Chapter 2

Evaluation of pro-atherogenic features in obese diabetic db/db mice

2.1 Introduction

Obesity has also been postulated as a disease of the innate immune system. There is increasing evidence that an ongoing cytokine-induced inflammatory response is related closely to the pathogenesis of type 2 DM and the associated complications such as dyslipidaemia, cardiovascular disease [Pickup, 2004; Adhikary et al., 2004, Madona et al., 2004]. Previous studies have indicated that the enhanced inflammation in obesity is associated with elevated levels of the prototypic inflammatory marker C-reactive protein (CRP) as well as the proinflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-6 [Madonna et al., 2004; Devaraj and Jialal, 2000]. In monocytes, these events can lead to increased production of inflammatory cytokines and chemokines. In fact, immature fat cells can transdifferentiate into macrophages both in vitro and in vivo. [Charriere et al., 2003]. Recent studies using large-scale genetic analyses to characterize gene expression patterns in adipose tissue from a variety of obese and lean mice have begun to clarify the role of the adipocyte as a hormone and cytokine secreting cell [Xu et al, 2003; Weisberg et al., 2003]. Surprising, in these studies, increasing adiposity in mice correlates very highly with the adipose tissue expression of a large cluster of genes characteristically expressed by macrophages, and both adipocyte size and total body weight are strong predictors of the number of mature macrophages found within adipose tissue, the correlation being even stronger for visceral than for subcutaneous fat. Furthermore, co-culture of differentiated 3T3-L1 adipocytes and macrophage cell line RAW264 results in the marked up regulation of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-alpha), and the down regulation of the anti-inflammatory cytokine adiponectin (Luo et al., 2010). Recent studies have demonstrated that treatment of cultured monocytes in vitro with free fatty acids mainly secreted from adipocytes can increase the production of various proinflammatory cytokines which are all associated with vascular inflammation. Formation of VLDL loaded foam cells resulted in a four-fold increase in basal IL-1β secretion [Persson et al., 2006]. Interestingly, these proinflammatory

cytokines also induced the binding of monocytes to VSMCs, suggesting a novel mechanism for subendothelial monocyte retention in the pathology of atherosclerosis [Cai et al., 2004]. Not only monocytes but also lymphocytes are playing major role in adipose inflammation. Lymphocytes are also increased with adiposity [Kintscher et al., 2008]. Recent studies have demonstrated that alteration of cyokines in lymphocytes of obese patients [O'Rourke et al., 2006]. Like monocyte, T cells can encounter signals that cause them to elaborate inflammatory cytokines such as interferon and lymphotoxin (tumor necrosis factor [TNF]–beta) that in turn can stimulate macrophages as well as vascular endothelial cells and SMCs [Hansson & Libby, 1996] These studies suggest that the proinflammatory milieu of obesity modulates signaling responses in monocytes or lymphocytes, leading to aberrant inflammatory gene expression and pathophysiological responses.

Elucidation of the in vivo relevance of these observations is crucial to our understanding of the increased risk of cardiovascular disease in insulin resistance and obesity. So here, I define the effects of endotoxemia on insulin sensitivity and focus on adipose inflammation in an animal model of obese diabetic db/db mice and compared with their age matched C57BL/6. Secondly, I examined the hypothesis that circulating PBMC from whole blood derived from db/db would exhibit increased expression of inflammatory genes and cellular activation relative to wild type control mice. I compared these parameters ex vivo challenged with LPS in whole blood cells derived from db/db mice, a well-characterized mouse model of type 2 diabetes, obesity, and insulin resistance [Halaas et al., 1995], relative to those from control non obese and non diabetic C57BL/6 mice. db/db mice have a mutation in the leptin-receptor gene, resulting in deletion of the cytoplasmic tail needed for intracellular signaling [Lee et al., 1996] and they develop hyperinsulinemia, hyperglycemia, and insulin resistance [Halaas et al., 1995; Lee et al., 1996]. One report showed that macrophages from db/db mice have some altered cytokine and nitric acid secretion in vitro [Zykova et al., 2000], whereas others demonstrated that in db/db mice, IL-1beta mediated

innate immunity is amplified [O'Connor et al., 2005] and endotoxin-induced lethality is increased [Faggioni et al., 1999], suggesting that these diabetic animals have dysregulated inflammatory and immune responses. They also have defective Janus-family tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) signaling, indicating that leptin signals through this pathway [Vaisse et al., 1999]. Furthermore, apolipoprotein E knockout mice crossed with db/db mice displayed increased rates of atherosclerosis [Wendt et al., 2006]. However, the impact of diabesity on cytokine/chemokine gene expression and associated signal transduction pathways has not been well characterized in both circulating PBMC (whole blood) and WAT of these db/db mice. We therefore measured inflammatory cytokine in adipocytes and whole blood cells during inflammation induced by lipopolysaccharide (LPS), a compound of the cell wall of gram-negative bacteria that has been demonstrated to induce inflammatory reactions.

Selection of animal model: LPS is a highly inflammatory constituent of the outer membrane of Gram-negative bacteria. When these bacteria multiply or lyse, LPS is released and then activates the toll-like receptor 4 (TLR4) receptor complex on endothelial cells, neutrophils, and macrophages, which results in a proinflammatory response through activation of the nuclear factor–kB pathway. In rodents, bacterial infection and LPS also promote atherogenesis [Campbell and Kuo, 2004;. Mussa et al., 2006; Nabipour et al., 2006].

2.2 Materials and Methods

2.2.1 Animals

Ten to 12-wk-old *db/db* (*C57BL/6J-leprdb/leprdb*) and their age-matched *C57BL/6J* littermates were purchased from the Jackson Laboratory. Mice were housed in I.V.C cages and given pelleted food (NIN diet, Hyderabad) and water *ad libitum* at a temperature (~25°C) and humidity (45–55%)-controlled environment with a 12-h/12-h dark-light cycle. All animal experiments were

carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, using protocols approved by the Institutional Animal Ethics Committee.

In vivo: Age matched C57BL/6 and db/db mice in the LPS group were injected intraperitonealy (i.p.) with 100µg/kg of Escherichia coli LPS (Sigma, USA), while control mice were injected with PBS (10 ml/kg). Animals were bled by retroorbital puncture at 0 (before) and 1, 4, 8h after the LPS administration. Serum was separated for estimation of biochemical parameters. In a similar study using C57BL/6 and db/db mice were euthanized in a CO_2 chamber at 0 (before), and 0.5, 1, 4 and 8h after LPS challenge and the white adipose tissue (WAT) were removed, immediately frozen in liquid nitrogen and stored at -70^{0}C for real time-PCR..

Ex vivo: Fresh whole blood was collected in the presence of heparin by retroorbital puncture from of C57BL/6 and db/db. Differential leukocyte count was made in the whole blood of three different strains using Cell dyne 3700 (Abbott, USA). A 500 µl aliquot of blood was mixed with 2 µl of lipopolysaccharide (dissolved in phosphate-buffered saline; final concentration of 10µg/ml) in microtubes. The blood mixture was incubated at 37^{0}C for 6 h, immediately chilled at 4^{0}C and then centrifuged at 3500g for 15 min. The plasma was stored at -70^{0}C until use. Concentrations of tumor necrosis factor-alpha, interleukin-1beta and interleukin-6 in the plasma were determined by enzyme-linked immunosorbent assays (BD Biosciences, USA).

2.2.2 RNA analysis and quantitative real-time PCR

Samples of WAT were homogenized in TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) using a Mixer 301 (Retsch, Haan, Germany) and total RNA was extracted following the manufacturer's protocol. Then, 1 µg total RNA from each sample was taken for first-strand cDNA synthesis using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). An equal amount of cDNA from each sample was taken for qRT-PCR using

ABIprism- 7300. FAM-labelled Taqman probes viz. PPAR gamma (Mm01184322), fatty acid binding protein (aP2) (Mm00495574_m1), lipoprotein lipase (LPL)(Mm00434764_m1), adiponectin (Mm00456425_m1), TLR-4 (Mm00445273_m1), COX-2 (Mm00478374_m1), TNF-alpha (Mm00443258_m1), IL-6 (Mm00446190_ m1) and MCP-1(Mm00441242_m1) (all from Applied Biosystems). Taqman Universal Mastermix (Applied Biosystems) was used for expression profiling of the aforementioned target genes. VIC-labelled mouse beta actin probe was co-amplified in each sample with every target gene(s) to normalize the results.

2.2.3 Serum measurements

Serum triglyceride (Pointe scientific, USA) levels were determined by the colorimetric method using biochemical kit. Serum glucose levels were determined by the glucose oxidase/peroxidase (GOD/POD) method using a commercially available kit (Ranbaxy Laboratories, Gurgaon, India). Insulin (Linco Research Inc., St Charles, MO, USA) TNF alpha (BD Biosciences, USA), IL-6 (BD Biosciences, USA), IL-1 beta (R & D Systems, USA) MCP-1 (BD Biosciences, USA), interferon gamma (BD Biosciences, USA), leptin and adiponectin (B Bridge, Mountain View, CA, USA) levels in the serum were determined by ELISA according to the manufacturers' protocols.

2.2.4 Data and Statistical Analysis

All the data has been expressed as Mean ± SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey test used to identify difference between all groups.

2.3 Results:

2.3.1 Expression of adipocytokines in the WAT of C57BL/6, and db/db mice:

The basal expression of inflammatory cytokines TNF-alpha, IL-6, MCP-1 and TLR-4 mRNA levels in the diabetic mice were significantly (7, 2.6, 1.5 and 3 folds

respectively) higher as compared to lean C57BL/6 mice (P< 0.05) (Fig 2.1). In contrast, adiponectin (Fig. 2.1E), PPAR gamma (Fig 2.3A), aP2 (Fig. 2.4A) and LPL (Fig. 2.4B) mRNA expression were found to be 8.4, 4.2, 4.0, 2.2 folds lower in db/db mice compared to C57BL/6 controls (P<0.05).

2.3.2 Endotoxemia induces adipose inflammation

We have further investigated the pro-inflammatory gene expressions in WAT of obese diabetic db/db mice and compared them with the lean C57BL/6 after LPS challenge. LPS induced endotoxemia enhanced the expression of all inflammatory genes in WAT in both C57BL/6 and db/db mice. Endotoxemia induced a robust innate inflammatory response in adipose of C57BL/6 and db/db with increases in mRNA levels of TNF (49 vs 214 fold, p<0.001) (Fig. 2.1A), IL-6 (8.1 vs 16.9 fold, p<0.001) (Fig. 2.1B). In parallel, adipose mRNA levels of MCP-1 in db/db mice, a chemokine implicated in adipose macrophage recruitment significantly (4.7 vs 9.4 fold) increased when compared to C57BL/6 mice. (Fig. 2.1C). Maximum expressions of TNF alpha was increased at 1 hour whereas IL-6 and MCP-1 at 4 hour after LPS challenge. As shown in Fig-2.1, TNF alpha, IL-6 and MCP-1 expressions were significantly (4.4, 2.1 and 2 folds) higher respectively in LPS challenged db/db mice when compared with LPS challenged C57BL/6 control mice (P< 0.05). Further, LPS administration in vivo increases the expression of TLR-4 in WAT of db/db mice. Adipose expression pattern of TLR-4 in db/db mice was similar with expression of LPS challenged C57BL/6 mice (Figure 2.1D). However, LPS administration decreases the mRNA expression of adiponectin in both the strain. A significantly lower expression of adiponectin in LPS challenged db/db mice was observed when compared with C57BL/6 mice after LPS challenge.

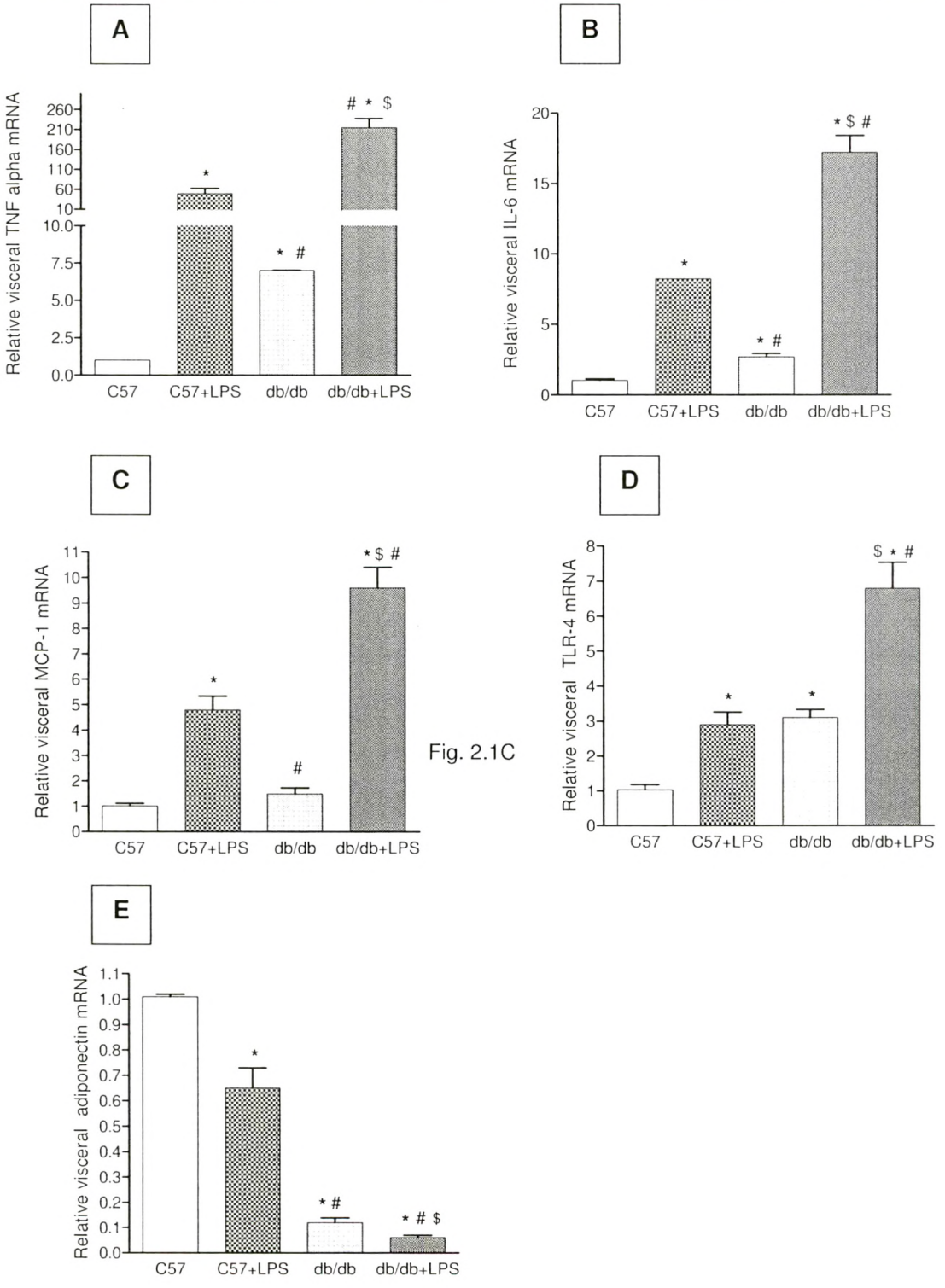


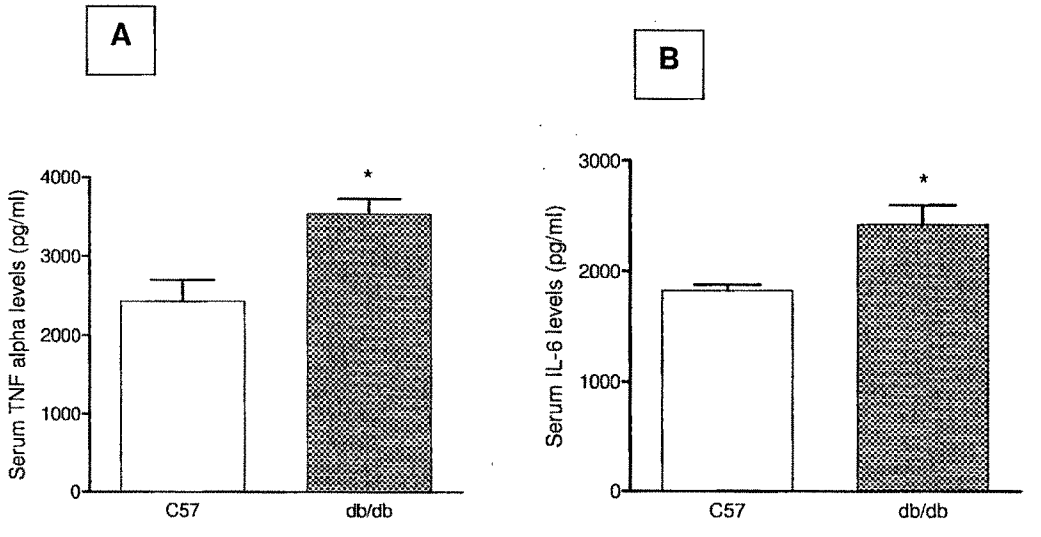
Fig. 2.1C

Fig. 2.1: Analysis of the expression of TNF alpha (A), IL-6 (B), MCP-1 (C) TLR-4 (D) adiponectin (E) mRNA in WAT of C57BL/6 and db/db mice after LPS challange. Fold change in C57BL/6

groups against other treatment is represented in the bar diagram. Each value represents mean ± S.E.M. (*n* = 6 mice/group). * P < 0.05 compared with C57BL/6 wild type mice. $ P < 0.05 compared with db/db mice and # P < 0.05 compared with LPS challenged C57BL/6 mice. (TNF alpha, IL-6, MCP-1, TLR-4 and adiponectin transcripts were significantly altered 0.5, 4, 4, 4 and 8 hour after LPS challenge and demonstrated in bar graph)

2.3.3 Alteration in the serum cytokines by LPS in mice:

Serum TNF alpha, IL-6, IL-1 beta, MCP-1 and interferon gamma levels were very low to detect in both db/db or in C57BL/6 mice. However, LPS challenge led to a dramatic rise in the serum TNF alpha, IL-6, MCP-1, interferon gamma and IL-1 beta levels after 1, 4, 4, 4 and 8 hr respectively in obese diabetic and lean mice. However, obese diabetic (db/db) animals showed remarkably higher TNF alpha, IL-6, MCP-1, interferon gamma and IL-1 beta levels when compared with C57BL/6 animals (Fig. 2.2). In contrast to other cytokine, adiponectin levels were detectable in both C57BL/6 and db/db mice. Serum adiponectin levels were decreased after LPS challenge in both db/db and C57BL/6 mice. Serum adiponectin levels were decreased after LPS challenge in C57BL/6 which was similar with the levels of db/db mice (Fig. 2.2F).



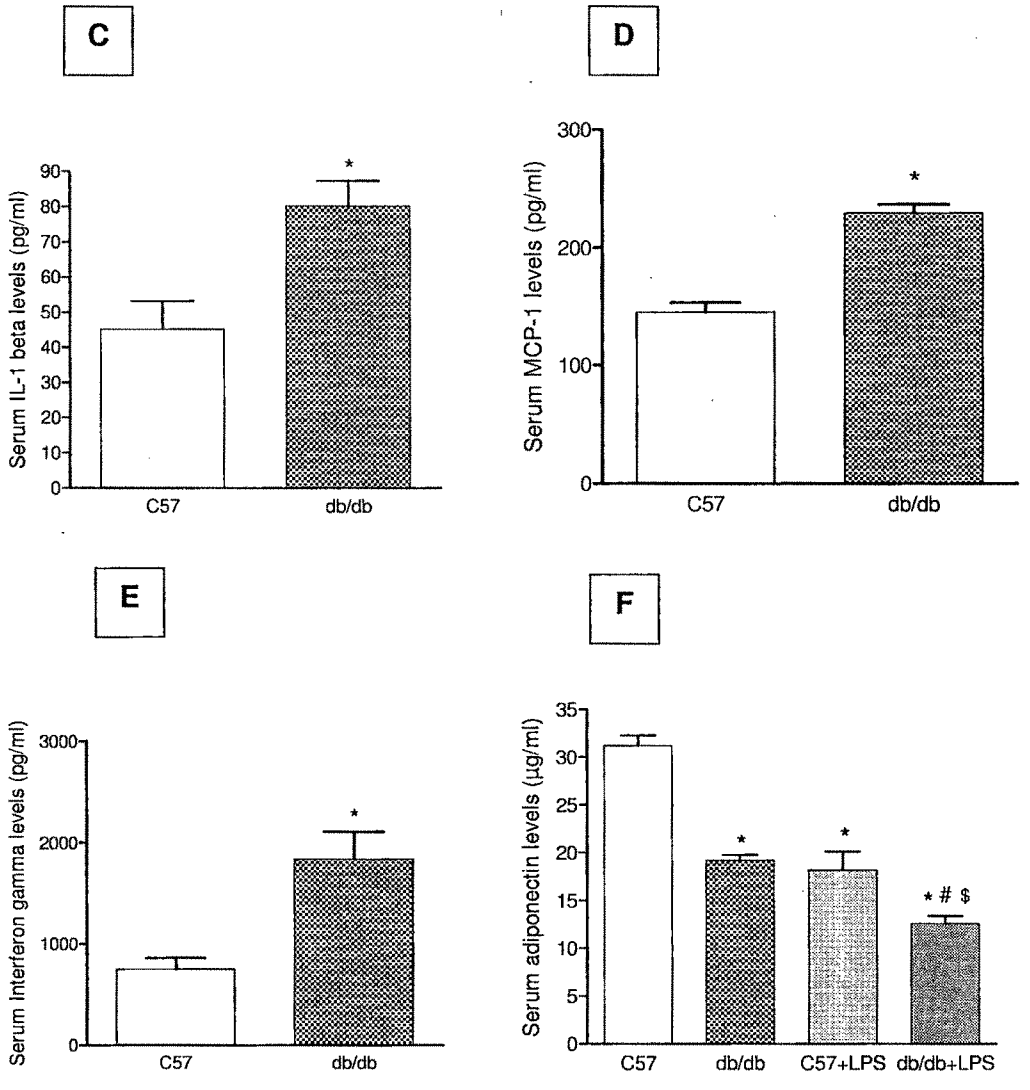


Fig 2.2: Analysis of the TNF alpha (A) IL-6 (B), MCP-1 (C) and IL-1 beta and interferon gamma (E) adiponectin (F) in serum of C57BL/6 and db/db mice after LPS challenge using ELISA. Serum levels of C57BL/6 and db/db after LPS challenge is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with C57BL/6 wild type mice.

2.3.4 Endotoxemia modulates the insulin signaling pathway in adipose

Sufficient evidence sought that adipose inflammation modulated insulin signaling pathways in adipose. SOCS family proteins inhibit tyrosine kinase receptor

signaling including the insulin receptor. At baseline prior to LPS, SOCS-3 mRNAs were the most abundant in adipose tissue. Following 8 hour after endotoxin, adipose SOCS-3 (approximately 2.5-fold) mRNAs increased markedly (Fig 2.3A). Adipose expression pattern of SOCS-3 in db/db mice was similar with expression of LPS challenged C57BL/6 mice. PPAR gamma is a nuclear receptor activators are key promoters of adipogenesis and improve insulin sensitivity was down regulated 8 hr after LPS administration (Fig. 2.3B).

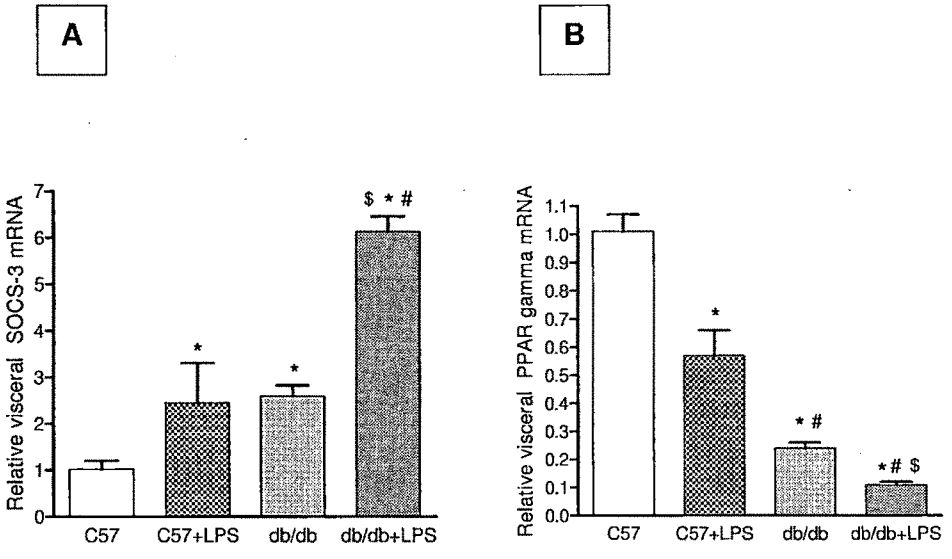


Fig 2.3 Analysis of the expression of SOCS-3 (A) and PPAR gamma (B) mRNA in WAT of C57BL/6 and db/db mice after LPS challange. Fold change in C57BL/6 groups against other treatment is represented in the bar diagram. Each value represents mean ± S.E.M. (n = 6 mice/group). * $P < 0.05$ compared with C57BL/6 wild type mice. \$ $P < 0.05$ compared with db/db mice and # $P < 0.05$ compared with LPS challenged C57BL/6 mice

2.3.5 Endotoxemia modulates the adipogenic gene expression in adipose

We investigated the mRNA expression of aP2 and LPL gene which are the major target gene for PPAR gamma are also down regulated in adipose tissue 8 hour after LPS challenge. Further we observed a similar adipose expression pattern of adipocyte marker aP2 and LPL between db/db mice and LPS challenged

C57BL/6 mice. There was no significant difference between db/db and LPS challenged C57BL/6 mice aP2 (Fig. 2.4A) and LPL (Fig. 2.4B) gene expression in WAT.

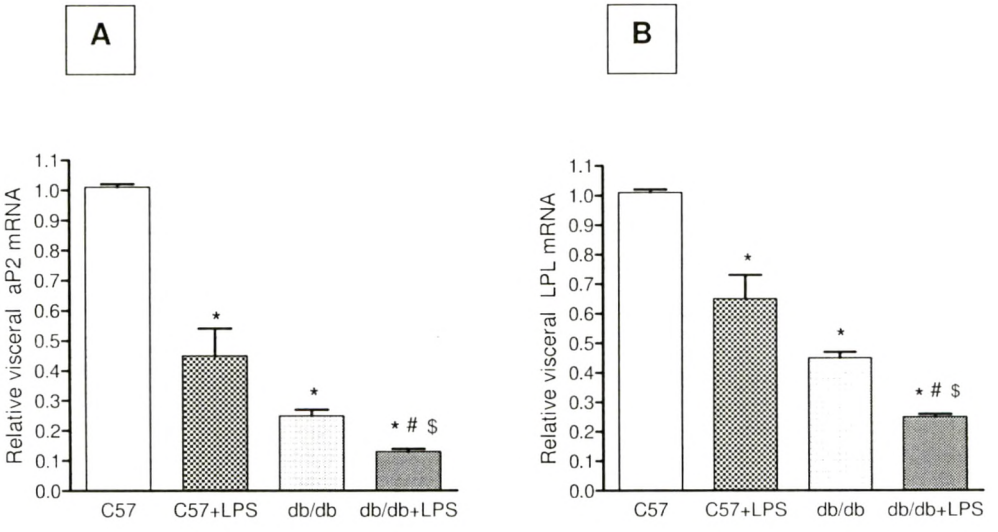


Fig 2.4: Analysis of the expression of aP2 (A) and LPL (B) mRNA in WAT of C57BL/6 and db/db mice after LPS challange. Fold change in C57BL/6 groups against other treatment is represented in the bar diagram. Each value represents mean ± S.E.M. (*n* = 6 mice/group). * P < 0.05 compared with C57BL/6 wild type mice. $ P < 0.05 compared with db/db mice and # P < 0.05 compared with LPS challenged C57BL/6 mice

2.3.6 Alteration of serum biochemical parameters in LPS challenged C57BL/6 and db/db mice

LPS significantly (P<0.05) decreases the triglyceride levels in db/db mice while there was a no significant change in C57BL/6 mice after two hr of the LPS administration. In contrast LPS significantly (P<0.05) decreases the FFAs levels in C57BL/6 mice while there was a no significant change was observed in db/db mice. Furthermore, LPS significantly (P<0.05) decreases the glucose levels but significant (P<0.05) increase in serum insulin levels, 8 hr after LPS administration in both the strain (C57BL/6 and db/db). There was remarkably increase in insulin

levels in C57BL/6 and db/db mice after LPS challenge. The increase in insulin levels after LPS challenge with C57BL/6 mice was similar with the basal levels of db/db mice (Table-2.1).

Table 2.1 Serum biochemical parameters of twelve weeks old C57BL/6 and db/db mice before and after LPS challange.

Metabolic Parameters	***C57BL/6 mice***		***db/db mice***	
	before	**after**	**before**	**after**
Triglyceride (mg/dl)	98.5 ± 7.1	87.5 ± 18.3	112.6 ± 11.4 *	78.1 ± 9.4 *#
FFA (mMol/L)	2.38 ± 0.27	1.61 ± 0.40 #	3.06 ± 0.38 *	2.70 ± 0.43 *
Glucose (mg/dl)	120.5 ± 6.2	72.9 ± 6.2 #	470.8 ± 21.4 *	262.1 ± 21.4 *#
Insulin (ng/ml)	0.21 ± 0.06	1.49 ± 0.21 #	1.79 ± 0.32 *	3.46 ± 0.28 *#
Leptin (ng/ml)	1.32 ± 0.3	4.52 ± 0.7 *#	110.1 ± 9.3	411.3 ± 73.6*#

Values are described as mean ± SEM. (n=6) * $P < 0.05$ as compared to C57BL/6 control. # $P < 0.05$ when compared with '0' (before) time point of their respective group. (TG, FFA were measured 2 hr and glucose, insulin and leptin were 8 hour after LPS challenge)

2.3.7 Ex vivo production of cytokines in db/db and C57BL/6 mice:

The above findings prompted us to investigate the immunocompetence of PBMC in the ex vivo production of inflammatory cytokines from whole blood in obese diabetic mice. When whole blood of db/db and C57BL/6 mice was challenged with LPS, TNF alpha (Fig. 2.5A), IL-6 (Fig. 2.5B), MCP-1 (Fig. 2.5C), IL-1 beta (Fig. 2.5D) and interferon gamma (Fig. 2.5E) levels were significantly lower. LPS challenge significantly increased the serum cytokines levels however whole blood cytokine levels were significantly lower in db/db mice when compared to C57BL/6 mice.

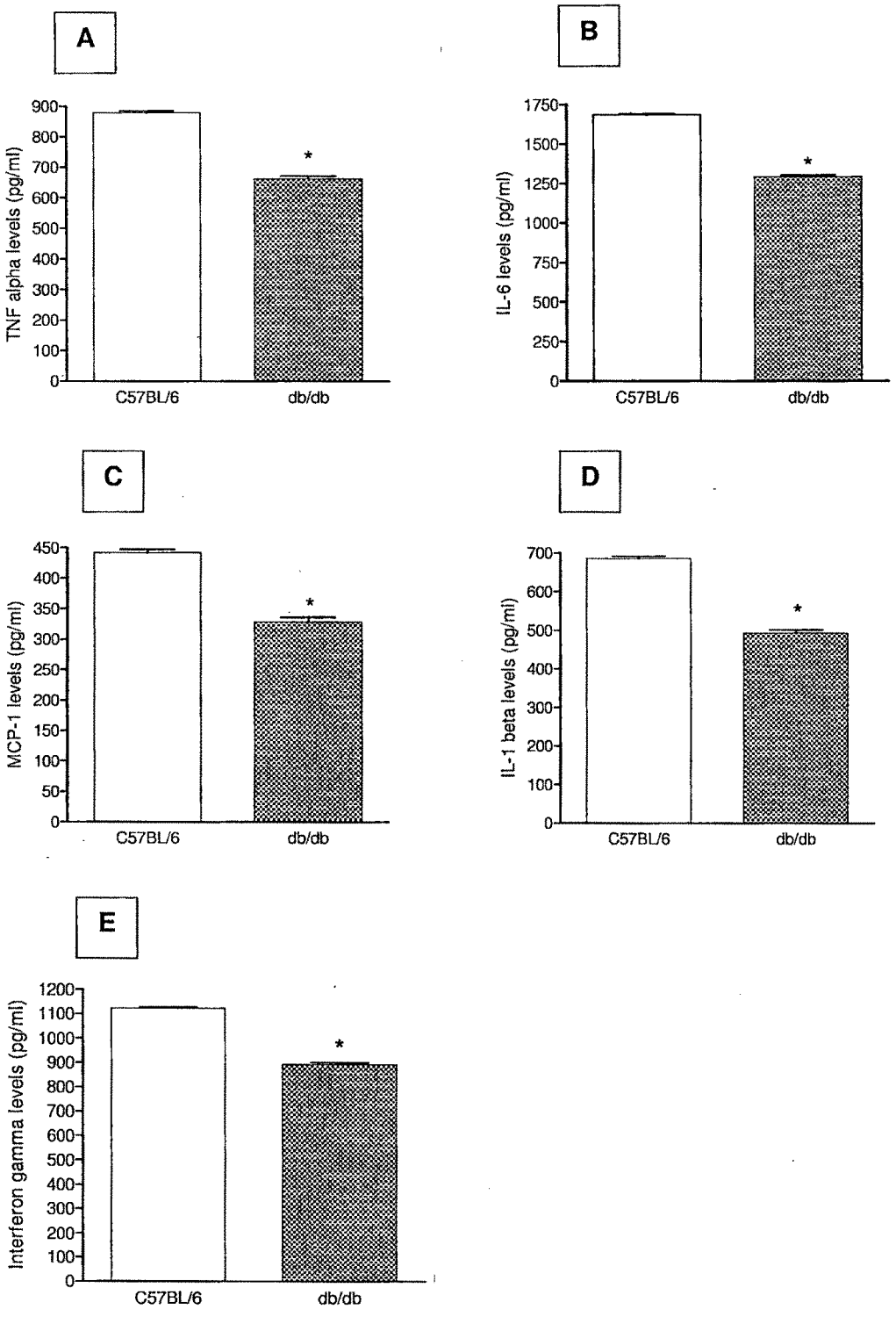


Fig. 2.5 Analysis of the TNF alpha (A) IL-6 (B), MCP-1 (C), IL-1 beta (D) and interferon gamma (E) in whole blood derived from C57BL/6 and db/db mice after LPS incubation using ELISA.

Cytokine levels of whole blood of C57BL/6 and db/db after LPS incubation is represented in the bar diagram. Each value represents mean ± S.E.M. (*n* = 6 mice/group). * P < 0.05 compared with C57BL/6 wild type mice.

2.3.8 Differential cell count in the whole blood of db/db and C57BL/6 mice:

Present study was undertaken to measure the differential blood cell count between the db/db and C57BL/6 mice to find out the presence of peripheral blood cells may give possible explanation for the discordant result between the whole blood cytokine and serum cytokines. In the present study WBC count in whole blood were significantly lower in the diabetic obese (db/db) mice as compared to C57BL/6. Higher monocyte and neutrophils and lower lymphocyte count were observed in the db/db mice when compared to control C57BL/6 (Table-2.2).

Table 2.2 Various cell composition (cells x 10^3±SEM) in blood of db/db and C57BL/6 mice.

Metabolic Parameters	***C57BL/6 mice***	***db/db mice***
WBC	12.1 ± 0.85	7.0 ± 0.94 *
Neutrophil	1.0 ± 0.05	1.9 ± 0.04 *
Lymphocyte	10.5 ± 0.99	4.3 ± 0.52 *
Monocyte	0.16 ± 0.06	0.66 ± 0.19 *
Eosinophil	0.009 ± 0.001	0.009 ± 0.002

Values are expressed as mean ± SEM (n=8). Statistical significance was assessed as *: p<0.05 Vs C57BL/6 control group.

2.4 Discussion:

In our earlier study, we have demonstrated that increased TLR-4 expression associated with the proinflammatory cytokines in adipose tissues of genetically altered obese diabetic (db/db) mice. TLRs are a family of pattern recognition receptors that are important in the regulation of immune function and inflammation [Li et al., 2007; Trinchieri et al., 2007; Stoll et al., 2006]. Their activation by various ligands trigger a signaling cascade leading to cytokine production and initiation of an adaptive immune response [Takeda et al., 2001]. TLRs 2 and 4 can recognize components of the bacterial cell wall such as lipopolysaccharide (LPS) and peptidoglycans. So in the present study, we demonstrate that modulation of key adipokine genes in visceral white adipose tissue in mice following the induction of endotoxaemia using LPS an agonist of Toll like receptor. The expression of several major inflammation-related adipokines was examined in WAT, and the largest response was observed with TNF alpha and IL-6 when compared with the control mice after LPS challange. The obese and diabetic animals showed significantly high levels of tissue and circulating cytokines compared to the control animals after LPS challenge. Studies in human subjects indicated TNF-alpha and IL-6 were produced by adipocytes as well as macrophages and there existed a positive correlation between their circulating levels and degree of obesity [Rajala and Scherer, 2003; Hotamisligil, 2003]. The administration of endotoxin, like LPS, a bacterial cell wall component serves as a well-established model for studying the acute inflammatory response. The administration of LPS triggers the inflammatory pathways in terms of early stimulation of TNF release, which is followed by IL-6 and IL-1beta up regulation. Furthermore, MCP-1 is a chemoattractant molecule whose increased production in obesity aids in the infiltration of macrophages into adipose tissue contributing to the inflammatory state. In the current study, we observed a significantly higher MCP-1 mRNA expression in WAT after LPS challenge in obese diabetic db/db mice, supporting a possible causative role of this chemokine in the observed macrophage infiltration. In addition to

inflammation, MCP-1, IL-6 and TNFα have been associated with the development of insulin resistance [Sartipy, 2003; Hotamisligil, et al., 1993; Moller, 2000; Rotter et al., 2003]. Sufficient evidence sought that adipose inflammation modulated insulin signaling pathways in adipose. SOCS family proteins inhibit tyrosine kinase receptor signaling including the insulin receptor. Following endotoxin, adipose SOCS-3 mRNAs increased markedly in both strains. Interestingly, SOCS-3 expression of db/db mice alone was similar pattern with LPS challenged C57BL/6.

Adiponectin, which is a key adipocyte-derived hormone, has multiple physiological effects, and these include anti-inflammatory, insulin sensitizing and antiatherogenic actions [Berg et al., 2001; Ouchi et al., 1999; Ouchi et al., 2003; Yamuchi et al., 2001; Luo et al., 2010]. In the present study, LPS down regulates the adiponectin along with major insulin sensitizing and anti-inflammatory nuclear receptor PPAR gamma mRNA expression in WAT of both db/db and control C57BL/6 mice. Thus, the major stimulation of the expression of these pro-inflammatory cytokines in response to LPS suggests that adipose tissue may contribute to insulin insensitivity and the dysregulation of glucose homeostasis in infection. Thus, an up-regulation of IL-6, MCP-1 and TNF-α expression may fall in expression of adiponectin along with major anti-inflammatory gene PPAR gamma expression in leading to a major pro-inflammatory response and insulin resistance in endotoxaemia. To elucidate the abnormalities of the activation of adipocyte in obesity, we have investigated the expression of Toll like receptor expression in WAT of obese diabetic as well as in normal C57/BLmice after LPS challenge. Results indicated that the TLR-4 expression in WAT of obese animals was significantly higher compared to controls. As TLR-4 is responsible for maintenance of preadipocyte status, differentiation of adipocytes and cytokine production [Poulain Godefroy and Froguel, 2007], the increased activation of TLR-4 in adipose tissues of C57BL/6 mice after LPS challenge are similar with the mRNA expression of obese diabetic db/db mice alone implies hyperactivation of inflammation in obesity. Overexpression of TLR-4 in WAT of

db/db mice may decrease the adipogenic potential as evidenced by the lower expression aP2 and LPL (adipogenic potential) which was similar with the expression of LPS challenged C57BL/6 mice.

Studies in human subjects indicated TNF-alpha and IL-6 were produced by adipocytes as well as macrophages and there existed a positive correlation between their circulating levels and degree of obesity [Rajala and Scherer, 2003; Hotamisligil, 2003] indicating the role of circulating proinflammatory cells in obesity. Evidence shows that monocytic cell line cultured with FFA enhances the proinflammatory state mimic an obese state of inflammation [Suganami et al., 2005]. There is increasing evidence that an ongoing cytokine-induced inflammatory response is related closely to the pathogenesis of type 2 DM and the associated complications such as dyslipidaemia, cardiovascular disease. To explore the role of Peripheral Blood Cells in obese state, we challenged LPS in whole blood derived from db/db mice, a model of obesity, insulin resistance, and type 2 diabetes, and compared with normal C57BL/6 mice to determine whether these key cells associated with vascular complications display a preactivated phenotype and altered pathophysiological responses. We found that the levels of key proinflammatory cytokines IL-1beta, IL-6, and TNF-alpha were decreased in whole blood from db/db mice after LPS challenge *ex vivo*. However, circulating cytokine levels were remarkably higher in db/db mice as compared to their age matched C57BL/6 control after intravenous administration of LPS indicates that circulating levels of different cytokines may obtained from adipose tissues. When we measure the WBC count in whole blood, there was significantly lower blood cells in db/db as compared to normal C57BL/6 mice and the percentage increase in cytokines per blood cell was enhanced in db/db mice emphasizing that PBMC are in activated state in obese condition. Furthermore, lower lymphocyte and higher monocyte count associated with db/db mice indicating that obesity has profound effects on immunity and inflammation. An elevated level of inflammatory cytokines in obesity is generally a strong predictor of diabetes and

cardiovascular complications. Furthermore, these cytokines also regulate the innate immune system and thus their dysregulated expression can impair the capacity of the immune system to appropriately regulate inflammation. Furthermore, elevated levels of the chemokine MCP-1 per blood cell was also found in the whole blood from db/db mice after LPS challenge. Chemokines play an important role in the development of atherosclerosis by inducing migration of leukocytes to the site of inflammation. Chemokines can also promote the migration of monocytes into the subendothelial spaces where they differentiate into macrophages. In addition, chemokines can induce the migration of VSMCs from the media to the intima, leading to the development of neointimal thickening and lesion formation. Interestingly, we also noted that the levels of key immunomodulatory cytokines IFN gamma was significantly increased in whole blood from db/db mice relative to those of control mice. Interferon gamma is a potent inflammatory and proatherogenic cytokine [Libby et al., 1996] that also plays an important role in regulating Th1 immune responses. Interferon gamma arising from the activated T lymphocytes in the plaque can halt collagen synthesis by SMCs, limiting its capacity to renew the collagen that reinforces the plaque [Libby et al., 1996; 2001]. Increased expression of Interferon gamma in obese diabetic mice may risk to cardiovascular disease. Similarly Li et al (2006) observed that the activation of CREB and NF-kB transcription factors was elevated in macrophages of db/db mice compared with those of db+ mice [Li et al., 2006]. Thus, the increased activation of signaling kinases and downstream oxidant-sensitive transcription factors in the basal state and in response to cellular stimuli might be key mechanisms for the augmented expression of inflammatory gene in these mice. Interestingly, they reported that VSMCs from db/db mice also showed enhanced expression of MCP-1 and IL-6. These cytokines/ chemokines are involved in VSMC proliferation and migration and can also attract monocytes to bind to VSMCs. In another very recent study, Reddy et al (2006) reported that VSMCs from db/db mice displayed increased Advanced Glycation End product receptor expression, Src kinase activation, and migration

[Reddy et al., 2006]. Thus, our new data along with those of others indicate that vascular cells including VSMCs, endothelial cells, and macrophages are in a preactivated state in db/db mice, and this could be a major underlying cause for the reported predisposition of db/db mice to develop accelerated atherosclerosis [Wendt et al., 2006]. Taken together, our results provide mechanistic insights for the augmented inflammation and accelerated cardiovascular complications associated with type 2 diabetes, insulin resistance, and atherosclerosis.

We have shown in the present study LPS augmenting the inflammatory response in WAT in mice and in PBMC in whole blood vitro. LPS induced infection which leads to a chronic inflammatory state that accelerates atherosclerosis in humans and rodents [Campbell and Kuo, 2004; Mussa et al., 2006; Nabipour et al., 2006; Hauer et al., 2006; Kiechl et al., 2001]. Chronic infection mimicked by exposure to lipopolysaccharide (LPS) increases the size of atherosclerosis lesions in apoE KO mice [Kleemann et al., 2007]. Atherosclerotic plaque disruption induced by lipopolysaccharide in apolipoprotein E knockout mice has also been reported [Ni et al., 2009]. The Bruneck study has provided the first epidemiologic evidence that circulating LPS levels as low as 50 pg/ml constitute a strong risk factor for the development of atherosclerosis, particularly among smokers [Wiedermann et al., 1999]. Furthermore, in rabbits on hypercholesterolemic diets, weekly injections of LPS accelerated the development of atherosclerotic lesions [Lehr et al., 2001]. These observations suggest that systemic proinflammatory mediators such as LPS may be pathogenically linked to the development and progression of atherosclerosis, and hence TLR mediated signaling, could play a mechanistic role in atherosclerosis. It is also possible that the effects are indirectly mediated, for example, by producing a generalized proinflammatory condition in which atherogenesis would be facilitated. However, very recent studies have now provided in vivo evidence for a direct mechanistic link between TLR4 signaling and innate immune system activation and atherogenesis [Li and Sun, 2007; Michelsen et al., 2004]. The

function of TLR4 in atherosclerosis has been investigated in mouse knockout studies and epidemiological studies of human TLR4 polymorphisms. These studies have shown that TLR4 function affects the initiation and progression of atherosclerosis. Upregulation of TLR-4 in visceral adiposity associated with inflammation may promote atherosclerosis environment and increase its expression in WAT after LPS challenge may further exaggerate the atherogenic condition.