependent manner and enhances the expression of cytokines such as TNF- α and IL-6 (Santos-Alvarez et al., 1999). Furthermore, decreased circulating leptin levels can lead to reduced lymphocyte cellularity in the thymus (Howard et al., 1999). Taken together, these data suggested that leptin can induce proliferation, differentiation and functional activation of hemopoietic cells.

A number of studies have shown that leptin is a potential regulator of cytokine production. Leptin regulates the production of several pro- and anti-inflammatory cytokines (Faggioni et al., 1998; Loffreda et al., 1998; Faggioni et al., 1999; Gabay et al., 2001). Conversely, pro-inflammatory cytokines (i.e. IL-1 and TNF) can increase leptin mRNA expression in adipose tissue (Grunfeld et al., 1996). Leptin was found to enhance the secretion of TNF- α , IL-6 in peritoneal macrophages (Loffreda et al., 1998) and IFN- γ in resting PBMCs (Zarkesh-Esfahani et al., 2001). Gabay et al. (2001) demonstrated that leptin stimulated the production of IL-1Ra in a dose-dependent manner in human monocytes. However, leptin had no effect on the secretion of IL-1 β , even at high doses of leptin (Gabay et al., 2001). In a recent study, leptin significantly up-regulated CC-chemokine ligands (CCLs, a group of chemokines which include CCL3, CCL4 and CCL5) gene expression in cultured murine macrophages (Kiguchi et al., 2009). Moreover, leptin regulates cytokine production in non-immune cells such as microglia, synovial fibroblasts and human keratinocytes (Tang et al., 2007; Kanda and Watanabe, 2008; Tong et al., 2008). Specifically, leptin increased IL-6 production in microglia (Tang et al., 2007) and IL-8 production in synovial fibroblasts (Tong et al., 2008) via the leptin receptor/IRS-1/PI3K/Akt/NF-κB and p300 signalling pathway. Furthermore, leptin enhances human beta-defensin (hBD)-2 production synergistically with IL-1 β in human keratinocytes (Kanda and Watanabe, 2008).

In response to LPS stimulation, leptin exhibits an enhancing effect on cytokine production. Faggioni et al. (1999) reported that ob/ob mice exhibit increased sensitivity to endotoxin-induced liver injury and lethality. *In vitro*, leptin promotes LPS-induced IL-1Ra in macrophages (Faggioni et al., 1999). Similarly, pre-treatment with leptin leads to a significant increase in the LPS-stimulated production of TNF- α , IL-6 and IL-12 by murine peritoneal macrophages (Loffreda et al., 1998).

IL-18 is а pro-inflammatory cytokine which is mainly produced by monocytes/macrophages (Orozco et al., 2007; Arend et al., 2008; Troseid et al., 2010). IL-18 synergizes with IL-12 to stimulate Th1-mediated immune responses through the production of IFN-y. In addition, IL-18 is required for priming NK cells in vivo to induce IFN- γ secretion in response to subsequent stimulation with IL-12 (Chaix et al., 2008). Interestingly, IL-18 alone can stimulate Th2 cytokine production as well as allergic inflammation (Nakanishi et al., 2001b). Thus, IL-18 plays an important role in Th1 and Th2 differentiation depending on the immunological context. Several reports associate an up-regulation of IL-18 to chronic inflammatory diseases, including rheumatoid arthritis, diabetes and periodontitis (Aso et al., 2003; Mosaad et al., 2003; Orozco et al., 2007; Altinova et al., 2008).

Diabetes is a potential risk factor for periodontal disease (Chavarry et al., 2009). Since increased levels of IL-18 and leptin are biomarkers of disease severity in patients with diabetes (Thorand et al., 2005; Welsh et al., 2009), understanding the relationship between these two mediators may provide a mechanism by which diabetes affects periodontal health. Only a few studies have addressed the interaction between leptin and IL-18 (Faggioni et al., 2000; Ahmed et al., 2007; Sennello et al., 2008). In an animal model, Faggioni et al. (2000) demonstrated that leptin-deficient (ob/ob) mice exhibit reduced circulating levels of TNF- α and IL-18 after administration of concanavalin A (Con A). In addition, exogenous leptin replacement completely restored Con A-induced serum IL-18 levels in ob/ob mice. These data suggested that there is a relationship between leptin and IL-18. *In vitro*, leptin was found in bovine monocyte/macrophages to increase expression of TNF- α , IL-12 p40 and caspase-1 that converts pro-IL-1 β and pro-IL-18 into active forms. However, the analysis of caspase-1 mRNA expression was measured by conventional RT-PCR which is only a semi-quantitative assay and this

144

study did not measure IL-18 secretion due to unavailability of bovine IL-18 assay (Ahmed et al., 2007). Conversely, IL-18 induces inflammation in obese leptin-deficient (ob/ob) mice. Injection of obese ob/ob mice with IL-12 plus IL-18 leads to acute pancreatitis as demonstrated by severe hypocalcemia and elevated acute-phase response (Sennello et al., 2008). These results also suggested that leptin deficiency may enhance the sensitivity to the toxic effect of IL-12 and IL-18 combination on pancreas.

Periodontal disease is a chronic inflammatory condition of the supporting tissue of the teeth which results from the interaction between host response and periodontal pathogens. P. gingivalis is a major bacterial species implicated in chronic periodontitis (Slots and Ting, 1999). Up-to date, only one study has reported the role of leptin in immune responses to periodontal pathogen such as P. gingivalis. The study demonstrated that leptin inhibits P. gingivalis LPS-induced decrease in salivary mucin synthesis (Slomiany and Slomiany, 2006). However, there is no detailed study has investigated the relationship between leptin and IL-18 in periodontal disease. Most of previous studies have reported the circulating and GCF levels of IL-18 and leptin to identify the possible link with periodontal disease progression. Serum IL-18 (Miranda et al., 2005) and GCF IL-18 (Johnson and Serio, 2005; Orozco et al., 2006; Figueredo et al., 2008b) levels are elevated in periodontal disease. In agreement of these reports, the present study (chapter 3) also demonstrated that serum IL-18 levels are higher in periodontitis patients compared to subjects with periodontal health. However, increased serum leptin but decreased GCF leptin were found in patients with periodontal disease and associated with disease severity (Johnson and Serio, 2001; Bozkurt et al., 2006; Karthikeyan and Pradeep, 2007b; a).

Although IL-18 and leptin levels have been suggested to be independently correlated with the progression of periodontal disease, the possible mechanistic link between these

two mediators has not been examined. Therefore, the aims of present study were to investigate the *in vitro* effect of leptin on the expression and secretion of IL-18 in the human monocytic cell line THP-1 and in primary monocytes. In addition, this study investigated whether leptin modulates *P.gingivalis* LPS-induced IL-18 secretion. Finally, the effect of leptin on the production of other cytokines (IL-1 β , IL-6 and IL-8) was also examined.

4.2 Results

4.2.1 Investigation of the effect of leptin on IL-18 release in human monocytes

To study the effect of leptin on IL-18 release by THP-1 monocytes, kinetic experiments were performed. THP-1 monocytes (5 x 10^5) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 4 different time points; 3 h, 6 h, 24 h and 48 h. Unstimulated cells served as a control. Supernatants were collected and assayed for IL-18 concentrations with ELISA as described in 2.3.1.

Figure 4.1 shows that leptin and *E.coli* LPS significantly increased IL-18 secretion in THP-1 monocytes after 3, 6, 24 and 48 h incubation (p < 0.05) compared to control. The levels of IL-18 (mean ± SD) in control cultures were 24 ± 7 pg/ml at 3 h stimulation and up to 101 ± 28 pg/ml at 48 h whereas IL-18 concentrations in leptin-stimulated cultures were 79 ± 43 pg/ml (p < 0.05, compared to controls) and 188 ± 87 pg/ml (p < 0.05, compared to controls) and 188 ± 87 pg/ml (p < 0.05, compared to controls) at 3 h and 48 h stimulation, respectively. *E.coli* LPS showed a more potent effect on IL-18 secretion and IL-18 concentrations of up to 528 ± 237 pg/ml were detected after 24 h stimulation (Figure 4.1).

Having found an effect of leptin on IL-18 production by THP-1 monocytes, a doseresponse study could be of interest. In order to investigate this issue, THP-1 monocytes (5×10^5) were treated with leptin (250-1000 ng/ml) for 24 h. The production of IL-18 in the supernatants was analysed using ELISA. As shown in Figure 4.2, leptin at doses of 250-1000 ng/ml significantly induced IL-18 release in THP-1 monocytes compared to controls (p < 0.05). However, there was no significantly difference between the mean levels of IL-18 secreted in response to the 3 different concentrations of leptin. Previous studies have only reported cytokine secretion in response to comparatively high concentrations (500, 1000 ng/ml) of leptin. (Loffreda et al., 1998; Faggioni et al., 1999), however; in this study IL-18 release by THP-1 monocytes can be seen at concentrations as low as 250 ng/ml. As the most substantial response was obtained at 1000 ng/ml leptin, this concentration was therefore used in all subsequent experiments.

To determine whether leptin was able to induce the production of IL-18 by primary human monocytes, PBMCs were isolated from healthy donors and monocytes were purified using the magnetic bead method as described in 2.1.2.1. Primary monocytes were cultured in the absence or presence of leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 h. During the last 30 min of stimulation time (Figure 4.3), the cells were exposed to ATP (6mM). Culture supernatants were collected for determination of IL-18 levels using ELISA. As shown in Figure 4.3, both leptin and *E.coli* LPS significantly enhanced IL-18 release in primary monocytes. It is noted that primary monocytes only secreted the low levels of IL-18 constitutively. The concentration of IL-18 in the unstimulated control was 3.8 ± 1.5 pg/ml. After leptin and *E.coli* LPS stimulation, the IL-18 concentrations were 12.1 ± 2.8 pg/ml and 10.9 ± 3.8 pg/ml (p < 0.05, compared to controls), respectively. Interestingly, ATP significantly enhanced leptin-induced and



Figure 4.1 Kinetics of IL-18 secretion in response to E.coli LPS and leptin

THP-1 monocytes (5 x 10^5) were cultured for different periods of time in the absence (controls) or presence of *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml). IL-18 levels in supernatants was measured by ELISA and compared with controls. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n = 6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA *: *p* < 0.05 compared with controls. Ec: *E.coli*.



Figure 4.2 Dose response of IL-18 secretion to leptin stimulation in THP-1 monocytes

THP-1 monocytes were stimulated with varying concentrations of leptin. IL-18 levels in supernatants was measured by ELISA and compared with controls. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n = 6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA, Student's *t*-test. *: *p* < 0.05 compared with controls.



Figure 4.3 Leptin induced IL-18 production in primary monocytes

Primary monocytes (5 x 10^5) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3h. ATP (6 mM) was added during the last 30 min of stimulation time. Un-stimulated cells served as control. IL-18 levels in supernatants was measured by ELISA and compared with controls. Each value is a mean ± SD of 3 experiments (each comprising duplicate cultures i.e. n = 6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA, Student's *t*-test. *: p < 0.05 compared with controls. Ec: *E.coli*.

4.2.2 The effect of leptin on IL-18 mRNA expression in THP-1 monocytes

To evaluate the effect of leptin on IL-18 production at the mRNA level, THP-1 monocytes (4×10^6) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 1, 3 and 24 h. Unstimulated cells served as the control. Total cellular RNA was extracted and reverse transcribed to produce cDNA and IL-18 gene expression investigated by conventional RT-PCR as described in 2.4.4.

As shown in Figure 4.4, THP-1 monocytes constitutively expressed IL-18 mRNA. The strong expression of IL-18 mRNA can be observed in control cultures and after *E.coli* LPS and leptin stimulation. Treatment with *E.coli* LPS or leptin did not alter the IL-18 mRNA level in THP-1 monocytes at 1, 3 and 24 h.

Because traditional PCR is only semi-quantitative at best, the quantification of IL-18 mRNA expression was then performed using Real-Time PCR as described in 2.4.5. Figure 4.5 shows the results from Real-Time PCR. Compared to control, both leptin and *E.coli* LPS had no effect on IL-18 mRNA expression at 1 and 3 h stimulation (p > 0.05). The expression of IL-18 mRNA was close to 1 in both leptin and *E.coli* LPS-treated cells indicating that there were no differences in IL-18 mRNA expression in those cells compared to control. However, *E.coli* LPS significantly down-regulated IL-18 mRNA expression still remained unchanged in leptin stimulation. Taken all together, the present study demonstrated that leptin enhances IL-18 release but does not regulate IL-18 mRNA expression in human monocytes.



Figure 4.4 The effect of leptin on IL-18 mRNA expression in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were co-cultured in the absence or presence of either leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of IL-18 and β 2M gene were analyzed by RT-PCR. Representative results of 3 independent experiments are shown.



Figure 4.5 IL-18 mRNA expression by THP-1 monocytes after stimulation with *E.coli* LPS and leptin

THP-1 monocytes (4 x 10^6 cells) were stimulated with *E.coli* LPS (100 ng/ml) and leptin (1000 ng/ml) for 1-24 h. The amount of IL-18 mRNA was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent experiments measured in 3 separate occasions. Statistical analysis was performed on δ Ct values using ANOVA. *: *p* < 0.05 compared with controls.

4.2.4 Investigation of the effect of leptin on IL-1β, IL-6 and IL-8 secretion in THP-1 monocytes

To examine the effect of leptin on IL-1 β , IL-6 and IL-8 release by THP-1 monocytes, THP-1 monocytes (5 x 10⁵) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Un-stimulated cells served as the control. Supernatants were collected and assayed for IL-1 β , IL-6 and IL-8 concentrations with ELISAs as described in 2.3.2. Compared to control, *E.coli* LPS significantly enhanced IL-1 β , IL-6 and IL-8 release in THP-1 monocytes (Figure 4.6, Figure 4.7 and Figure 4.8, respectively).The concentrations of IL-1 β (mean ± SD) were 340 ± 69 pg/ml (*E.coli* LPS) vs 1 ± 1 pg/ml (control). The concentrations of IL-6 (mean ± SD) were 49 ± 3 pg/ml (*E.coli* LPS) vs 2 ± 0.4 pg/ml (control). The concentrations of IL-8 (mean ± SD) were 1478 ± 346 pg/ml (*E.coli* LPS) vs 73 ± 16 pg/ml (control). However, leptin significantly induced IL-8 release of 189 ± 78 pg/ml (Figure 4.8, *p* < 0.05) but had no effect on IL-1 β (Figure 4.6) and IL-6 (Figure 4.7) secretion in THP-1 monocytes.



Figure 4.6 The effect of leptin on IL-1β secretion in THP-1 monocytes

THP-1 monocytes (5 x 10^6) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml). IL-1 β levels in supernatants were measured by ELISA and compared with unstimulated controls. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: Student's *t*-test. *: *p* < 0.05 compared with controls. Ec: *E.coli*.



Figure 4.7 The effect of leptin on IL-6 secretion in THP-1 monocytes

THP-1 monocytes (5 x 10⁶) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml). IL-6 levels in supernatants were measured by ELISA and compared with unstimulated controls. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: Student's *t*-test. *: *p* < 0.05 compared with controls. Ec: *E.coli*.



Figure 4.8 The effect of leptin on IL-8 secretion in THP-1 monocytes

THP-1 monocytes (5 x 10^6) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml). IL-8 levels in supernatants were measured by ELISA and compared with unstimulated controls. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: Student's *t*-test. *: *p* < 0.05 compared with controls. Ec: *E.coli*.

4.2.5 Investigation of the effect of leptin on IL-1β expression in THP-1 monocytes

IL-18 is a member of IL-1 cytokine family. However, the present study demonstrated that leptin enhanced IL-18 production at protein level but had no effect on IL-1 β secretion in THP-1 monocytes. To investigate whether leptin regulates IL-1 β at transcriptional level, the IL-1 β mRNA expression was analysed by conventional RT-PCR and Real-Time PCR in comparable experimental setup as described in 4.2.2.

Figure 4.9 and Figure 4.10 show the results from conventional RT-PCR and Real-Time PCR, respectively. The up-regulation of IL-1 β mRNA expression was detected after treatment with *E.coli* LPS for 1, 3 and 24 h (Figure 4.9). In fact, *E.coli* LPS significantly induced a 650-fold increase in IL-1 β mRNA expression at 3 h stimulation (Figure 4.10, *p* < 0.05). This study demonstrated that *E.coli* LPS not only regulates IL-1 β secretion but also enhances IL-1 β mRNA expression. As shown in Figure 4.9, IL-1 β mRNA expression remained unchanged in leptin-treated cells. Additionally, Real-Time PCR analysis demonstrated that IL-1 β mRNA expression in leptin-stimulated cells were comparable to unstimulated control (Figure 4.10). In summary, leptin had no effect on IL-1 β production at both mRNA and protein levels. Although leptin did not induce IL-6 secretion, leptin significantly enhanced IL-8 release in THP-1 monocytes.



Figure 4.9 The effect of leptin on IL-1β mRNA expression in THP-1 monocytes

THP-1 of 4 x 10^6 cells were co-cultured in the absence or presence of either leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of IL-1 β and β 2M gene were analysed by RT-PCR. Representative results of 3 independent experiments are shown.



Figure 4.10 IL-1 β mRNA expression by THP-1 monocytes after stimulation with *E.coli* LPS and leptin

THP-1 monocytes (4 x 10⁶) were stimulated with *E.coli* LPS (100 ng/ml) and leptin (1000 ng/ml) for 1-24 h. The amount of IL-1 β mRNA was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent experiments measured in 3 separate occasions. Statistical analysis was performed on δ Ct values using ANOVA. *: *p* < 0.05 compared with controls. Ec: *E.coli*.

4.2.6 Leptin enhances IL-18 release in *P.gingivalis* LPS treated cells but not in *E.coli* LPS treated cells

To investigate the role of leptin on IL-18 production by THP-1 monocytes in response to *P.gingivalis* LPS, THP-1 monocytes (0.5×10^6) were pre-incubated with leptin (1000 ng/ml) for 5 h. LPS from *E.coli* or *P.gingivalis* (1 ng/ml) were then added for 2 h. Unstimulated cells and cells stimulated with leptin or LPS alone served as control. Supernatants were collected and assayed for IL-18 concentrations with ELISA.

As shown in Figure 4.11, *E.coli* LPS, *P.gingivalis* LPS as well as leptin alone enhanced IL-18 release in monocytes. The IL-18 level (mean \pm SD) in unstimulated controls was 14 \pm 4 pg/ml. After leptin, *E.coli* LPS and *P.gingivalis* LPS stimulation, the IL-18 concentrations increased to 24 \pm 7 pg/ml, 81 \pm 15 pg/ml and 74 \pm 9 pg/ml, respectively. Interestingly, leptin significantly promoted IL-18 release in *P.gingivalis* LPS treated cells (*P.gingivalis* LPS; 74 \pm 9 pg/ml vs leptin plus *P.gingivalis* LPS 103 \pm 12 pg/ml, *p* < 0.05) but not in *E.coli* LPS treated cells. As *E.coli* LPS stimulates TLR4 pathway whereas *P.gingivalis* LPS appears to stimulate TLR2 pathway (Correia et al., 2001; Hirschfeld et al., 2001). These data suggested that leptin may have differential effect on TLR2 and TLR4 signalling pathways.



Figure 4.11 Pre-treatment monocyte with leptin enhances IL-18 release in response to *P.gingivalis* LPS but not to *E.coli* LPS

THP-1 monocytes (5 x 10⁵) were stimulated with leptin for 5 h, followed by stimulation with *E.coli* LPS or *P.gingivalis* LPS for 2 h. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA, *: *p* < 0.05 compared with controls. Ec: *E.coli*. Pg: *P.gingivalis*.

4.3 Discussion

Since obesity is considered an important risk factor for diabetes (Wild et al., 2004; Mealey and Ocampo, 2007), understanding the effect of obesity related cytokines such as leptin on cellular immune responses may provide an insight in the mechanistic link between obesity and diabetes. In addition, this mechanism may also involve in the development of major complications of diabetes such as cardiovascular disease and periodontal disease. Leptin, initially discovered as a regulator of food intake and energy expenditure, has emerged as a pleiotropic molecule involved in a variety of functions in immunity and inflammation (Fantuzzi, 2005; Lago et al., 2007a; Fernandez-Riejos et al., 2010). Alterations in leptin levels and/or in the responsiveness to leptin have been reported not only in obese individuals, but also in patients affected by diabetes, renal failure and periodontal disease (Daschner et al., 1998; Ahmed et al., 2001; Karthikeyan and Pradeep, 2007a). A number of studies suggested that leptin activates immune response through cytokine up-regulation (Loffreda et al., 1998; Martin-Romero et al., 2000; Gabay et al., 2001; Kanda and Watanabe, 2008). In addition, a recent study demonstrated that leptin activates human peripheral blood B cells to induce secretion of IL-6, IL-10, and TNF- α (Agrawal et al., 2011). However, the effect of leptin on the regulation of IL-18 production in human monocytes has not been investigated.

The present study demonstrated for the first time that leptin enhances IL-18 release in human monocytes. However, leptin had no effect on IL-18 mRNA expression. In addition, leptin significantly enhanced IL-8 secretion but did not induce IL-1 β and IL-6 production in THP-1 monocytes. Finally, this study also demonstrated that leptin significantly increased IL-18 release in *P. gingivalis* LPS treated monocytes but not in *E.coli* LPS treated-monocytes.

The results from this study showed that leptin enhances IL-18 secretion in human monocytes which supports the concept that leptin regulates immune responses. It was previously reported that leptin enhanced the production of several pro-inflammatory mediators (Fernandez-Riejos et al., 2010). For example, leptin up-regulates phagocytic activity and enhances the secretion of inflammatory cytokines such as TNF- α and IL-6 from rat isolated macrophages (Loffreda et al., 1998) and human monocytes in response to LPS (Santos-Alvarez et al., 1999; Zarkesh-Esfahani et al., 2001). In addition, leptin increases the proliferation of naive T cells, increases Th1 (IFN-y and IL-2) and suppresses Th2 (IL-4) cytokine production (Lord et al., 1998). Moreover, leptin markedly increases IFN-y-induced NO and PGE₂ production in a concentrationdependent manner by increasing NOS and cyclooxygenase-2 expression in macrophages (Raso et al., 2002). Furthermore, leptin activated microglia, which are phagocytic and antigen presenting cells, and induced pro-inflammatory cytokines, such as IL-1β, through the JAK2–STAT3 pathway (Pinteaux et al., 2007). In some studies, it is noted that leptin alone can induce cytokine production such as IL-6 in microglia (Tang et al., 2007), IL-8 in synovial fibroblasts (Tong et al., 2008), or IL-1Ra in human monocytes (Gabay et al., 2001). However, the other studies demonstrated that leptin requires a pre-treatment or co-stimulation with LPS in order to significantly induce the secretion of TNF- α and IL-6 in human monocytes (Santos-Alvarez et al., 1999) and murine macrophages (Loffreda et al., 1998). The difference in results may be explained by the differential regulation of cytokines in different cell types. To date, the knowledge regarding the relationships between leptin and IL-18 is very limited (Faggioni et al., 2000; Ahmed et al., 2007). Faggioni et al. (2000) found that leptin-deficient (ob/ob) mice showed a lower production of IL-18 in response to Con A suggesting the association between leptin and IL-18. Another study attempted to examine the IL-18
mRNA expression in bovine monocyte/ mcacrophages after leptin stimulation and suggested that leptin could induce IL-18 release through the induction of caspase-1 expression (Ahmed et al., 2007).

The present study is the first study to show that leptin alone can induce the secretion of IL-18 in human monocytes. Although the effect of leptin on IL-18 secretion is not comparable to LPS, the significant up-regulation of IL-18 release was observed at all time points (3, 6, 24 and 48 h stimulation) compared with unstimulated controls. Previous studies found that high concentrations (500, 1000 ng/ml) of leptin are required to stimulate TNF- α and IL-6 secretion by murine macrophages (Loffreda et al., 1998) and IL-1Ra secretion in macrophages (Faggioni et al., 1999). However, in the present study, lower concentration of leptin (250 ng/ml) also shows a significant increase in IL-18 secretion by THP-1 monocytes.

Contamination of any component of cell incubation by endotoxin could activate cells and induce the production of cytokines. In the present study, the effect of leptin on IL-18 secretion is unlikely due to any LPS contamination, as evidenced by significantly low amount of LPS in recombinant leptin tested by LAL assay as well as the lack of an appreciable effect of leptin on IL-1 β production at mRNA and protein levels. In addition, leptin showed no effect on IL-6 secretion in THP-1 monocytes. Furthermore, our group currently showed that leptin lacks of effect on TNF- α secretion by human monocytes (Jaedicke, 2010). Because IL-1 β , TNF- α and IL-6 are one of the sensitive cytokines in response to LPS by monocytes and the present study showed that leptin did not induce the secretion of these cytokines. Thus, the effect of leptin on IL-18 release in the present study is independent on contaminating LPS.

The enhancing effect of leptin on IL-18 release was further confirmed in human primary monocytes isolated by CD14 coated magnetic beads. However, it is likely that primary

monocytes are less sensitive than THP-1 monocytes as lower levels of IL-18 were detected in primary monocytes even in LPS-treated cells. Mehta et al. (2001) used *E.coli* LPS (1 ng/ml) to stimulate primary monocytes and found no difference in IL-18 release between unstimulated control and *E.coli* LPS-stimulate cells. However, Piccini et al. (2008) showed that human primary monocytes release significantly elevated levels of IL-18 in response to *E.coli* LPS (1 µg/ml). These data suggested that IL-18 release in primary monocytes may require higher concentration of LPS reflecting the restrictive regulation of this cytokine in inflammatory responses. However, it should be noted that discrepancies between studies may be also due to differences in method of monocyte isolation, number of cells and incubation time. Interestingly, the present study showed that ATP significantly promoted IL-18 release in both *E.coli* LPS and leptin-pre-treated cells. Similarly, it was previously demonstrated that ATP induces the activation of caspase-1 which subsequently enhances the processing and release of IL-18 in primary monocytes (Perregaux et al., 2000; Mehta et al., 2001).

The effect of leptin on IL-18 production was further assessed at mRNA level. However, leptin had no effect on IL-18 mRNA expression in monocytes as shown by the results from conventional and Real-Time PCR analysis. Monocytes are one of major sources of IL-18 (Dinarello et al., 1998). Previous studies have reported that the monocytes/macrophages produce IL-18 in response to inflammatory stimuli such as LPS (Perregaux et al., 2000; Seki et al., 2001; Foster et al., 2007; Piccini et al., 2008; Yamauchi et al., 2008). Interestingly, at the mRNA level, IL-18 is constitutively expressed in many cell types including THP-1 monocytes (Akita et al., 1997; Tone et al., 1997; Puren et al., 1999; Gracie et al., 2003; Yamauchi et al., 2008). In agreement with previous studies, the present study demonstrated the constitutive expression of IL-18 in THP-1 monocytes. No significant changes in IL-18 expression were detected after

stimulation with *E.coli* LPS or leptin for 1 and 3 h. In fact, the Real-Time PCR analysis showed that *E.coli* LPS down-regulated IL-18 mRNA expression at 24 h.

In PBMCs, Puren et al. (1999) found that there was no significant enhancement of IL-18 mRNA expression after 1-6 h LPS stimulation and, in addition, there appeared to be a down-regulation of mRNA below basal levels by 24 h which is similar to the results of the present study. However, the data from this study are limited from conventional RT-PCR which is only semi-quantitative analysis (Puren et al., 1999). In contrast, Hamedi et al. (2009) reported that purified *P.gingivalis* LPS enhanced IL-18 expression Monomac-6 monocytic cells. Although differences could be due to the different source of cells and LPS used in the experiments, it should be noted that the overall expression of IL-18 mRNA was at a considerably low level compared to IL-1 β mRNA expression and any observed up-regulation is unlikely to be of biological relevance (Hamedi et al., 2009). In the present study, leptin was found to enhance IL-18 release in human monocytes but shows no effect on IL-18 mRNA. These findings likely reflect the fact that leptin regulates IL-18 release by modulation of the post-transcriptional regulation of IL-18 synthesis.

Another interesting observation in the present study is that leptin had no effect on IL-1 β expression and secretion in THP-1 monocytes which is in agreement with a previous study (Gabay et al., 2001). IL-18 is biologically and structurally related to IL-1 β . Like IL-1 β , IL-18 is produced as a pro-form lacking a secretory signal peptide. In order to be secreted in its active form, IL-18 requires cleavage by caspase-1 (Mariathasan and Monack, 2007). However, whether IL-18 processing and secretion are regulated by the same mechanisms that control IL-1 β is unclear. Notably, the results from the present study also endorsed the view that the regulation of IL-18 expression and secretion is distinct from that of IL-1 β in human monocytes as demonstrated by the differential

response to leptin. Additionally, *E.coli* LPS significantly enhanced the up-regulation of IL-1 β expression and secretion in THP-1 monocytes, whereas this was not observed in unstimulated control and leptin stimulation. Similarly, it was suggested that LPS regulates IL-1 β at both mRNA and protein levels whereas IL-18 is constitutively expressed in monocytes and LPS only enhances IL-18 protein secretion (Puren et al., 1999; Mariathasan and Monack, 2007; Piccini et al., 2008). Taken together, these data demonstrated the differential regulation of IL-18 and IL-1 β production in monocytes. Different mechanisms are involved at transcriptional level (Puren et al., 1999; van de Veerdonk et al., 2011). However, as the processing and release of these two cytokines are mediated by the activation of caspase-1 inflammasome, whether this level is regulated by the same mechanisms remains to be determined.

Previous studies demonstrated that leptin enhances IL-6 production in microglia (Tang et al., 2007) and in PBMCs (Zarkesh-Esfahani et al., 2001). In addition, leptin induces IL-8 production in synovial fibroblasts (Tong et al., 2008). Interestingly, the present study demonstrated for the first time that leptin significantly enhanced IL-8 release in THP-1 monocytes. However, leptin appeared to have no effect on IL-6 secretion. Although leptin has been shown to up-regulate intracellular level of IL-6 in human monocytes (Santos-Alvarez et al., 1999), the effect of leptin on IL-6 secretion was not measured and remains unclear. Another study showed that leptin alone failed to induce the detectable amount of IL-6 in murine macrophages. However, leptin pre-stimulation leads to a significant increase in the LPS-stimulated secretion of IL-6 from such macrophages (Loffreda et al., 1998). These data likely suggest that leptin may regulate IL-6 expression but not secretion in monocytes.

IL-8 is a chemokine that plays an important role in controlling neutrophil and monocyte chemotaxis toward sites of infection and induced inflammation (Zlotnik and Yoshie,

2000). The finding of the present study supports the role of leptin in immunity. Leptin induces not only IL-18 but also IL-8 release from monocytes. In addition, the fact that leptin has no effect on IL-1 β and IL-6 release suggested that leptin has a specific role in the differential regulation of cytokines. Leptin signalling may mediate diverse signalling pathways which lead to activation of transcription factors such as NF- κ B and AP-1. Such transcriptional factors are responsible for the production of IL-1, IL-6, TNF- α or IL-8 in different cell types (Gonzalez et al., 2004; Tang et al., 2007; Tong et al., 2008). Growing in the knowledge of the leptin signalling pathways may offer an insight into mechanisms which leptin regulates the production of different cytokines.

In periodontal disease, it has been reported that GCF leptin levels are elevated (Karthikeyan and Pradeep, 2007a). A recent study from our group (Jaedicke, 2010) found that serum levels of leptin were significantly elevated in male T2DM patients who had gingivitis or periodontitis compared to the non-diabetic control subjects. *In vitro*, leptin enhances *P.gingivalis* LPS-induced TNF- α mRNA expression in human monocytes (Jaedicke, 2010). Therefore, the interaction between leptin with periodontal bacteria such as *P.gingivalis* may exist. The results from this study demonstrated that stimulation monocytes with leptin plus *P.gingivalis* LPS significantly enhanced IL-18 release compared with cells stimulated with leptin or *P.gingivalis* LPS alone. However, leptin shows no enhancing effect on *E.coli* LPS-stimulated IL-18 release in THP-1 monocytes. These data suggested that leptin has differential effect on TLR2 and TLR4 signalling pathways. Interestingly, leptin appears to up-regulate TLR-2 expression but does not up-regulate TLR-4 expression in monocytes (Jaedicke, 2010). In liver cells from *ob/ob* and in adipocytes from both *ob/ob* and *db/db* mice, no difference in TLR2 or TLR4 mRNA expression was detected compared to wild type mice (Romics et al.,

2005; Batra et al., 2007). However, higher TLR2 and TLR4 mRNA expression was observed in pre-adipocytes from *ob/ob* and *db/db* mice (Batra et al., 2007).

The innate recognition pathway for *E.coli* LPS is well-characterized through TLR4 signalling pathway (Correia et al., 2001). However, the immune recognition of *P.gingivalis* LPS is very controversial. *P.gingivalis* LPS has been shown to signal through TLR2 or TLR4 to activate different cell types to produce cytokines (Sandros et al., 2000; Hirschfeld et al., 2001; Wang and Ohura, 2002). In THP-1 monocytes, TLR2 signalling pathway was demonstrated to be the main pathway of *P.gingivalis* LPS induced cytokine production (Zhang et al., 2008). In addition, LPS from *P.gingivalis* has ability to stimulate the production of IL-18 in monocytes (Foster et al., 2007). The present study found that leptin enhanced *P.gingivalis* LPS-induced IL-18 in THP-1 monocytes suggesting the interaction between adipose tissue-derived cytokine leptin and *P.gingivalis* LPS. Indeed, previous studies revealed that leptin and *P.gingivalis* LPS may share a common downstream signalling pathways such as ERK (Wang and Ohura, 2002) and PI3K pathways (Slomiany and Slomiany, 2005) which represent a critical signalling target for leptin in the LPS-induced production of cytokines.

In conclusion, the present study supports that leptin functions as a hormone and a cytokine which potentiates and induces inflammatory responses through cytokine up-regulation. The pro-inflammatory effect of leptin on IL-18 synthesis may play a role in the cross-susceptibility between diabetes and periodontal disease.

Chapter 5 Analysis of caspase-1 inflammasome activation in leptin-stimulated monocytes

5.1 Introduction

Like IL-1 β , pro-IL-18 is processed intracellularly into active IL-18 by the action of caspase-1 associated with the inflammasome (Dinarello, 2007). It has been suggested that two steps control activation of these cytokines (Mariathasan and Monack, 2007). The first step involves in the transcription and translation of their mRNA into the proform proteins (pro-IL-1 β , pro-IL-18) which is regulated by classical pro-inflammatory stimuli such as TLR agonists. The second step is required for their secretion and is mediated by the activation of the inflammasome by danger signals resulting in the activation of caspase-1 and cleaving of the pro-cytokines into mature bioactive IL-1 β and IL-18 (Mariathasan and Monack, 2007). As a key regulator of the processing of the two important pro-inflammatory cytokines, the investigation of the regulation of caspase-1 activation has become an extensive area of research (Latz, 2010; Tschopp and Schroder, 2010).

Caspase-1 is constitutively expressed as procaspase-1 (Thornberry et al., 1992; Martinon et al., 2002). Upon the inflammasome activation, caspase-1 undergoes autocleavage into p20 and p10 fragments. These free p20 and p10 fragments assemble into an active heterotetramer that acts as a highly efficient IL-1 β converting enzyme (Yamin et al., 1996). Active caspase-1 has been purified from THP-1 monocytic cells and has been shown to consist of an equimolar ratio of p10 and p20 subunits (Miller et al., 1993). In addition, LPS-induced IL-1 β release has been described to be associated with caspase-1 activation in monocytic THP-1 monocytes (Schumann et al., 1998). However, initial studies found that LPS stimulation of human monocytes or THP-1 cells fails to change the amount of p45 or its activity and does not induce appearance of detectable p20 caspase-1 (Thornberry et al., 1992). Additionally, the activation of caspase-1 by LPS appeared not to result from modulation of caspase-1 transcription (Schumann et al., 1998). The difficulties in isolation, purification and measuring caspase-1 activity in cells and cell lysates could be due to low-level of expression and instability of intracellular caspase-1 (Ayala et al., 1994). The activation of caspase-1 was only demonstrated by the inhibition of caspase-1 activity by caspase-1 inhibitors, YVAD-CHO and YVAD-CMK (Schumann et al., 1998). Secondary stimuli such as ATP, nigericin and other bacterial pore-forming toxins have been frequently used to activate caspase-1 and boost IL-1 β and IL-18 release following pre-stimulation of monocytes or macrophages with LPS (Perregaux et al., 1992; Perregaux and Gabel, 1994; Cheneval et al., 1998; Watanabe et al., 1998; Mehta et al., 2001). For example, nigericin is able to increase the active caspase-1, which in turn results in the release of mature IL-1ß in murine macrophages (Perregaux and Gabel, 1994) and THP-1 monocytes (Cheneval et al., 1998).

In primary monocytes, although ATP was found to activate caspase-1, ATP or LPS alone is insufficient to induce IL-1 β and IL-18 release suggesting that LPS priming is necessary for IL-1 β and IL-18 release in primary monocytes (Mehta et al., 2001). The LPS-priming step is essential for the synthesis of pro-IL-1 β and may also provide additional signals for these secondary stimuli to achieve their effect. Unlike IL-1 β , IL-18 is constitutively expressed in many cell types and IL-18 release may be stimulated by single stimulation such as LPS alone. Subsequent studies showed that THP-1 monocytes display caspase-1 activation in response to LPS alone and do not require

costimulation with ATP (Martinon et al., 2004; Sarkar et al., 2006) suggesting that single signal may be sufficient to regulate IL-18 production.

The inflammasome plays a key role in caspase-1 activation (Latz, 2010). The inflammasome is an intracellular multi-protein complex consisting of 3 main components which are a nucleotide-binding domain leucine-rich repeat (NLR) protein, the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) domain and the effector protein caspase-1 (Martinon et al., 2002; van de Veerdonk et al., 2011). In response to specific pathogens or host danger signals, inflammasomes are assembled from self-oligomerizing scaffold proteins resulting in caspase-1 autoactivation (Schroder and Tschopp, 2010). This activation of caspase-1 subsequently leads to cleavage of IL-1 β and IL-18 (Dinarello, 1996; Ghayur et al., 1997).

ASC, also known as TMS1, is an important component of the inflammasome and plays a key role in caspase-1 activation. ASC deficient macrophages are unable to activate caspase-1 in response to a number of stimuli (Mariathasan et al., 2004). ASC is one of the few proteins in the human genome that contains both an N-terminal YD and a Cterminal CARD. ASC is predominantly expressed in monocytes and mucosal epithelial cells (Masumoto et al., 2001). ASC interacts with the CARD of procaspase-1 and induces formation of the protein complex inflammasome, thereby regulating activation of caspase-1 and secretion of IL-1 β (Stehlik et al., 2003).

Another important component of the inflammasome is the NLR protein. Many NLRs, including NLRP3 (formerly cryopyrin, CIAS1, and NALP3), have been implicated in several different inflammatory signalling pathways (Ting et al., 2006; Ting et al., 2008). The NLRP3 is expressed in monocytes and macrophages (Feldmann et al., 2002; Dowds et al., 2004). Mutations in gene encoding the NLRP3 are associated with hyperactive inflammatory signalling characterized by excessive IL-1β (Hoffman et al.,

2001; Dowds et al., 2004). Thus, the NLRP3 has emerged as a critical mediator of

inflammation. NLRP3 and ASC, together with procaspase-1, form one of the most important inflammasomes called the NLRP3 inflammasome (Petrilli et al., 2005; Pedra et al., 2009). The NLRP3 inflammasome is the best characterized inflammasome. A wide range of stimuli have been identified for the activation of the NLRP3 inflammasome including microbe-associated molecular patterns (MAMPS) and other molecular signs of danger, known as danger-associated molecular patterns (DAMPS). Amongst these are Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria* monocytogenes, Gram-negative bacteria Shigella flexneri, as well as ATP, maitotoxin, and uric acid crystals (Mariathasan et al., 2006; Martinon et al., 2006; Willingham et al., 2007; Craven et al., 2009). NLRP3 inflammasome has also been demonstrated to regulate caspase-1 mediated cytokine secretion in response to environmental pollutants such as asbestos and silica, as well as particulate adjuvants and β -amyloid (Dostert et al., 2008; Eisenbarth et al., 2008; Halle et al., 2008; Hornung et al., 2008; Li et al., 2008). Recently, cholesterol crystals have been demonstrated to activate the NLRP3 inflammasome in phagocytes in a process that involves phagolysosomal damage (Duewell et al., 2010).

The regulation of IL-18 maturation and release is not completely understood. A number of studies have reported the role of caspase-1 and NALP3 inflammasome activation in IL-18 processing and release (Sutterwala et al., 2006; Petrilli et al., 2007a; Duncan et al., 2009; Kankkunen et al., 2009). Like IL-1 β , pro-IL-18 requires cleavage by caspase-1 in order to be secreted as a biologically active IL-18 (Ghayur et al., 1997). IL-18 is secreted by many cell types in response to different stimuli such as LPS and cytokines (Mehta et al., 2001; Cheng et al., 2005; Dinarello, 2007; Foster et al., 2007; Yamauchi et al., 2008). Mehta et al. (2001) suggested that LPS priming is necessary for ATP to induce IL-18 release by primary monocytes. The same study showed that ATP stimulation alone promotes caspase-1 activation without inducing processing of pro-IL-18. However, ATP stimulation after LPS priming induces both caspase-1 activation and IL-18 processing and release (Mehta et al., 2001). In contrast, Hentze et al. (2003) demonstrated that in the absence of LPS priming, a potassium ionophore nigericin alone activates caspase-1 and initiates the processing of release of mature IL-18 from THP-1 monocytes (Hentze et al., 2003). IL-18 release has been linked to the activation of caspase-1 and NLRP3 inflammasome induced by various stimuli. For example, monosodium urate crystals mediated IL-18 processing is dependent on caspase-1 activating NALP3 inflammasome in THP-1 derived macrophages (Martinon et al., 2006). In murine macrophages, NLRP3 (cryopyrin) is essential for inflammasome mediating IL-1 β and IL-18 release in response to signalling pathways triggered specifically by ATP, nigericin, maitotoxin, Staphylococcus aureus, Listeria monocytogenes (Mariathasan et al., 2006). Additionally, ASC-deficient macrophages exhibited defective maturation of IL-1ß and IL-18 in response to TLR agonists and extracellular ATP (Mariathasan et al., 2004). In response to bacterial RNA and small antiviral compounds, NLRP3 and ASC are essential for caspase-1 activation and IL-1ß and IL-18 secretion by murine peritoneal and bone marrow macrophages (Kanneganti et al., 2006b). Similarly, NLRP3 and ASC are also required for IL-1B and IL-18 secretion by murine macrophages in response to double-stranded RNA and viral infection (Kanneganti et al., 2006a). In addition, Neisseria gonorrhoeae-induced IL-18 and IL-18 production requires inflammasome components ASC and NLRP3 as demonstrated by THP-1 cells with knocked down expression of NLRP3 or ASC failed to produce IL-18 in response to N. gonorrhoeae (Duncan et al., 2009). Consistent with that observation, N. gonorrhoeae induced the formation of caspase-1 p10 subunit indicating that N.

gonorrhoeae-induced IL-18 release is associated with caspase-1 activation. *In vivo*, mice reconstituted with NLRP3-deficient or ASC-deficient bone marrow showed significantly lower plasma levels of IL-18 compared to wild type (Duewell et al., 2010). Furthermore, in human macrophages, *Stachybotrys chartarum* and *E.coli* LPS had a strong synergistic effect on IL-1 β and IL-18 mRNA expression and release in human macrophages (Kankkunen et al., 2009). This is associated with the caspase-1 activation as clearly shown by the up-regulation of caspase-1 p20 subunit. This study suggested that TLR signalling is probably needed for expression of essential inflammasome components. However, conflicting results have also been reported. For example, NLRP3-deficient macrophages were found to activate caspase-1 and secreted normal levels of IL-1 β and IL-18 when infected with Gram-negative *Salmonella typhimurium* or *Francisella tularensis* (Mariathasan et al., 2006). Another study showed that LPS alone failed to activate the formation of caspase-1 p20 in macrophages, which in turn was not capable of processing pro-IL-1 β or pro-IL-18 into the biologically active forms of the cytokines (Kankkunen et al., 2009).

Another stimulus that activates caspase-1 and IL-18 through the NLRP3 inflammasome is aluminium hydroxide (alum), the most widely used adjuvant, (Lindblad, 2004). Aluminium adjuvants enhanced LPS-induced IL-1 β and IL-18 release from wild-type primary murine macrophages. Additionally, macrophages from mice deficient in NLRP3, ASC or caspase-1 failed to produce IL-1 β and IL-18 on stimulation with aluminium adjuvant (Eisenbarth et al., 2008). Another study also demonstrated that IL-18 release by human peripheral blood mononuclear cells in response to alum is associated with caspase-1 activation because it was inhibited by caspase-1 inhibitor Z-YVAD-Fmk (Li et al., 2008). In addition, the level of IL-18 present in the peritoneal lavage of mice injected with alum for 24 h was significantly higher in wild-type mice than in NLRP3 deficient mice (Li et al., 2008). Collectively, these data likely suggest that the activation of the NLRP3 inflammasome and subsequent IL-1 β and IL-18 release require specific signals in response to different stimuli by monocytes/macrophages. Although a number of stimuli have been described to activate the NLRP3 inflammasome, the precise mechanisms remain unclear.

Leptin plays an important role not only in energy expenditure but also in immune responses. Leptin exerts its proinflammatory effect by stimulating the production of several cytokines such as TNF-α, IL-6 and IL-8 (Loffreda et al., 1998; Zarkesh-Esfahani et al., 2001; Tong et al., 2008; Fernandez-Riejos et al., 2010; Agrawal et al., 2011). Interestingly, the present study (Chapter 4) demonstrated that leptin induces IL-18 release in human monocytes. The fact that leptin had no effect on IL-18 mRNA expression allows the speculation to be made that leptin may regulate IL-18 production at post-translational level involving the activation of caspase-1 inflammasome. However, the knowledge regarding the role of leptin in caspase-1 mediated IL-18 release is very limited. Only one study thus far published has shown that leptin induces expression of caspase-1 mRNA in monocytes and these experiments were carried out using bovine cells (Ahmed et al., 2007). Furthermore, the data was derived from conventional RT-PCR analysis which is only semi-quantitative and the IL-18 protein secretion was not measured due to unavailability of bovine IL-18 assay. Another study found that leptin induces IL-1ß release in rat microglia. However, this was not associated with caspase-1 pathway as leptin-induced IL-1ß release was not inhibited by the caspase 1 inhibitor (BOC-D-FMK and Ac-YVAD-CHO) (Pinteaux et al., 2007).

The activation of caspase-1 in inflammasome is not completely understood. Because leptin stimulation induces IL-18 release in monocytes, it is possible that caspase-1 mediated IL-18 release is regulated by leptin. To test this, the present study aimed to

investigate the effect of leptin on the activation of caspase-1 inflammasome in human monocytes. In parallel, the effect of leptin on IL-18 processing was also investigated.

5.2 Results

5.2.1 The investigation of the effect of leptin on the expression of the inflammasome components

Because secretion of mature IL-18 has been shown to require activation of caspase-1 mediated by NLRP3 inflammasome, the present study investigated whether the secretion of IL-18 also requires NLRP3 and ASC. To evaluate the effect of leptin on the expression of NLRP3, ASC and caspase-1 mRNA, THP-1 monocytes (4×10^6) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 1, 3 and 24 h. Unstimulated cells were served as control. Total cellular RNA was extracted and reverse transcribed to produce cDNA. The cDNAs were analysed for the expression of NLRP3, ASC and caspase-1 mRNA by conventional RT-PCR as described in 2.4.4.

As shown in Figure 5.1, THP-1 monocytes constitutively expressed the NLRP3, ASC and caspase-1 mRNA. The strong expression of NLRP3, ASC and caspase-1 mRNA can be observed in control cultures and after *E.coli* LPS and leptin stimulation. No observable change in the expression of NLRP3, ASC and caspase-1 mRNA was observed after treatment with *E.coli* LPS or leptin at 1, 3 and 24 h.

In addition, NLRP3 mRNA expression was further analysed by Real-Time PCR. Figure 5.2 shows the results from Real-Time PCR. Compared to control, leptin had no effect on NLRP3 mRNA expression. The expression of NLRP3 mRNA remained unchanged after stimulation with leptin for 1-24 h (p > 0.05). *E.coli* LPS stimulated a very slight up-regulation of NLRP-3 expression at 1 h stimulation and a decline at 3 h stimulation

although these changes were not statistically significant. However, *E.coli* LPS significantly down-regulated NLRP3 mRNA expression at 24 h stimulation (p < 0.05).



Figure 5.1 Effect of leptin on mRNA expression of caspase-1 inflammasome components in THP-1 monocytes

THP-1 monocytes (4 x 10^6) were co-cultured in the absence or presence of either leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of caspase-1, ASC, NLRP3, and β 2M were analyzed by RT-PCR. The results shown are representative of 3 independent experiments.



Figure 5.2 NLRP3 mRNA expression in THP-1 monocytes after stimulation with *E.coli* LPS and leptin

THP-1 monocytes (4 x 10⁶) were stimulated with *E.coli* LPS (100 ng/ml) and leptin (1000 ng/ml) for 1-24 h. The NLRP3 mRNA expression was quantified by Real-Time PCR. The data are expressed as mean fold-induction of 3 independent experiments measured in 3 separate occasions. Statistical analysis was performed on δ Ct values using ANOVA.*: *p* < 0.05 compared with controls. Ec: *E.coli*.

5.2.2 The effect of leptin on the caspase-1 activity

The findings that leptin induces IL-18 release in monocytes but has no effect on the expression of IL-18 mRNA allowed me to speculate that leptin may have a role as an activator of caspase-1. To test this hypothesis, caspase-1 activity in stimulated cells was measured by using caspase-1 colorimetric assay as described in 2.5 (Ghayur et al., 1997). As shown in Figure 5.3, the activity of caspase-1 was readily detectable in unstimulated control cells (unstimulated control; 11.17 ± 2.29 (mean \pm SD) pmol/min/mg protein). Leptin significantly increased caspase-1 activity in THP-1 moncytes (leptin; 19.24 ± 3.96 pmol/min/mg protein, p < 0.05 compared to control). In fact, leptin enhanced caspase-1 activity to the same levels as observed in *E.coli* LPS or *P.gingivalis* LPS treated cells (*E.coli* LPS; 18.30 ± 3.84 pmol/min/mg protein, *P.gingivalis* LPS; 17.84 ± 1.77 pmol/min/mg protein).



Figure 5.3 Leptin as well as *E.coli* LPS and *P.gingivalis* LPS up-regulate caspase-1 activity

THP-1 monocytes (4 x 10^6) were stimulated with leptin (1000 ng/ml) or *E.coli* LPS and *P.gingivalis* LPS (100 ng/ml) for 3 h. Unstimulated cells were served as control. Cells were lysed and caspase-1 activity was assessed by caspase-1 colorimetric assay. Statistics: ANOVA. * *p* < 0.05 compared with controls. Ec: *E.coli*. Pg: *P.gingivalis*.

5.2.3 Investigation of the effect of leptin on caspase-1 activation

To further test whether leptin-induced IL-18 release was also associated with activation of caspase-1, Western blot analysis was performed using anti-caspase-1 p10 antibody. THP-1 monocytes (4 x 10^6 cells) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Unstimulated cells and recombinant caspase-1 were served as negative and positive control, respectively. Cell pellets were lysed and blotted for caspase-1 as described in 2.6.4.

Figure 5.4 shows the results for caspase-1 p10 Western blot. The stimulation with *E.coli* LPS and leptin had no effect on either precursor form of caspase-1 (p45) or the intermediate form (p30). Compared to control, stronger bands of active caspase-1 (p10) in leptin treated cells were observed suggesting that leptin may increase production of active caspase-1. Equal protein loading was confirmed by assessing the protein level of β -actin.

However, the detection of caspase-1 p10 subunit appeared not to be reproducible in the subsequent Western blots. Repeated experiments obtained relatively faint bands of caspase-1 p10 which could not allow any comparison to be made amongst stimulations. A variety of adaptations to Western blot protocols were performed including increasing the amount of total protein, increasing of antibody concentration and using an alternative percentage of pre-cast gel. However, none of these techniques was able to achieve a significantly improvement as these techniques could not reproducibly detect the active caspase-1 p10 subunit.

In an attempt to improve the detection of active caspase-1 p10 subunit, immunoprecipitation using caspase-1 p10 antibody-followed by Western blotting was performed. The first immunoprecipitation experiment was performed to test whether the protocol is efficient. The lysis buffer was spiked with two volumes of recombinant caspase-1 (5 μ l and 20 μ l) to be used as samples for immunoprecipitation. The spiked samples were immunoprecipitated with caspase-1 p10 antibody as described in 2.6.5. The immunoprecipitates were then analysed for caspase-1 p10 with Western blotting using the same caspase-1 antibody as for immunoprecipitation. The recombinant caspase-1 served as a positive control. As shown in Figure 5.5A, the immunoprecipitation enhanced the signal of caspase-1 p10 subunit. Compared with control (lane 3), the stronger band of caspase-1 p10 can be detected in the immunoprecipitates of the lysates spiked with recombinant caspase-1 (lanes 1 and 2) indicating that this technique is efficient to detect the signal of caspase-1 p10 subunit. In addition, a strong band of heavy IgG chain (50 kDa) of the caspase-1 anti- human rabbit antibody was also clearly detected. However, the band of the light chain IgG (25 kDa) was undetectable.

The immunoprecipitation/Western blot procedure was subsequently used to detect caspase-1 p10 subunit in cell stimulation experiment. THP-1 monocytes (4 x 10^6 cells) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Unstimulated cells were served as control. Cells were lysed and immunoprecipitated for caspase-1 p10 using the same protocol as in the first immunoprecipitation experiment. As seen in Figure 5.5B, the immunoprecipitation/Western blot could not detect the p10 subunit of the activated caspase-1 in either unstimulated cells or LPS and leptin-treated cells. Immunoprecipitates from unstimulated cells (lane 1), *E.coli* LPS-treated cells (lane 2) and leptin treated cells (lane 3) showed no band of active caspase-1 p10 subunit. The clear bands of heavy chain of IgG (50 kDa) and light chain of IgG (25 kDa) were observed, indicating that the immunoprecipitation was efficient. Additionally, the observed clear band of caspase-1 p10 in lane 4 which is a positive

recombinant caspase-1 control confirmed that Western blot was also efficient. While the data from Western blot analysis demonstrated an irreproducible increased caspase-1 p10 after leptin stimulation, the immunoprecipitation experiment failed to show any activation of caspase-1. However, the experiment was conducted at only one time point (3 h) and may not represent the relevant time of caspase-1 activation. In summary, these data do not allow for any conclusion to be made concerning the effect of leptin or LPS on the level of intracellular caspase-1 p10 subunit.



Figure 5.4 Western blot analysis for caspase-1 p10 subunit

THP-1 monocytes (4 x 10^6) were stimulated with 1000 ng /ml leptin or 100 ng/ml *E.coli* LPS for 3 h. Human recombinant caspase-1 served as positive control. Cell lysates were separated by SDS-PAGE on a 10-20% gradient gel. After blotting, the proteins were probed with antibody against the p10 subunit of caspase-1. Equal protein loading was assessed by detecting the protein of β -actin.

A)



B)





A) Immunoprecipitates of recombinant caspase-1 was analysed to test the established protocol. Lane 1, recombinant caspase-1 (5 μ l); lane 2, recombinant caspase-1 (20 μ l); lane 3, recombinant caspase-1 positive control. **B**) THP-1 monocytes (4 x 10⁶) were stimulated with 1000 ng/ml leptin or 100 ng/ml *E.coli* LPS for 3 h. Cell lysates were immunoprecipitated with antibodies for caspase-1 p10 subunit. Lane 1, unstimulated control; lane 2, *E.coli* LPS; lane 3, leptin; lane 4, recombinant caspase-1 positive control.

5.2.4 The effect of leptin on caspase-1 p20 subunit

Western blotting of caspase-1 p20 was also performed to investigate whether leptin induces IL-18 release through the activation of caspase-1. The experiments were similar to those performed for analysis for caspase-1 p10 with exception that primary antibody used was anti-caspase-1 p20. THP-1 monocytes (4 x 10^6 cells) were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 1 and 3 h. Unstimulated cells served as the control. Cell pellets were lysed and blotted for caspase-1 p20 as described in 2.6.4.

As shown in Figure 5.6 A, Western blot experiments revealed that the intracellular protein levels of the p20 subunit of caspase-1 was constitutively expressed in THP-1 monocytes. The clear band of caspase-1 p20 subunit was observed in unstimulated control at 1 and 3 h stimulation. After 1 h stimulation, no observable changes were apparent in either LPS-treated cells or leptin-treated cells compared with control. However, after 3 h stimulation, *E.coli* LPS and leptin seemed to decrease the protein level of the caspase-1 p20 subunit compared to control. Similar results were obtained on each of three separate experiments. These data suggested that leptin-induced IL-18 release may be associated with the function of caspase-1 as demonstrated by the change in the level of caspase-1 p20 subunit.

The caspase-1 p20 Western blot experiment was repeated in primary monocytes. Primary monocytes were isolated and purified using the anti-CD14 coated magnetic beads as described in 2.1.2.1. Primary monocytes (4 x 10^6 cells) were cultured in the absence or presence of leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 h. Cells were collected and analysed for caspase-1 p20 by Western blotting. For reasons of cost, only one experiment was performed and the results were shown in Figure 5.6 B. Only

faint bands were detected at the correct size of caspase-1 p20 subunit (20 kDa). The markedly strong band of recombinant caspase-1 was seen as a result of increased developing and detection time. Although differences between unstimulated control and leptin can be observed, the signal is too weak to allow any conclusion to be made regarding the effect of leptin on the level of caspase-1 p20 subunit in primary monocytes.

A) THP-1 monocytes



B) Primary monocytes



Figure 5.6 Western blot analysis for an active caspase-1 p20 subunit

A) THP-1 monocytes (4 x 10^6) were stimulated with 1000 ng /ml leptin or 100 ng/ml *E.coli* LPS for 1 and 3 h. Human recombinant caspase-1 served as positive control. Cell lysates were separated by SDS-PAGE on a 10-20% gradient gel. After blotting, the proteins were probed with antibody against the p20 subunit of caspase-1. The blot shown is representative from three independent experiments. Equal protein loading was assessed by detecting the protein of β -actin. **B**) The caspase-1 p20 Western blot was repeated in primary monocytes using a comparable experimental setup.

5.2.5 The inhibition of leptin-induced IL-18 release by caspase-1 inhibitor (Ac-YVAD)

Further investigation of whether leptin-induced IL-18 release through caspase-1 dependent pathway was carried out by using a specific caspase-1 inhibitor (Ac-YVAD) (Schumann et al., 1998; Mehta et al., 2001). THP-1 monocytes (5 x 10^5) were pre-treated with Ac-YVAD (100 µM) for 1 h, followed by the stimulation with either of 100 ng/ml *E.coli* LPS or 1000 ng/ml leptin for 3 h. Supernatants were collected and levels of IL-18 were determined using ELISA kits. As shown in Figure 5.7, the induction of IL-18 was significantly reduced by the inhibitor (p < 0.05) in *E.coli* LPS and leptin treated cells, whereas DMSO solvent had no effect. To exclude cytotoxic effects, cell viability was assessed by a cell proliferation assay and no cytotoxicity of both DMSO solvent and Ac-YVAD could be observed as previously shown in 2.2.5. Collectively, this experiment demonstrated that both *E.coli* LPS and leptin-induced IL-18 release can be blocked specifically by the caspase-1 inhibitor Ac-YVAD and that leptin-induced release of IL-18 is dependent on caspase-1 activity.





THP-1 monocytes (5 x 10⁵) were pre-treated with caspase-1 inhibitor, Ac-YVAD (100 μ M) for 1 h, followed by the stimulation with either of 100 ng/ml *E.coli* LPS or 1000 ng/ml leptin for 3 h. Supernatants were collected and levels of IL-18 were determined using ELISA kits. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA. * *p* < 0.05.

5.2.6 The analysis of IL-18 processing after leptin stimulation

In order to determine if the increase in caspase-1 activity seen in leptin-treated cells was paralleled by an increased processing of IL-18, the cleavage of intracellular pro-IL-18 into active IL-18 was examined by Western blot analysis. THP-1 or primary monocytes $(4 \times 10^6 \text{ cells})$ were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Unstimulated cells were served as control. Cell pellets were lysed and blotted for IL-18 as described in 2.6.4.

A representative blot from three experiments is shown in Figure 5.8 A, the unstimulated control showed a clear band of the proform of IL-18 (24 kDa). After 3 h stimulation, *E.coli* LPS and leptin decreased the intracellular protein level of pro-IL-18. However, only a faint band corresponding to active IL-18 (18 kDa) was detected in unstimulated control. No band of active IL-18 can be seen in either *E.coli* LPS-treated cells or leptin-treated cells. There was no observable difference in the protein level of β -actin indicating equal protein loading.

Figure 5.8 B shows the results from primary monocytes. Only faint bands were detected at the correct size of pro-IL-18 and active IL-18 in all cell cultures. In addition, no clear differences in band intensity can be observed. It is difficult to draw any conclusion regarding the effect of leptin on IL-18 processing in primary monocytes from this single experiment.



Figure 5.8 The effect of leptin on IL-18 processing in human monocytes

A) THP-1 monocytes or **B**) primary monocytes (4 x 10^6) were stimulated with 1000 ng /ml leptin or 100 ng/ml *E.coli* LPS for 3 h. Cell lysates were separated by SDS-PAGE on a 10-20% gradient gel. After blotting, the processing of pro IL-18 into active IL-18 was detected using antibody recognizing both pro-IL-18 and mature IL-18. Equal protein loading was confirmed by incubation with an anti- β -actin antibody.

5.3 Discussion

Caspase-1 and its associated NLRP3 inflammasome complex have been implicated in inflammatory responses by monocyte-derived cells to a variety of stimuli (Martinon et al., 2004; Martinon et al., 2006; Mariathasan and Monack, 2007; Latz, 2010). The present study has now shown for the first time that stimulation with leptin can activate the caspase-1 inflammasome and this activation is essential for the release of IL-18 by human monocytes. The NLRP3-inflammasome is a signalling multi-protein complex that activates procaspase-1 and induces processing of proinflammatory cytokines, IL-1 β and IL-18 (Mariathasan and Monack, 2007). Data presented in the previous chapter suggests that leptin regulates IL-18 synthesis and secretion at different levels. Leptin increases IL-18 release at protein level but has no effect on IL-18 mRNA expression. This could indicate that IL-18 regulation by leptin may be involved in the posttranslational step which is mediated by the activation of the NLRP3-inflammasome (Pedra et al., 2009). Indeed, this present study provides the explanation for leptin stimulation of IL-18 release. Leptin enhances the activity of caspase-1 and modulates the production of active caspase-1 p20 subunit which is associated with the activation of NLRP3-inflammasome. Additionally, this association was further confirmed by the finding that leptin-induced IL-18 release was significantly blocked by caspase-1 inhibitor (Ac-YVAD).

The caspase-1 and NLRP3-inflammasome are activated in response to a myriad of proinflammatory stimuli, including pathogen-derived molecules, endogenous inducers of (sterile) inflammation and non-pathogenic microbial pore-performing toxins, including nigericin and maitotoxin (Kanneganti et al., 2006a; Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006; Franchi et al., 2009a). Interestingly, it has been demonstrated that the activation of caspase-1 and IL-1β secretion by microbial ligands such as LPS require a brief stimulation with high dose concentration of ATP (Kahlenberg et al., 2005). Moreover, Piccini et al (2008) showed that microbial components and uric acid are able to induce maturation and secretion of IL-1 β and IL-18 by inducing active release of endogenous ATP (Piccini et al., 2008). There is, however, disagreement in the literature concerning the requirements for activation of the inflammasome and whether pattern recognition receptor (PRR) activation alone is sufficient to activate caspase-1 (Mehta et al., 2001; Miggin et al., 2007). The present study showed that leptin alone is able to activate the action of caspase-1. In agreement with the previous studies, ultrapure LPS alone can activate caspase-1 in murine macrophages (Martinon et al., 2002) or in certain cell lines such as THP-1 monocytes (Kanneganti et al., 2006b).

ASC and NLRP3 are two important components of the NLRP3 inflammasome. The interaction between these components results in the activation of caspase-1 and thereby plays a key role in the processing of IL-1 β and IL-18 (Mariathasan et al., 2004; Mariathasan et al., 2006; Sarkar et al., 2006; Hornung et al., 2008; Duewell et al., 2010). To date, only one study investigated the effect of leptin on caspase-1 mRNA expression and demonstrated that leptin enhanced caspase-1 mRNA expression in bovine monocytes (Ahmed et al., 2007). The present study is the first study investigating the relationship between leptin and the inflammasome components in human monocytes. However, neither leptin nor *E.coli* LPS has effect on NLRP3, ASC and caspase-1 expression. Transcriptional induction of caspase-1 gene does not account for LPS-induced caspase-1 activation in monocytes (Schumann et al., 1998). Another study also found that Langerhans cells and epidermal-derived dendritic cell lines constitutively expressed levels of caspase-1 mRNA that are not significantly enhanced by LPS (Ariizumi et al., 1995). The Real-Time PCR results of the present study

confirmed the findings that NLRP3 mRNA expression remains unchanged after leptin stimulation 1-24 h. These data suggested that leptin-induced IL-18 release may not involve in the transcription of the NLRP3 inflammasome components. Interestingly, a slight up-regulation of NLRP3 mRNA expression was observed after 1 h of *E.coli* LPS stimulation (albeit not statistically significant) and NLRP3 mRNA expression started to decline at 3 h stimulation. This data suggests that LPS may play a role in caspase-1 activation by regulating the transcription of NLRP3 inflammasome components (Mariathasan et al., 2006). In agreement with this observation, single signals from different TLRs induce not only synthesis but also processing and secretion of IL-1 β and IL-18 in human monocytes (Piccini et al., 2008). These data also allow speculation to be made that the induction of NLRP3 may rapidly occur at the early event and TLR signalling is probably needed for expression of particular inflammasome components. The dependency on the inflammasome components for leptin-induced IL-18 release in human monocytes could possibly be further investigated using more robust models such as NLRP3 gene silencing experiments by siRNA (Netea et al., 2009) and in vitro studies using bone marrow-derived macrophages derived from mice deficient in inflammasome components such as caspase-1, ASC and NLRP3 (Martinon et al., 2006; Gross et al., 2009).

The induction of caspase-1 activity is an important indication of caspase-1 activation (Gu et al., 1997; Schumann et al., 1998). The data from caspase-1 colorimetric assays revealed that leptin significantly enhanced caspase-1 activity. Interestingly, the levels of caspase-1 activity in leptin-treated monocytes are comparable to of those LPS-treated monocytes. The kinetics of caspase-1 activity directly relates to secretion of IL-1 β and IL-18 (Gu et al., 1997; Schumann et al., 1998). In parallel to previous studies (Ghayur et al., 1997; Gu et al., 1997; Sutterwala et al., 2006), the findings that leptin up-

regulated caspase-1 activity may indicate that leptin induced IL-18 release is associated with caspase-1 pathway.

Another indication of the activation of caspase-1 is the autocatalytic processing of procaspase-1 into its p20 and p10 subunits. Although leptin increases the caspase-1 activity, the present study was unable to demonstrate reproducible up-regulation of active caspase-1 p10 subunit by Western blot analysis. Interestingly, the formation of the active caspase-1 p20 subunit in THP-1 monocytes was demonstrated. The constitutive levels of intracellular caspase-1 p20 were observed. Compared to unstimulated control, no significant difference was detected in leptin or LPS-treated cells after 1 h stimulation. However, E.coli LPS and leptin down-regulated caspase-1 p20 subunit after 3 h stimulation. The present study provided the findings which were consistent with previous reports (Martinon et al., 2004; Mariathasan et al., 2006; Netea et al., 2009). The precursor of caspase-1 p45 was identified in THP-1 cell lysates but any fractions of active caspase-1 (p10 and p20) were undetectable (Miller et al., 1993; Ayala et al., 1994). It has been demonstrated that caspase-1 activation is difficult to detect in cell extracts because active caspase-1 is rapidly secreted upon inflammasome assembly (Martinon et al., 2002; Mariathasan et al., 2004; Martinon et al., 2006). Indeed, caspase-1 was present in the supernatant and that active form of caspase-1 was rapidly released into the supernatant following LPS stimulation of differentiated THP-1 monocytes (Martinon et al., 2002; Martinon et al., 2006). Additionally, Western blotting revealed that caspase-1 p10 subunit was detected in supernatants of murine macrophages infected with Salmonella typhimurium (Mariathasan et al., 2006). In primary monocytes, stimulation with LPS plus ATP induced a significant decrease in intracellular caspase-1 p10 and p45, consistent with a release of the inflammasome components into the supernatant (Netea et al., 2009). The inflammasome is a crucial regulator of IL-1 β and IL-18 release. In intact cells, the quantities of the inflammasome complex must be minute or the inflammasome activation must be transient and tightly controlled (Martinon et al., 2002), possibly explaining why it was not possible to detect the up-regulation of active caspase-1 in cell extracts of stimulated monocytes. Therefore, the failure to see the up-regulation of any active caspase-1 in activated cell lysates does not exclude the possibility that the caspase-1 activation may have a role in leptin-induced IL-18 release.

Attempts to detect the up-regulation of active caspase-1 inside activated THP-1 monocytes addressed in the including were present study, the immunoprecipitation/Western blot technique. The presence of caspase-1 p10 subunit was successfully detected in THP-1 monocytes stimulated with N. gonorrhoeae using immunoprecipitation/immunoblot (Duncan et al., 2009). Although the protocol was successfully established and efficient for recombinant caspase-1 positive control in this study, this technique failed to detect any active caspase-1 p10 in stimulated THP-1 monocytes. Because the precise protocol was not mentioned in detail (Duncan et al., 2009), the reason for these discrepancies is not clear, but it may be explained at least in part by different cell preparation, different time schedules of treatment and concentrations of LPS or due to the diverse immunological assay variations. In order to confirm the effect of leptin on caspase-1 activation in further studies, analysing the production of activated caspase-1 could be achieved by adopted techniques of the published studies (Martinon et al., 2006; Halle et al., 2008). The first technique using intracellular antibodies and confocal microscopy could allow us to detect the intracellular caspase-1 p10 subunit (Halle et al., 2008). Another possibility is using a commercially available caspase-1 ELISA to determine the effect of leptin on extracellular caspase-1 because caspase-1 is activated in coupling of IL-18 processing and
rapidly released into the supernatant following LPS stimulation of differentiated THP-1 monocytes (Martinon et al., 2002; Martinon et al., 2006). The present study has attempted to use the caspase-1 ELISA. However, the time limit and unavailability of this assay have not allowed the investigation to be carried out.

The evidence for the role of caspase-1 in biological processes such as processing of cytokines and apoptosis was initially obtained using potent peptide-based and macromolecular inhibitors. For example, the finding that AC-YVAD-CHO, a potent caspase-1 inhibitor, prevented the release of IL-1 β from monocytes, suggested that caspase-1 was the pro-IL1 β -processing enzyme (Thornberry et al., 1992). In addition, *Shigella flexneri*-induced IL-18 release is significantly blocked in THP-1 monocytes treated with caspase-1 inhibitor, YVAD-CHO (Willingham et al., 2007). The caspase-1 inhibitor experiments revealed that leptin-induced IL-18 release was clearly inhibited by caspase-1 inhibitor. This data provides evidence supporting that the effect of leptin on IL-18 release is dependent on caspase-1 pathway.

A further confirmation that leptin-induced IL-18 release is associated with caspase-1 activation is the presence of pro-IL-18 processing into its active IL-18. Although leptin and *E.coli* LPS were found to decrease the levels of immature pro-IL-18, the present study failed to detect intracellular mature IL-18 (18 kDa) after 3 h leptin or LPS stimulation. In agreement with previous study (Mehta et al., 2001), it was noted that it is difficult to document the presence of mature IL-18 inside LPS-stimulated monocytes. This data suggested that the processing and release of IL-18 are rapid and probably concurrent events (Mehta et al., 2001).

In conclusion, the data presented in this study demonstrate a novel role for leptin in the activation of caspase-1 mediated IL-18 release by human monocytes. Although leptin

has no effect on IL-18 mRNA, leptin up-regulates caspase-1 activity and modulates levels of active caspase-1 p20, indicating that leptin enhances inflammasome function. Furthermore, leptin-induced IL-18 release was significantly inhibited by caspase-1 inhibitor (Ac-YVAD). Thus, leptin stimulates IL-18 release by modulating the posttranslational regulation of IL-18 synthesis. However, additional work is needed to understand the discrepancies in the experiments and physiological relevance of caspase-1 activation in IL-18 synthesis regulated by leptin. Alternative techniques such as using siRNA or animal models to block this pathway could be useful to confirm this finding.

Chapter 6 An investigation into signalling mechanisms that regulate leptin-induced IL-18 release in monocytes

6.1 Introduction

Leptin is mainly produced by adipocytes and released into circulation to exert its peripheral and central biological effects (Fernandez-Riejos et al., 2010). Multiple isoforms of leptin receptors (ObRs) are expressed in many tissues (Tsiotra et al., 2000; Schroeter et al., 2007). For example, the long isoform of leptin receptor (ObR-b) is highly expressed in the hypothalamus whereas the short isoform (ObR-a) is more abundant in choroid plexus where it is believed to play a role in the transport of leptin across the blood-brain barrier (Tartaglia et al., 1995). In addition, leptin receptors are found in a variety of immune cells including monocytes (Sanchez-Margalet and Martin-Romero, 2001; Sanchez-Margalet et al., 2003; La Cava and Matarese, 2004). The ObRb is known to be predominantly responsible for active signal transduction (Myers, 2004). Upon binding to ObR-b, leptin activates JAK-2, which then initiates downstream signalling including members of STAT family of transcription factors (Kloek et al., 2002). The downstream JAK-2-STAT3 signalling by leptin has been demonstrated to regulate several cellular functions including cytokine up-regulation (Tang et al., 2007; Kanda and Watanabe, 2008; Kiguchi et al., 2009). In addition, the activation of leptin receptor is able to phosphorylate insulin receptor substrate (IRS) protein and induce the IRS-PI3K signalling pathway (Szanto and Kahn, 2000; Niswender et al., 2001; Tong et al., 2008). Moreover, leptin signal transduction is also associated with the activation of the mitogen-activated protein kinase (MAPK) and the p38 pathways (Lam et al., 2007).

IL-18 is essential for host defence and mediates the pathogenesis of many inflammatory disorders (Dinarello, 2007). IL-18 is produced as an immature cytoplasmic precursor. The NLRP3 inflammasome and caspase-1 are key regulators of IL-18 processing and release (Mariathasan and Monack, 2007). Although the NLRP3 inflammasome is activated by a broad range of stimuli (Tschopp and Schroder, 2010), the precise mechanism that initiates the assembly of NLRP3 inflammasome and the subsequent activation of caspase-1 is unclear. It has been suggested that the activation of NLRP3 inflammasome may not result from direct interaction between NLRP3 and pathogens or specific ligands because NLRP3 inflammasome is able to be activated by a range of stimuli with different molecular structures (Latz, 2010; Tschopp and Schroder, 2010). To date, three major mechanisms have been proposed to describe the NLRP3 inflammasome activation (Franchi et al., 2009a). The first suggests that extracellular ATP stimulates the purogenic P2X7 receptor (Ferrari et al., 2006), which acts as a cation channel to rapidly induce the release of intracellular potassium (K^{+}) , which is then necessary for inflammasome activation (Perregaux and Gabel, 1994; Ferrari et al., 2006). In addition, extracellular ATP also results in the opening of a pore mediated by pannexin-1 (Kanneganti et al., 2007), which induces the translocation of bacterial molecules from an intracellular vesicular compartment to the cytosol, where it induces caspase-1 activation via NLRP3 (Marina-Garcia et al., 2008). Furthermore, exogenous ATP enhances IL-1 β processing and secretion through Ca²⁺ influx and activation of phospholipases in LPS-stimulated primary human monocytes (Walev et al., 2000; Andrei et al., 2004; Ferrari et al., 2006).

Previous studies suggested that even though procaspase-1 and P2X7 receptor (P2X7R) are constitutively expressed monocytes/macrophages, LPS priming is necessary for

ATP-mediated caspase-1 activation (Mariathasan et al., 2004; Yamamoto et al., 2004; Kahlenberg et al., 2005). For example, microbial ligands such as LPS induce robust caspase-1 activation and IL-1 β secretion in macrophages after brief stimulation of P2X7R by ATP (Kahlenberg et al., 2005). In mouse macrophages, caspase-1 is efficiently activated in response to LPS or infection with extracellular bacteria such as *S. aureus* and *E.coli* only after addition of ATP (Franchi et al., 2007). However, in human monocytes, agonists of different PRRs, including LPS alone, triggers the release of endogenous ATP which is responsible for the maturation and secretion of IL-1 β and IL-18 (Piccini et al., 2008) suggesting that single stimulation is sufficient to activate caspase-1 and subsequent IL-1 β and IL-18 release in monocytes. These data also suggested the a differential requirement for robust caspase-1 activation between

monocytes and macrophages (van de Veerdonk et al., 2011).

NLRP3 appears to be a key inflammasome component for ATP-induced caspase-1 activation because macrophages from NLRP3 knock-out mice did not cleave pro-IL-1 β in response to LPS and ATP (Mariathasan et al., 2006). However, a study demonstrated that blocking of the ATP receptor (P2X7R) had no effect on monosodium urate-driven inflammasome activation. This finding suggested that monosodium urate crystals can induce inflammasome activation via P2X7R independent pathway (Martinon et al., 2006). Collectively, these data suggested that other inflammasome-activating pathways may exist and ATP may modulate IL-1 β secretion in a certain context. Indeed, the second mechanism proposes that phagocytes directly engage and engulf specific stimuli such as DAMPs. As a result of lysosomal damage and rupture, lysosomal enzymes such as cathepsin B are released into the cytoplasm which thereby triggers NLRP3 activation (Halle et al., 2008; Hornung et al., 2008). Finally, the production of reactive oxygen species (ROS) is another possible mechanism: the NLRP3 inflammasome activation and

subsequent IL-1 β release are triggered by ROS which is a product produced after

phagocytosis of pathogenic substances such as asbestos and silica (Dostert et al., 2008).

Up to now, there is no information regarding regulation of caspase-1 activation in human monocytes and the underlying mechanism. A study by Mirshamsi et al. (2004) showed that leptin increases the activity of K_{ATP} channels in neuron cells (Mirshamsi et al., 2004) suggesting the role of leptin on K_{ATP} channels. This data also allows the speculation to be made that leptin-induced the activation of caspase-1 inflammasome could be mediated by the indirect effect of leptin on endogenous ATP production. The data in previous chapters have demonstrated that leptin is involved in regulation of IL-18 release in monocytes. Leptin induces IL-18 release via caspase-1 dependent pathway. Therefore, in this chapter, the present study aimed to investigate the intracellular signalling pathways that contribute to leptin-induced IL-18 production in THP-1 monocytes. The involvement of JAK-2 signalling in leptin-induced IL-18 release and the role of leptin in the production of endogenous ATP were addressed in this study.

6.2 Results

6.2.1 Analysis of ObR mRNA expression in THP-1 monocytes

To investigate the role of leptin receptors in leptin-mediated increase of IL-18 production, the expression of leptin receptors in THP-1 monocytes was assessed by conventional RT-PCR analysis. PCR products for both short (ObR-a) and long (ObR-b) isoforms of leptin receptors were constitutively expressed in THP-1 monocytes (Figure 6.1). However, the intensity of the PCR products remained unchanged after treatment with *E.coli* LPS and leptin 1-24 h suggesting that mRNA expression of ObRs was not influenced by these treatments. These findings confirmed that leptin receptors are

expressed in THP-1 monocytes. The signalling through leptin receptors may be associated with leptin-induced IL-18 release by THP-1 monocytes.



Figure 6.1 The ObR mRNA expression in THP-1 monocytes

THP-1 of 4 x 10^6 cells were co-cultured in the absence or presence of either leptin (1000 ng/ml) or E.coli LPS (100 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of ObR-a, ObR-b and β 2M gene were analysed by RT-PCR. Negative control is water. The results shown are representative of 3 independent experiments.

6.2.2 The effect of JAK-2 inhibitor on leptin-induced IL-18 release

One of the important pathways mediates leptin action is the JAK-2 pathway (Niswender et al., 2001). To examine whether JAK-2 activation is involved in the signal transduction pathway leading to IL-18 secretion by leptin, the JAK-2 inhibitor (AG490) was used to specifically inhibit JAK-2 signalling. THP-1 monocytes (5 x 10^6 cells) were pretreated with AG490 (5 μ M) for 30 min. The cells were washed and the medium was changed. Subsequently, the cells were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Supernatants were collected and analysed for IL-18 concentration by ELISA.

As shown in Figure 6.2, the leptin-induced IL-18 release was significantly suppressed by the JAK-2 inhibitor AG490. The mean level of IL-18 release in leptin stimulation was 41.3 \pm 6.8 pg/ml whereas the mean IL-18 concentration in AG490 with leptin treated cells was 22.5 \pm 7.1 pg/ml (p < 0.05). It has been demonstrated that there is a crosstalk between JAK-2 pathway and TLR4 signalling (Hu et al., 2007; Handfield et al., 2008); therefore, this may explain the reduction of IL-18 release by *E.coli* LPS which was also observed in AG490 pretreated cells. In addition, AG490 and DMSO solvent alone has no effect on IL-18 release as demonstrated by the comparable levels of IL-18 to unstimulated control. To exclude cytotoxic effects, cell viability was assessed by a cell proliferation assay and no cytotoxicity of both DMSO solvent and the inhibitor AG490 could be observed, as previously shown in 2.2.5. Taken together, these results indicate the involvement of JAK-2 signalling in the stimulation of IL-18 release by leptin in THP-1 monocytes.



Figure 6.2 The effect of JAK-2 inhibitor on leptin-induced IL-18 release

THP-1 monocytes (5 x 10^6 cells) were pretreated with the inhibitor AG490 (5 μ M) for 30 min. The cells were washed and the medium was changed. Subsequently, the cells were stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Supernatants were collected and analysed for IL-18 concentration by ELISA. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA *: *p* < 0.05. Ec: *E.coli*.

6.2.3 The effect of ATP on IL-18 release in monocyte treated with leptin

ATP acts as an agonist of IL-18 post-translational processing by binding to the P2X7 receptor (Piccini et al., 2008). Therefore, monocytes were stimulated in the presence or absence of ATP to determine the additional effect of ATP on IL-18 release induced by leptin. THP-1 or primary monocytes (5 x 10^5 cells) were cultured in the absence or presence of leptin (1000 ng/ml) or *E.coli* LPS (100 ng/ml) for 3 h. During the last 30 min of stimulation time, the cells were exposed to ATP (6 mM). Unstimulated cells served as control. Culture supernatants were collected for determination of IL-18 levels using ELISA.

As shown in Figure 6.3, ATP, *E.coli* LPS and leptin alone induce IL-18 release in THP-1 monocytes. Interestingly, ATP significantly enhanced IL-18 release in both *E.coli* LPS and leptin treated cells compared with *E.coli* LPS alone or leptin alone, respectively (p < 0.05). The mean IL-18 release in leptin-treated cells was 64.8 ± 24.2 pg/ml whereas ATP significantly promoted IL-18 release in THP-1 monocytes by leptin (676.8 ± 218.5 pg/ml, p < 0.05). In addition, the same pattern was observed in *E.coli* LPS-treated cells (*E.coli* LPS, 213.1 ± 62.8 pg/ml vs. *E.coli* LPS plus ATP, 1507.6 \pm 331.3 pg/ml).

The results from similar experiments carried out using primary monocytes are shown in Figure 6.4. The concentration of IL-18 (mean \pm SD) in the unstimulated control cultures was 3.8 ± 1.5 pg/ml. After leptin and *E.coli* LPS stimulation, the IL-18 concentrations were 12.1 ± 2.8 pg/ml and 10.9 ± 3.8 pg/ml, respectively. ATP significantly enhanced leptin-induced and *E.coli* LPS-induced IL-18 release in primary monocytes (p < 0.05). The IL-18 concentration (mean \pm SD) in the cultures stimulated with leptin plus ATP was 41.4 ± 6.6 pg/ml. Additionally, *E.coli* LPS plus ATP induced a drastic release of

IL-18 (330.2 \pm 45.5 pg/ml) in primary monocytes at 3 h stimulation. Taken, together, these data indicate that IL-18 secretion in leptin-treated cells is significantly enhanced by ATP. This suggests a synergy effect between ATP and leptin signaling on IL-18 release in human monocytes.



Figure 6.3 IL-18 secretion in leptin treated cells is significantly enhanced by ATP

THP-1 monocytes were stimulated with either of 1 ng/ml Ec LPS or 1000 ng/ml leptin for 3 h, followed by stimulation with ATP (6 mM) at the last 30 min of LPS or leptin stimulation. Supernatants were collected and analysed for IL-18 concentration by ELISA. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA *: *p* < 0.05. Ec: *E.coli*.



Figure 6.4 The effect of ATP on leptin-induced IL-18 release in primary monocytes

Primary monocytes were stimulated with either of 1 ng/ml Ec LPS or 1000 ng/ml leptin for 3 h, followed by stimulation with ATP (6 mM) at the last 30 min of LPS or leptin stimulation. Supernatants were collected and analysed for IL-18 concentration by ELISA. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA *: *p* < 0.05. Ec: *E.coli*.

6.2.4 The determination of endogenous ATP production in leptintreated monocytes

In order to test the hypothesis that leptin-induced IL-18 release might be mediated by endogenous ATP released by activated monocytes, the levels of ATP produced by monocytes were determined using an ATP assay (see 2.7). THP-1 monocytes (5 x 10^5 cells) were pre-incubated with ecto-ATPase inhibitor ARL (200 μ M) for 30 min. The cells were then stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h (Figure 6.5). Unstimulated cells were served as control. Culture supernatants were collected for assay of ATP levels.

As shown in Figure 6.5, *E.coli* LPS and leptin significantly induced ATP release by THP-1 monocytes. The levels of ATP (mean \pm SD) in *E.coli* LPS-treated cells and leptin-treated cells were 17.2 \pm 4.7 nM and 16.4 \pm 3.8 nM, respectively whereas the ATP release in the unstimulated control was 6.5 \pm 2.4 nM (p < 0.05).



Figure 6.5 Leptin induces the production of endogenous ATP in THP-1 monocytes

THP-1 monocytes (5 x 10^5 cells) were pre-incubated with ecto-ATPase inhibitor ARL (200 μ M) for 30 min. The cells were then stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. Unstimulated cells served as control. Culture supernatants were collected for determination of ATP levels using ATP determination kit. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). These experiments were carried out on 3 different occasions. Statistics: ANOVA *: *p* < 0.05. Ec: *E.coli*.

6.2.5 The effect of P2X7R inhibitor (PPADS) on leptin-induced IL-18 release

To further investigate the role of ATP signalling in leptin-induced IL-18 release, experiments using an ATP inhibitor were carried out. As shown in Figure 6.5, THP-1 monocytes (5 x 10^5 cells) were pre-incubated with ATP inhibitor PPADS (20 μ M) for 30 min. The cells were then stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. During the last 30 min of stimulation time, the cells were exposed to ATP (6 mM). Unstimulated cells were served as negative control. Culture supernatants were collected and analysed for IL-18 concentrations using ELISA.

The results are shown in Figure 6.6 and Figure 6.7. The ATP inhibitor PPADS significantly inhibited ATP-induced IL-18 release in THP-1 monocytes. The level of IL-18 (mean \pm SD) in ATP stimulation alone was 105.5 \pm 67.0 pg/ml whereas stimulation with PPADS plus ATP reduced IL-18 release to 52.6 \pm 43.7 pg/ml (p < 0.05). The PPADS also significantly blocked the enhancing effect of ATP on both *E.coli* LPS and leptin-induced IL-18 release. The IL-18 release in cells stimulated with *E.coli* LPS plus ATP was 647.3 \pm 219.7 pg/ml whereas the combination of PPADS, *E.coli* LPS and ATP stimulated IL-18 release at 260.9 \pm 47.3 pg/ml (p < 0.05, Figure 6.6). Additionally, the level of IL-18 secretion in the leptin plus ATP stimulated cell cultures was 253.2 \pm 125.3 pg/ml whereas the combination of PPADS, leptin and ATP resulted in IL-18 levels of 100.0 \pm 40.7 pg/ml (p < 0.05, Figure 6.7).

However, the ATP inhibitor PPADS only slightly decreased IL-18 release in THP-1 monocytes stimulated with *E.coli* LPS or leptin in the absence of exogenous ATP. The IL-18 concentration after *E.coli* LPS stimulation alone was 221.3 ± 60.0 pg/ml whereas the PPADS plus *E.coli* LPS resulted in IL-18 levels of 214.9 ± 47.3 pg/ml. The IL-18 concentrations in these two cultures were not significantly different from each other (*p*)

> 0.05, Figure 6.6). In addition, leptin induced IL-18 release at 41.8 ± 15.5 pg/ml. Only a modest level of inhibition of IL-18 release was observed after adding the inhibitor PPADS to leptin-treated cells (37.1 ± 11.7 pg/ml, p > 0.05, Figure 6.7). In summary, the inhibitor of P2X7 receptor (PPADS) does not significantly reduce the effect of leptin on IL-18 release suggesting that leptin-induced IL-18 release may be independent on ATP signalling pathway.



Figure 6.6 The effect of ATP inhibitor (PPADS) on *E.coli* LPS-induced IL-18 release

THP-1 monocytes (5 x 10^5 cells) were pre-incubated with ATP inhibitor PPADS (20 μ M) for 30 min. The cells were then stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. At the last 30 min of stimulation time, the cells were exposed to ATP (6 mM). Unstimulated cells served as negative control. Culture supernatants were collected and analysed for IL-18 concentrations using ELISA. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). * *p* < 0.05. NS: no significant. Ec.: *E.coli*.



Figure 6.7 The effect of ATP inhibitor (PPADS) on leptin-induced IL-18 release

THP-1 monocytes (5 x 10^5 cells) were pre-incubated with ATP inhibitor PPADS (20 μ M) for 30 min. The cells were then stimulated with *E.coli* LPS (100 ng/ml) or leptin (1000 ng/ml) for 3 h. At the last 30 min of stimulation time, the cells were exposed to ATP (6 mM). Unstimulated cells served as negative control. Culture supernatants were collected and analysed for IL-18 concentrations using ELISA. Each value is a mean \pm SD of 3 experiments (each comprising duplicate cultures i.e. n=6 in total). * *p* < 0.05. NS: no significant. Ec.: *E.coli*.

6.3 Discussion

The present study demonstrated that the inhibition of JAK-2 tyrosine kinase prevents leptin-induced IL-18 release in THP-1 monocytes, thereby suggesting a downstream signalling pathway for the previously observed leptin activation of monocytes to secrete IL-18. Interestingly, addition of exogenous ATP significantly enhanced IL-18 release in leptin-stimulated monocytes. Moreover, this study found that leptin enhanced the endogenous ATP production, suggesting that this mechanism may play a role in the activation of caspase-1 inflammasome.

The leptin receptor is related to gp130 and therefore belongs to the class I cytokine receptor superfamily (Tartaglia et al., 1995). It has been reported that leptin receptors are expressed in THP-1 monocytes (Gabay et al., 2001) and human primary monocytes (Cioffi et al., 1996; Zarkesh-Esfahani et al., 2001; Schroeter et al., 2007). In addition, leptin receptors are found in other cell types including fibroblasts, T and B lymphocytes and macrophages (Loffreda et al., 1998; Martin-Romero et al., 2000; Tsiotra et al., 2000; Ahmed et al., 2007; Tong et al., 2008; Agrawal et al., 2011). In agreement with previous studies, the present study found that THP-1 monocytes express both ObR-a and ObR-b at the mRNA level (Gabay et al., 2001; Zarkesh-Esfahani et al., 2001). However, the mRNA expression of ObR-a and ObR-b seemingly remains unchanged after treatment THP-1 monocytes with E.coli LPS or leptin for 1, 3 and 24 h. As a member of class I cytokine family, the leptin receptor is known to stimulate tyrosine phosphorylation of STAT proteins by activating JAK kinases, which are associated with the intracellular part of the transmembrane receptor (Kishimoto et al., 1994; Baumann et al., 1996; Vaisse et al., 1996; Fernandez-Riejos et al., 2010). In a previous study, the long isoform of the leptin receptor (ObR-b) has been shown to activate JAK-2 in human peripheral blood mononuclear cells which may mediate the action of leptin on cytokine production (Sanchez-Margalet and Martin-Romero, 2001; Sanchez-Margalet et al., 2003). The present study demonstrated that JAK-2 activation is associated with leptininduced IL-18 release in THP-1 monocytes. The AG490, as a JAK-2 inhibitor significantly suppressed leptin-induced IL-18 release in THP-1 monocytes. In addition, the proliferation assays showed that no significant difference in cell number between AG490-treated monocytes and non-stimulated control indicating that the inhibition of leptin-induced IL-18 release is not due to cytotoxicity of the inhibitor AG490. It would be interesting to inhibit the leptin receptor (Ob-Rb) to further confirm the effect of leptin on IL-18 release. The potential model that could determine this issue is an animal model. Loffreda et al. (1998) revealed that rodents with genetic abnormalities in leptin (ob/ob) and leptin receptor (db/db) have an impaired ability in macrophage phagocytosis and the expression of cytokines such as TNF-a and IL-6 both in vivo and in vitro. This model could be applied to investigate whether the effect of leptin on IL-18 release is mediated directly by the leptin receptor. For example, an experiment using monocytes isolated from db/db mice and their wild-type littermates stimulated with exogenous leptin could enable dissection of the precise role of the leptin receptor in leptin-mediated IL-18 release.

The exogenous ATP is one of the well-characterized inflammasome-activating signals (Latz, 2010; van de Veerdonk et al., 2011). ATP-driven maturation and release of IL-1 β and IL-18 are specifically mediated by the P2X7 receptor (Perregaux et al., 2000; Ferrari et al., 2006; Piccini et al., 2008). The P2X7 receptor is expressed on the cell surface of macrophages and THP-1 monocytes (Humphreys and Dubyak, 1998; North and Surprenant, 2000; Into et al., 2002). Previous studies demonstrated that short-term (5 min) stimulation of macrophages with ATP results in the processing and release of IL-1 β to the extracellular medium within 15 min (Perregaux and Gabel, 1994;

Kahlenberg and Dubyak, 2004; Kahlenberg et al., 2005). However, because IL-1 β production is regulated by induced expression as well as post-transcriptional processes, such studies suggested that LPS-priming is necessary to up-regulate the transcription and translation of IL-1 β before acute stimulation of P2X7 receptor by ATP addition in monocyte/macrophages. Unlike IL-1 β , IL-18 is constitutively expressed in human monocytes (Puren et al., 1999; Dinarello, 2007) and ATP alone was found to be able to induce IL-18 release (Perregaux et al., 2000). In agreement with the latter study, the present study demonstrated that ATP (6 mM) significantly enhanced IL-18 release in THP-1 and primary monocytes after 30 min stimulation. Furthermore, this study also demonstrated that ATP significantly enhances IL-18 release in both *E.coli* LPS and leptin treated monocytes. Therefore, it could be concluded that although leptin and LPS alone will stimulate IL-18 release, the response can be significantly amplified by exogenous factors such as ATP.

The source of extracellular ATP for IL-1 β and IL-18 release *in vivo* is not completely known. It is suggested that at the site of inflammation DAMPs derived from injured or bystander cells can passively induce ATP production into extracellular space (Piccini et al., 2008). *In vitro*, a study by Ferrari et al. (1997) was the first study to demonstrate that microglia and monocyte-derived human macrophages release ATP when stimulated with LPS. This study also showed that the activation of ATP receptor (P2X7R) is involved in IL-1 β release stimulated by LPS (Ferrari et al., 1997). Another study supported this finding showing that different TLR agonists trigger the secretion of endogenous ATP that activates P2X7 receptor through an autocrine loop resulting in secretion of IL-1 β and IL-18 in human monocytes (Piccini et al., 2008). The present study has now demonstrated that leptin stimulates the production of endogenous ATP they are present that the autocrine stimulation of P2X7

receptor by the released ATP may be one possible mechanism responsible for the secretion of IL-18 induced by leptin. To further test this hypothesis, monocyte stimulations were carried out in the presence of P2X7 receptor inhibitor (PPADS). Although, blocking the P2X7 receptor by the inhibitor PPADS effectively inhibited the enhancing effect of exogenous ATP on IL-18 release, the inhibitor PPADS did not significantly prevent *E.coli* LPS and leptin-induced IL-18 release. Only a slight reduction of IL-18 release was observed in *E.coli* LPS and leptin-stimulated monocytes.

The reason for these results is unclear but a possible explanation is that the concentration of inhibitor PPADS (20 μ M) used in the experiment may be not sufficient to block the LPS or leptin-induced IL-18 release. Although a lower concentration of PPADS (10 μ M) has been shown to block both DBATP-induced currents, and YO-PRO1 influx in HEK-293 cells expressing the recombinant human P2X7 receptor (Chessell et al., 1998), higher concentrations of PPADS (100, 500 μ M) were used in previous studies to inhibit LPS-induced IL-1 β release in THP-1 monocytes and human primary monocytes (Grahames et al., 1999) and macrophages (Into et al., 2002).

The finding that the inhibitor PPADS failed to show a significant reduction in IL-18 release in leptin-stimulated monocytes raises the possibility that leptin-induced IL-18 release may be independent on the ATP signalling pathway. Mechanisms have been proposed to describe the NLRP3 inflammasome activation which subsequently leads to IL-1 β and IL-18 release (Latz, 2010; Tschopp and Schroder, 2010; van de Veerdonk et al., 2011). One of the potential mechanisms for NLRP3 activation is the generation of reactive oxygen species (ROS) (Tschopp and Schroder, 2010). The NLRP3 inflammasome-activating ligands, including ATP, asbestos and silica can induce the generation of short-lived ROS. In addition, IL-1 β production in monocytes was impaired in response to asbestos, MSU and ATP when pre-treatment cells with ROS

inhibitors (Cruz et al., 2007; Petrilli et al., 2007b; Dostert et al., 2008). Leptin was found to be one of factors that can induce oxygen radical formation by monocytes (Sanchez-Pozo et al., 2003). Thus, it is likely that leptin-mediated ROS production or other signalling pathways could potentially contribute to leptin-induced IL-18 release.

In addition, it should be noted that the cellular ATP concentrations $(16.4 \pm 3.8 \text{ nM})$ secreted by THP-1 monocytes is well below the concentration of exogenous ATP (6 mM) used to stimulate IL-18 release and therefore one could argue that such endogenous ATP concentrations are not able to provide their own signal for IL-18 secretion. However, this finding was previously reported in other experimental systems such as human monocytes (Piccini et al., 2008) astrocytes (Joseph et al., 2003) and renal glomeruli (Karczewska et al., 2007). Because cell-derived ATP is rapidly hydrolysed it is very difficult to estimate the actual amount of ATP released at the cell surface and so the ATP assay measurements of culture supernatants may be an underestimate of the true levels of biologically active ATP in this system (Joseph et al., 2003; Karczewska et al., 2007). In addition, the ecto-nucleotidases play a key role in hydrolysis of the released ATP (Joseph et al., 2003; Karczewska et al., 2007). The present study found that the concentrations of ATP are detected in culture media when monocytes are pretreated with ecto-ATPase inhibitors. On the other hand, it could be possible that an autocrine ATP production may only provide limiting signal in inflammasome-mediated IL-1β and IL-18 secretion as different TLR agonists are capable of inducing different degree of endogenous ATP production (Piccini et al., 2008). Since experiments performed in vivo in P2X7 receptor knockout mice conclusively demonstrated ATPmediated IL-1ß release through the P2X7 receptor (Solle et al., 2001; Labasi et al., 2002), further studies using this animal model may be useful to further investigate the role of P2X7 receptor-mediating the effect of leptin on IL-18 release in monocytes.

In conclusion, the present study provides molecular mechanisms responsible for the regulation of leptin-induced IL-18 release in human monocytes. Several important pathways are involved in these processes: leptin receptors are expressed in THP-1 monocytes and leptin activates IL-18 release through JAK-2 signalling. This study also demonstrates an important role of ATP in IL-18 secretion by leptin. Exogenous ATP significantly enhanced IL-18 release in leptin-treated monocytes and leptin was found to promote the production of endogenous ATP. However, the inhibition of P2X7 receptor does not significantly alter the effect of leptin on IL-18 release. These data question the role of ATP-dependent IL-18 release by leptin and likely suggest that other mechanisms may be involved in this process.

Chapter 7 General discussion

Cytokines play a central role in the function of effective immune responses. The actions of pro-inflammatory cytokines underpin the pathophysiology of chronic inflammatory diseases and may mediate the pathological link between disorders such as diabetes and periodontal disease (Donath and Shoelson, 2011; Preshaw and Taylor, 2011). Periodontal disease results from the complex interaction between periodontal pathogens and host responses. The cellular processes that drive the immune responses are mediated by numerous cytokines functioning as a network. The bacterial products such as LPS are known to stimulate the expression and secretion of a number of cytokines and chemokines from periodontal resident and immune cells (Liu et al., 2010; Taylor, 2010; Preshaw and Taylor, 2011). As a result, these cytokines form a network, acting in an autocrine and paracrine manner to control infection. On the other hand, the unbalanced/excessive production of pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6 may lead to periodontal tissue destruction (Graves and Cochran, 2003). Cytokines do not function as a sole mediator but rather as a complex number of cytokines working with each other as a system. Increasing knowledge of cytokine interactions and cytokine-mediated links between innate and adaptive immune responses is therefore important to provide a better understanding of the pathogenesis of periodontal disease. Up to date, a number of studies suggest the pivotal role for IL-1 β , TNF-α, IL-6 and RANK/RANKL in periodontal inflammation (Deo and Bhongade, 2010; Liu et al., 2010; Taylor, 2010).

IL-18 is another important cytokine that may contribute to the inflammatory process (Delaleu and Bickel, 2004; Orozco et al., 2007). The pro-inflammatory cytokine IL-18 is fundamentally important in the activation of Th1 responses (Nakanishi et al., 2001b).

In addition, IL-18 drives a wide range of pro-inflammatory effector networks in many cell types (Dai et al., 2007; Dinarello, 2007). The levels of IL-18 are elevated in the diseased periodontium, and its unbalanced production may be contribute to the initiation and progression of periodontal disease (Orozco et al., 2006; 2007; Figueredo et al., 2008b; Pradeep et al., 2009b). Moreover, the serum levels of IL-18 have been reported to be closely associated with the development of type 2 diabetes (Troseid et al., 2010). The present study demonstrated for the first time that serum IL-18 is significantly elevated in T2DM subjects with periodontitis compared to non-diabetic periodontally healthy controls suggesting that IL-18 could be a potential biomarker in cross-susceptibility between T2DM and periodontal disease.

To improve understanding the role of IL-18 in periodontal disease, it is interesting to investigate the interaction of IL-18 functions in the cytokine network existing in periodontal inflammation. Most previous publications in periodontal research have focused on single mediators for reasons of cost and technical difficulties (Preshaw and Taylor, 2011). In addition, because cytokine-mediated immune responses are complex, it is difficult to dissect and prioritise the role of specific cytokines in driving tissue destruction. Fortunately, recent advances have enabled us to examine cytokine responses in a holistic approach. For example, analysis of gene expression patterns in periodontal cells and tissues on a genome-wide basis has been demonstrated by using microarray technology (Mans et al., 2010; Taylor, 2010). The overall findings suggested that host and microbiota interactions leads to the up-regulation of cytokine genes in periodontal inflammation (Handfield et al., 2008). However, the microarray required a highly appropriate experimental design to obtain high quality datasets and the data does not take into account post-transcriptional events. Extension of these studies with a more

robust technology and experimental system will expand our fundamental understanding of the cytokine networks in periodontal disease.

The complex cytokine networks that play diverse roles in the progression of inflammatory diseases suggest that manipulation of anti-cytokines may be a beneficial therapeutic modality for periodontitis. Because cytokines do not function in isolation, identification of key mediators which play a regulatory role in this complex may offer a logical target as a therapeutic manipulation. For example, IL-1 β and TNF- α are considered to be key cytokines in the development of chronic periodontitis (Graves and Cochran, 2003; Liu et al., 2010). These cytokines are produced by activated monocytes, macrophages and fibroblasts within periodontal lesions. IL-1 β and TNF- α promote critical inflammatory responses during periodontal disease. In addition, IL-1ß and TNF- α stimulate the production of other inflammatory enzymes and mediators such as MMPs, RANK/RANKL and PGE₂ which result in periodontal tissue destruction (Ejeil et al., 2003; Kinane et al., 2007). Studies have demonstrated that IL-1 β and TNF- α are elevated in patients with periodontal disease compared with healthy subjects and are associated with the clinical signs of periodontitis such as bone resorption, increased probing depth and attachment loss (Stashenko et al., 1991; Lee et al., 1995; Gamonal et al., 2000; Gorska et al., 2003). Moreover, successful periodontal treatment often results in reductions in levels of IL-1 β and TNF- α (Gamonal et al., 2000; Al-Shammari et al., 2001). These studies suggest that IL-1 β and TNF- α could be potential targets for therapeutic intervention in periodontal inflammation. Blocking TNF- α and IL-1 β has shown promising results in animal models of periodontitis (Zhang et al., 2004; Di Paola et al., 2007). For example, injection of animals with IL-1 β and TNF- α antagonists results in a significant improvement in periodontal healing as demonstrated by a reduction of inflammatory cell infiltration and postsurgical osteoclastogenesis in deep

gingival tissue (Zhang et al., 2004). Histological analysis revealed that IL-1 β and TNF- α antagonists significantly reduced the loss of connective tissue attachment by approximately 51% and the loss of alveolar bone height by almost 91% in a primate model of experimental periodontitis (Delima et al., 2001). However, the use of anticytokine therapy in periodontal inflammation is still in at an early stage and its impact on periodontal disease has not yet investigated in humans.

Although chronic periodontitis and rheumatoid arthritis develop from different aetiologies, these two diseases share common pathophysiological pathways which lead ultimately to connective tissue and bone destruction (Detert et al., 2010). Therefore, the proven therapies in rheumatoid arthritis could be considered useful to treat periodontitis (Kinane et al., 2011). Biological agents that target IL-1 β and TNF- α have demonstrated efficacy in patients with rheumatoid arthritis (Gabay and McInnes, 2009; Kapoor et al., 2011). Interestingly, studies have demonstrated that IL-18 plays an important role in pathological processes during experimental and clinical rheumatoid arthritis (Gracie et al., 2003; Rooney et al., 2004; van Kuijk et al., 2006; Dai et al., 2007; Dinarello, 2007). Therapeutic agents for rheumatoid arthritis targeting IL-18 neutralisation are in clinical development. In a phase I study, although recombinant human IL-18BP has a favourable safety profile and is well-tolerated in both healthy volunteers and in subjects with rheumatoid arthritis, laboratory analysis indicated an increase in the serum concentration of IL-18 after recombinant human IL-18BP treatment in healthy volunteers and in subjects with rheumatoid arthritis (Tak et al., 2006). The reason for this failure of efficacy is unclear but it could be explained by the biological compensation and redundancy of IL-18 functions by alternative pathways, making the inhibition of IL-18 by recombinant human IL-18 BP very limited. There are currently no clinical trials ongoing employing anti-IL-18 therapies although IL-18 itself is being

used in clinical trials for cancer immunotherapy (Sims and Smith, 2010). Further studies are needed to develop proper monoclonal antibodies for mature IL-18 and to define the biologic role for IL-18 as a therapeutic intervention. An interesting approach is to modulate the synthesis and release of IL-18. The maturation of proIL-18 and subsequent IL-18 release are dependent on the action of caspase-1 associated with the inflammasome. Inhibition of the caspase-1 pathway and the P2X7 receptor may provide an opportunity to block not only IL-18 but also IL-1β effector function, which could potentially be a possible therapeutic manipulation. Small molecular inhibitors of inflammasome activity have recently been identified but there have only been a very limited number of animal and human trials of these compounds (Juliana et al., 2010; Mitroulis et al., 2010). Taken together, it has become evident that cytokine networks are central to the pathogenesis of inflammatory diseases such as rheumatoid arthritis and periodontal disease. Single-cytokine targeting has proven useful in rheumatoid arthritis. A major challenge is to define the most plausible cytokines and their signalling pathways which control the pathogenesis of disease. Moreover, since periodontal inflammation is mediated by multi-cytokines functioning in networks, rational targeting of combinations of multiple cytokines may become possible to achieve successful treatment outcomes. In addition to IL-1 β and TNF- α , IL-18 could also serve as a novel therapeutic target.

Epidemiological studies suggest that periodontal disease is associated with systemic diseases such as type 2 diabetes, cardiovascular disease and obesity (Beck and Offenbacher, 2005; Pischon et al., 2007; Schallhorn et al., 2010). The strongest evidence has been established to demonstrate the inter-relationship between periodontal disease and type 2 diabetes (Taylor and Borgnakke, 2008; Chavarry et al., 2009; Preshaw, 2009). Clinical studies demonstrated that the existence of type 2 diabetes or

obesity promote the initiation and progression of periodontal disease (Pischon et al., 2007; Chavarry et al., 2009). On the other hand, there is evidence that periodontal disease can influence systemic diseases (Shimada et al., 2010; Simpson et al., 2010; Sun et al., 2010). Inflammation appears to play a key role in this relationship. Investigations in inflammatory processes reveal many mechanisms that link the pathogenesis of periodontal disease and systemic diseases (King, 2008; Preshaw, 2009; Donath and Shoelson, 2011; Ouchi et al., 2011). Nevertheless, the precise mechanisms have not been fully elucidated.

Obesity is a major public health problem and it is an important risk factor for type 2 diabetes and cardiovascular disease (Mokdad et al., 2003; Pischon et al., 2007; Shoelson et al., 2007; Hyde, 2008). A number of studies suggest that obesity has an effect on the immune responses and promotes inflammation, and that obesity-induced inflammation potentially contributes to the pathogenesis of the complications of obesity (Fernandez-Riejos et al., 2010; Donath and Shoelson, 2011; Ouchi et al., 2011). Adipose tissues have emerged as an active secretory organ, producing a variety of adipokines which influence the function of immune systems (Antuna-Puente et al., 2008; Fernandez-Riejos et al., 2010). Increasing numbers of adipokines have been identified to be secreted by adjpose tissue, for example, leptin, adjponectin, resistin, TNF- α and IL-6 (Ouchi et al., 2011). Accumulating evidence suggests that obesity represents a lowgrade inflammation state which is mediated by the dysregulation of adipokine production (Kralisch et al., 2007a; Shoelson et al., 2007; Shoelson and Goldfine, 2009; Kim, 2010a). This could be a potential mechanism that leads to the initiation and progression of obesity-induced metabolic disorders, type 2 diabetes and cardiovascular complications.

The function of adipokines could potentially link the causal relationship between diabetes and periodontitis (Pischon et al., 2007; Ouchi et al., 2011). Indeed, the finding in the present study that serum IL-18 level is positively correlated with BMI suggests a role for IL-18 in a relationship between diabetes and obesity. In vitro, it has been shown that adipocytes are also a source of IL-18 (Skurk et al., 2005). Since IL-18 is produced mainly by monocytes and macrophages, adipokines or other adipocyte-derived cytokines would be likely to stimulate IL-18 secretion by these cell types. However, effects of the adipokines on IL-18 secretion by cultured monocytes or macrophages have not been reported, so such an *in vitro* study would be helpful to clarify the role of IL-18 in the inter-relationship between diabetes and obesity. The present study demonstrated, for the first time, that leptin enhances IL-18 release in human monocytes. These novel data provide an insight into a possible cellular mechanism suggesting how adipose tissue-derived cytokines might contribute to immune responses. The present study also expands our knowledge of the molecular interaction between leptin and IL-18 regulation. A schematic diagram of proposed effects of leptin on IL-18 release in monocytes is shown in Figure 7.1. The present study has investigated processes by which leptin may induce IL-18 release from human monocytes. The most intriguing finding is that leptin-induced IL-18 release is mediated by activation of caspase-1 inflammasome. The proposed mechanisms for leptin-induced activation of the caspase-1 inflammasome are also shown in Figure 7.1.

It is becoming clear that leptin plays an important role in the regulation of energy homeostasis, neuroendocrine function, metabolism and immune function (Fernandez-Riejos et al., 2010). Animals or individuals with leptin deficiency present with dysfunction in these systems that can be restored by leptin treatment (Dardeno et al., 2010).



Figure 7.1 The schematic diagram of proposed effect of leptin on IL-18 production

Leptin activates the JAK-STAT signalling and other related pathways including IRS, PI3K and MAPK, which in turn, may result in production of IL-18. Leptin has no effect on IL-18 mRNA expression. In addition, leptin is found to mediate the activation of NLRP3 inflammasome either by directly enhancing the action of caspase-1 (1) or by indirectly inducing the production of endogenous ATP (2) which subsequently lead to IL-18 processing and release. Moreover, leptin may modulate the expression and the assembly of other components of the inflammasome (3).

For example, exogenous leptin administration enhances weight loss and reverses the metabolic, endocrine and immune disturbances in ob/ob mice (Harris et al., 1998; Loffreda et al., 1998; Hsu et al., 2007). In obese children with complete congenital leptin deficiency, leptin replacement has beneficial effects on appetite, fat mass, hyperinsulinemia, and hyperlipidemia. Leptin therapy also resulted in appropriate pubertal development and restored immune function by enhancing numbers of circulating CD4+ T cells and T cell proliferation as well as the production of cytokine (Farooqi et al., 2002). Clinical studies have attempted to use leptin as a therapeutic agent for obesity (Heymsfield et al., 1999; Fogteloo et al., 2003; Lejeune et al., 2003); however, the results from these clinical trials showed that the effect of leptin on weight loss maintenance is relatively modest. The reason for this may be due to the leptin resistance state presented in obese individuals (Considine et al., 1996). Development of insulin sensitizers targeting signalling mechanisms of leptin resistance to use in conjunction with leptin for weight control in obese individuals is currently under investigation (Roth et al., 2008; Dardeno et al., 2010). Thus, further studies are required to address the role of key adipokines and their signalling mechanisms to understand the pathogenesis of obesity-linked disorders. Moreover, adipokine-targeting therapy could be a useful strategy to prevent and/or treat obesity-linked inflammatory disorders such as type 2 diabetes and cardiovascular diseases.

Inflammation appears to be a shared common process between periodontal disease and diabetes (King, 2008). IL-1 β and IL-18 play a key role in the inflammatory process. Given its importance to the regulation of these two cytokines, inflammasome-mediated inflammation might underpin the cellular processes that contribute to the cross-susceptibility between periodontal disease and diabetes. Recently, Bostanci et al. (2009) have investigated the role of NLRP3 inflammasome in periodontal disease. NLRP3 are

expressed at significantly higher levels in diseased periodontal tissues compared to health. In vitro, NLRP3 expression was elevated significantly in monocytic cell line after *P. gingivalis* challenge, in parallel with the expression of IL-1 β and IL-18 (Bostanci et al., 2009). Another study found that gingival epithelial cells express a functional NLRP3 inflammasome (Yilmaz et al., 2010). P. gingivalis-infected gingival epithelial cells required the addition of ATP in order to secrete IL-1B. In addition, blocking NLRP3 with siRNA inhibited the effect of ATP on IL-1β secretion by infected cells (Yilmaz et al., 2010). In type 2 diabetes, the expression of IL-1Ra is reduced in the pancreatic islets. In addition, glucose was found to stimulate IL-1^β production which leads to decreased cell proliferation and apoptosis (Welsh et al., 2005). Interestingly, use of recombinant IL-Ra (Anakinra) has been shown to decrease HbA1c levels and increase insulin production in T2DM patients (Larsen et al., 2007). Moreover, the finding that glyburide (a sulfonylurea drug used for the treatment of type 2 diabetes) prevents the activation of NLRP3 inflammasome and the subsequent IL-1ß and IL-18 release suggested the considerable therapeutic promise of the NLRP3 inflammasomeinhibiting drug (Lamkanfi et al., 2009). Indeed, the potential of NLRP3 inflammasome as a therapeutic target for diverse diseases has been currently reviewed (Cook et al., 2010) although in limited trials currently available caspase-1 inhibitors are not efficacious due to high toxicity (Mitroulis et al., 2010). Increasing knowledge of the role of the NLRP3 inflammasome in the pathogenesis of periodontal disease could potentially offer a novel therapeutic approach in the future.

Difficulties in demonstrating the activation of caspase-1 and the lack of inhibitory effect by P2X7R inhibitor on leptin-mediated IL-18 release in the present study highlight the importance of further investigating the post-transcriptional regulation of IL-18 release by leptin in human monocytes. A study by Martin et al. (2009) suggested an interesting
model system which could be used to determine leptin effects on cytokine secretion. In this paper, bone marrow-derived macrophages (BMDMs) were engineered to overexpress IL-1F6 by retroviral transduction; cells overexpressing green fluorescent protein (GFP) were generated to determine whether the release mechanism is a general pathway or unique to cytokines. The engineered cells constitutively expressed IL-1F6 and GFP, but they did not constitutively release these polypeptides to the medium which would allow us to look at the pathways that activate cytokine release in isolation rather than the regulation of gene transcription. This study found that LPS/ATP (which activates the P2X7 receptor) did not selectively induce IL-1F6 release by engineered BMDMs as a noncytokine (GFP) shows similar behaviour (Martin et al., 2009). Likewise, using this approach we could investigate the effect of leptin or leptin/ATP on IL-18 release (that is secretion) rather than transcription, translation or processing. In addition, this model can also be used to investigate the biological relevance of ATP concentrations for IL-18 release.

While a range of evidence regarding the production, signalling pathway and biological effects of IL-18 is available, further experimental studies are needed to understand the role of IL-18 in the pathogenesis of periodontal disease. Only a few *in vitro* studies are available regarding the biological role of IL-18 related to periodontal disease. For example, IL-18 together with IL-12 was found to decrease osteoclastic bone-resorbing activity (Yamada et al., 2002). Foster et al (2007) demonstrated that *P. gingivalis* LPS enhanced IL-18 release in human monocytes (Foster et al., 2007). No study has demonstrated any direct effect of IL-18 which contributes to the pathogenesis of periodontitis. Such studies investigating the periodontal cellular response to IL-18 could be of interest.

In conclusion, the present study demonstrates a role of IL-18 as a potential mediator linking type 2 diabetes and periodontal disease. The up-regulation of serum IL-18 levels may be directly relevant to periodontal destruction in diabetic individuals. In addition, the present study demonstrates a novel role for leptin in immune responses by monocytes. Leptin enhances IL-18 secretion via modulation of the caspase-1 inflammasome function. The leptin signalling pathway may therefore be crucial in the regulatory control of inflammatory responses in periodontal diseases and may also contribute to the cross-susceptibility between T2DM and periodontal diseases.

Future work

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

- 1. Analysis of serum and GCF IL-18 in a robust case-controlled study matching other confounding factors such as BMI and HbA1c.
- 2. An investigation into the dependency of the leptin receptor (ObR) on leptininduced IL-18 release using leptin receptor (db/db) deficient mice.
- 3. Further investigation of the effect of leptin on caspase-1 activation in THP-1 and primary human monocytes using a caspase-1 ELISA.
- 4. An investigation into the molecular relationship between leptin-induced inflammasome activation and IL-18 processing and release using siRNA gene silencing or monocytes/macrophages derived from mice deficient in caspase-1, ASC, NLRP3 and P2X7 receptor.
- 5. An investigation into the role of IL-18 in the regulation of cytokine production by periodontal cells such as gingival fibroblasts or epithelial cells.

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