# Role of myeloid cells specific PDK-1 in inflammation and obesity induced insulin resistance

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# Abbreviations

| | |
|---|---|
| A | Adenosine |
| aa | Amino acid |
| Akt | Proteinkinase B |
| Avertin | Tribromoethyl alcohol and *tert*-amyl alcohol |
| β-me | β-mercaptoethanol |
| bp | Base pair |
| C | Cytosine |
| CAP | Cbl associated protein |
| °C | Degrees Celsius |
| cpm | Counts per minute |
| Cre | Site-specific recombinase |
| Ctrl | Control |
| $ddH_2O$ | Double distilled water |
| DEPC | Diethylpyrocarbonate |
| dNTP | Deoxynucleotide-triphosphate |
| DMSO | Dimethylsulfoxide |
| DNA | Desoxyribonucleic acid |
| DTT | 1,4-Dithio-DL-threitol |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylene-diaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| EtBr | Ethidium Bromide |
| FOXO | Forkhead transcription factor |
| G | Guanine |
| Glut | Glucose transporter |
| Grb | Growth factor receptor binding protein |

| | |
|---|---|
| GSK | Glycogen synthase kinase |
| GTT | Glucose tolerance test |
| h | Hour |
| HEPES | Hydroxy-ethylpiperazine-ethansulfonic acid |
| i.p. | Intraperitoneal |
| IL | Interleukin |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| ITT | Insulin tolerance test |
| kDa | Kilodalton |
| loxP | Locus of x (crossing) over of P1 |
| MCP-1 | Macrophage chemoattractant protein 1 |
| min | Minute |
| mTOR | Mammalian target of rapamycin |
| NaCl | Sodium Chloride |
| NaOH | Sodium hydroxide |
| OD | Optical Density |
| p70S6K | p70 S6 kinase |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PDK | Phosphoinositide-dependent kinase |
| PH | Pleckstrin homology |
| PI3K | Phosphatidylinositol-3 kinase |
| $PIP_2$ | Phosphatidylinositol-4,5-biphosphate |
| $PIP_3$ | Phosphatidylinositol-3,4,5-triphosphate |
| PKA | Proteinkinase A |
| PKC | Proteinkinase C |
| PTB | Phosphotyrosine binding |
| Ras | Rat sarcoma |
| Raf | v-raf-leukemia viral oncogene |

| | |
|---|---|
| RNA | Ribonucleic acid |
| Rpm | Rounds per minute |
| RT | Room temperature |
| sec | Second |
| SDS | Sodium dodecyl sulfate |
| SH | Src homology |
| SOS | Son of sevenless |
| SSC | Sodium chloride/ sodium citrate buffer |
| TAE | Tris-acetic acid-EDTA buffer |
| Taq Pol | Polymerase from *Thermus aquaticus* |
| TE | Tris-EDTA buffer |
| TNF | Tumor necrosis factor |
| Tris | 2-amino-2-(hydroxymethyl-)1,3-propandiole |
| TWEEN | Polyoxethylene-sorbitan-monolaureate |
| U | Units |
| v/v | Volume per volume |
| WAT | White adipose tissue |
| w/v | Weight per volume |
| wt | Wildtype |
| 5’ | Five prime end of DNA sequences |
| 3’ | Three prime end of DNA sequences |

# 1 Introduction

## 1.1 Obesity

Obesity is characterized by abnormal or excessive fat accumulation leading to negative impact on one's health (Spiegelman and Flier 2001). Obesity is of growing concerns in the western world mainly for its association with a numerous diseases such as, diabetes mellitus type 2, cardiovascular diseases, osteoarthritis and various forms of cancer (Spiegelman and Flier 2001; Haslam and James 2005). Thus, obesity is an independent factor for the development of cardiovascular diseases (Poirier, Giles et al. 2006). Hence obesity is increasingly being associated with an enhanced risk of morbidity and mortality as well as reduced life expectancy (Poirier, Giles et al. 2006).

With the growing trend in sedentary life style across the globe obesity has started to become a global epidemic, and in the past ten years there has been a tremendous increase in obesity among children and adults living in United States (Flegal, Carroll et al. 1998; Flegal, Carroll et al. 2002; Engeland, Bjorge et al. 2003; Eckel, York et al. 2004). (10). According to recent reports published by world health organization (WHO) in 2005, approximately 1.6 billion adults were overweight and at least 400 million were obese. Moreover according to WHO projections these numbers are expected to double by the year 2015 (WHO 2005). Moreover the health service use and medical costs associated with obesity and related diseases have risen dramatically among adults in United States and are expected to continue to rise, thus leading to an increase burden on countries health care system (Yang and Hall 2007).

The fundamental cause of obesity and weight gain is an energy imbalance i.e. caused by chronic consumption of energy in excess than that used by the body (Nestle and Jacobson 2000). Although body weight is best determined by interaction between genetic, environmental and psychological factors acting through physiological mediators of energy intake and expenditure, the rising prevalence of obesity is attributed to behavioral and environmental changes resulting from technological advances (Kopelman 2000). However the combination of an excessive and energy-dense nutrient intake combined with reduced physical activity is largely accepted as a cause for the rapid acceleration of obesity in industrialized societies (Hill and Peters 1998).

Overweight and obesity are currently classified by body mass index (BMI) which is defined as weight in kilograms (kg) divided by square of individual's height in meters (m) ($kg/m^2$). In adults, overweight is defined as a BMI of 25.0 to 29.9 $kg/m^2$, whereas obesity is defined as a BMI $\geq$30.0 $kg/m^2$ (Bleich, Cutler et al. 2007). Increasing body fatness is accompanied by profound changes in physiological function. These changes to a certain extent are dependent on the regional distribution of adipose tissue. Adipose tissue in turn secretes leptin (an adipocyte derived cytokine) which acts in the brain, informing the brain, by its suppression that the body is starving, thereby initiating an appetite (Flier 2004). However apart from secreting leptin, adipocytes also secrete other cytokine such as TNFα, IL6, MCP-1 and adiponectin to name a few, which appear to be responsible for the onset of medical problems associated with obesity.

## 1.2 Diabetes mellitus

Diabetes mellitus is a disease in which the pancreas either does not release insulin or insulin sensitive tissues such as liver, muscle and fat do not apparantely respond to this hormone (Bergman, Ider et al. 1979; Topp, Promislow et al. 2000). The long term effects associated with diabetes include diabetic retinopathy, nephropathy, peripheral neuropathy and development of atherosclerosis (Derouich and Boutayeb 2002).

Diabetes is classified in two forms: type 1 and 2 diabetes mellitus (Rother 2007). Type 1diabetes also called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes results from the loss of insulin producing pancreatic β cells as a consequence of autoimmune attack (Kloppel, Lohr et al. 1985). Type 1 diabetes account for 5 to 10% of all the cases diagnosed with diabetes (Porksen 2002). Type 2 diabetes also called as non-insulin-dependent diabetes mellitus (NIDDM) is prevalent among adults and largely caused by insulin resistance (Rother 2007). However, the way insulin resistance triggers β cell failure remains obscure (Kloppel, Lohr et al. 1985). 90-95% of all the diagnosed diabetes cases account for type 2 diabetes.

Due to the large population of diabetic patients in the world and the big health expenses, many researchers are motivated to study the glucose-insulin endocrine metabolic regulatory system so that we can better understand how the mechanism functions (Bergman, Finegood et al. 2002;

Porksen 2002), hence eventually resulting in providing more effective, efficient and financially reasonable treatments against diabetes.

## 1.3 Insulin and Insulin signaling

Insulin is a small peptide hormone, with a molecular weight of about 6kd. It is composed of two chains held together by disulphide bonds. The amino acid sequence is highly conserved among vertebrates. Insulin is synthesized in significant quantities only in pancreatic β-cells. The insulin mRNA is translated as a single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin (Steiner, Cunningham et al. 1967).

Proinsulin consist of three domains: an amino- terminal B chain, a carboxy- terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases, which excise the C-peptide, thereby generating the mature form of insulin (Orci, Ravazzola et al. 1985). Insulin and free peptide are packaged in the golgi into secretory granules which accumulate in the cytoplasm (Davidson, Rhodes et al. 1988). In response to elevated plasma glucose level, insulin is secreted from the cell by exocytosis and diffuses into the capillary blood. The secreted insulin thereon maintains glucose homestasis by either acting on the peripheral tissues, skeletal muscle and adipose tissue to mediate the glucose uptake or by inhibiting release of lipids from the adipose tissue. In addition insulin acts on the liver to inhibit de novo glucose production (gluconeogenesis) (Docherty and Hutton 1983; Davidson and Hutton 1987).

Like receptors for other protein hormones, the receptor for insulin is embedded in the plasma membrane. The insulin receptor is composed of two α and two β subunits which are linked by a s disulphide bridges. The alpha chains are entirely extracellular and house insulin binding domain, while the linked beta chains penetrate through the plasma membrane and bear an intrinsic protein kinase activity (Saltiel 1996). Receptor occupancy by insulin-induces conformational changes in the receptor, which is transduced across the membrane, resulting in activation of intracellular β

subunit kinases. The insulin receptor kinase then phosphorylates a number of proteins on tyrosine residues, allowing for their interaction with numerous downstream target enzymes, resulting in the amplification and branching of the initial insulin signal (Saltiel 1996).

There are two main limbs that propagate the signal generated through insulin receptor: the insulin receptor substrates/phosphatidylinositol 3-kinase pathway (IRS/PI3-K) and Ras/ mitogen-acctivated protein kinase (MAPK) pathway (Fig 1) (Di Guglielmo, Drake et al. 1998; Ogawa, Matozaki et al. 1998; White 1998). The IRS/PI3-K pathway leads to the generation of phosphatidylinositol 3-phosphate, and the consequent activation of PI-dependent kinases, such as the PI-dependent kinases 1 and 2 (PDK-1, PDK-2) and protein kinase C (PKC). Some of these kinases may be required to activate downstream kinases, such as the serine/threonine kinase AKT. It has been demonstrated that AKT may directly phosphorylate and inactivate glycogen synthase kinase 3, thus leading to dephosphorylation and activation of glycogen synthase and increased glycogen synthesis. Moreover, there is also clear evidence linking AKT to translocation of glucose transporters. Insulin, through the formation of complexes between the exchange factor SOS (son of sevenless) and growth factor receptor- binding protein 2 (GRB-2), can activate the Ras/MAPK pathway. GRB-2 can be activated by IRS or SHC, two direct substrates of the insulin receptor kinase. Thus IRS/PI3-K pathway plays role in metabolism whereas Ras/MAPK pathway may play a role in certain tissues to stimulate the long term effects of insulin on growth and proliferation (Fig 1). In addition the insulin signaling activation leads to the translocation of Glucose transporters (GLUT) by an additional signaling pathway engaging adaptor protein with a pleckstrin homology and an Src homology 2 domain (APS), Casitas B-lineage lymphoma (Cbl) and Cbl associated protein (CAP) which forms a trimeric complex (Bastard, Maachi et al. 2002). Upon tyrosine phosphorylation of Cbl, it recruits CrKII-C3G complex to the plasma membrane, thereby leading to the activation of downstream molecule TC10 which in turn regulate the translocation of GLUT to the plasma membrane (Trischitta, Brunetti et al. 1989; Chiang, Baumann et al. 2001; Patki, Buxton et al. 2001; Liu, Kimura et al. 2002; JeBailey, Rudich et al. 2004).

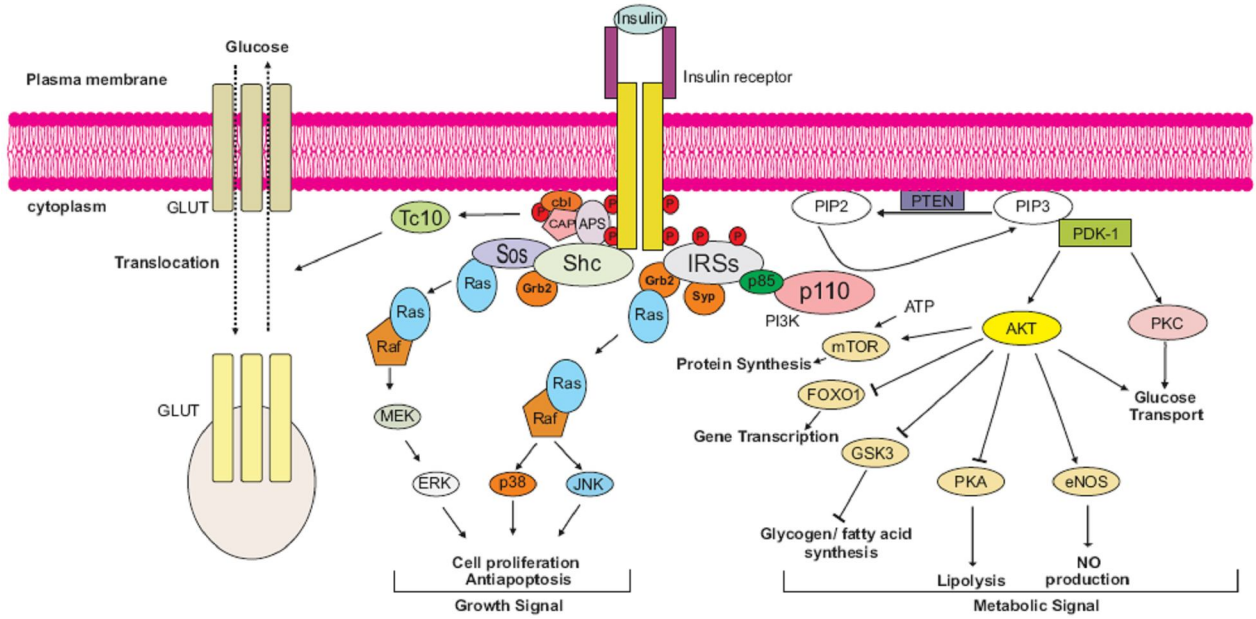



**Fig 1. Intracellular Insulin signaling.**

Insulin binding to the insulin receptor results in receptor auto-phosphorylation and its trans-activation leading to the recruitment and subsequent tyrosine phosphorylation of insulin receptor substrates. This enables the binding of SH-2 domain containing proteins, which ultimately leads to the activation of downstream signaling pathways such as the PI3K or the Ras/Raf MAPK signaling pathway. (Abbreviations: Akt: Proteinkinase B, APS: adapter protein with a pleckstrin homology and an Src homology 2 domain, CAP: cbl-associated protein, SOS: son of sevenless, Raf: v-raf-leukemia viral oncogene, Ras: rat sarcoma, ERK: extracellular signal-regulated kinase, FOXO: Forkhead transcription factor , Glut: glucose transporter , Grb2: growth factor receptor binding protein 2, Gsk3: glycogen synthase kinase 3, IR: insulin receptor, IRS: insulin receptor substrate, mTOR: mammalian target of rapamycin, p70S6K: p70 S6 kinase, PI3K: phosphatidylinositol-3 kinase, PIP: phosphatidylinositol phosphate, Pdk: phosphoinositide-dependent kinase, PKA: Proteinkinase A, PKC: Proteinkinase C,).

## 1.4 Phosphoionositide dependent kinase-1 (PDK-1)

The phosphoinositide-3 kinase (PI-3K) signaling pathway is one of the key regulatory pathways activated upon extracellular stimulation of growth factor receptors, such as the insulin receptor (Wick and Liu 2001). The activation of the phosphoinositide-3 kinase (PI-3K) pathway

constitutes one of the most important mechanisms that regulate important cellular functions such as gene expression, cell cycle progression and differentiation (Dygas and Baranska 2001). Apart from this, PI-3K is also known to be critical for numerous insulin specific actions such as glucose uptake, glycogen, lipid and protein synthesis (Wick and Liu 2001).

PI-3K mediates the transfer of phosphate groups to phosphatidylinositol residues located in the plasma membrane. There are several kinases including protein kinase B (PKB/Akt) and protein kinase C (PKC) zeta that are activated by rise in PI-3 phosphate. These kinases, which primarily include members of AGC kinase family, are recognized as key mediators of the PI-3K signal. Activation of Akt and other PI-3K downstream kinase is mediated by 3'-phosphoinositide dependent kinase 1 (PDK1) (Kikani, Dong et al. 2005). PDK1 has been found to act as the upstream kinase for a number of protein kinases, including p70 ribosomal S6 kinase (S6K), PKC isoforms, serum and glucocorticoid regulated kinase (SGK), p90 ribosomal S6 kinase (RSK), protein kinase C- related kinase 1 and 2 (PRK1/2) and p21-activated kinase 1 (PAK1) (Dygas and Baranska 2001; Wick and Liu 2001; Kikani, Dong et al. 2005).

PDK1 is a 63 kDa protein kinase, consisting of an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain. The PH domain of PDK1 bind to the PI-3K products phosphoinositol (3, 4, 5) phosphate (PIP3), and phosphoinositol (3, 4) phosphate (PIP2), which target PDK1 to the plasma membrane where PDK1 phosphorylate the threonine 308 in the primary sequence of thee Akt serine/threonine kinase in the activation loop . Other substrates of PDK1 lacking a PH domain are activated by direct association with PDK1. The role of PDK1 in insulin signalling is as follows: Insulin binding to its receptor stimulates its phosphorylation by tyrosine phosphorylation. Activated receptors then recruit and activate insulin receptor substrates 1/2 (IRS 1/2), leading to the activation of PI-3K and subsequent generation of inositols lipid, PIP3. PIP3 then targets PDK1 and Akt on to plasma membrane where PDK1 activates Akt by phosphorylating the kinase in the activation loop (Kikani, Dong et al. 2005) (Fig. 1).

## 1.5 Nutrient sensing and chronic inflammation under obesity

Metabolic functions and the immune system are among the basic requirements across the animal kingdom. Moreover, these two pathways interact with each other at many levels (Hotamisligil and Erbay 2008). Although required for homeostasis, the interaction between these two can also

lead to metabolic dysregulation under conditions of chronic stress (Wellen, Fucho et al. 2007). The association between inflammation and metabolism is also present at the organ level of higher organisms, as in case of adipose tissue and liver wherein immune cells (macrophages) are found along side with adipocytes and hepatocytes respectively (Chung, Lapoint et al. 2006). Moreover the cells from either of the systems are closely related and share similar functions, for example they both are capable of being activated by pathogen- associated component and can also secrete cytokines (Hotamisligil 2006).

Functionally either limitation of nutrient can lead to immune suppression resulting in enhanced susceptibility to infection or an excess in nutrient avaliability leads to the development of obesity, which in turn results in hyperactivation of immune system, Thereby increasing risk for the development of inflammatory associated diseases such as diabetes, atherosclerosis and fatty liver diseases in obese people (Chandra 1996; Blackburn 2001).

In presence of an excess of nutrient, the inflammatory potential of the metabolically important tissues is activated, which leads as a source of the inflammatory cytokines and inflammatory factors (Uysal, Wiesbrock et al. 1997; Hotamisligil 2003). This leads to the development of chronic inflammatory state under obesity (excess nutrient) (Shoelson, Lee et al. 2006). And hence, this can lead to insulin resistance when inflammatory pathways interfere with insulin signaling (Hotamisligil, Shargill et al. 1993).

The first molecular link between obesity and inflammation was established more than a decade ago when enhanced expression of tumor necrosis factor alpha (TNFα) in adipose tissue of obese mouse and skeletal muscle of obese patients was demonstrated(Hotamisligil, Arner et al. 1995; Saghizadeh, Ong et al. 1996; Sethi and Hotamisligil 1999; Shoelson, Lee et al. 2006). Moreover it was shown that TNFα impairs insulin action both *in vitro* or when administered to mice *in vivo* (Uysal, Wiesbrock et al. 1997; Vozarova, Weyer et al. 2001). Obesity not only induces a low inflammatory state in different tissues but also leads to increased plasma levels of pro-inflammatory molecules, whereas levels of anti-inflammatory proteins such as adiponectin are decreased (Berg, Combs et al. 2002; Chiellini, Bertacca et al. 2002; Sartipy and Loskutoff 2003; Xu, Barnes et al. 2003). Obesity is characterized by adipocyte hypertrophy leading to the release

of key chemokines such as CCL2 (MCP1) and cytokines such as TNFα, which thereby function to promote macrophage infiltration into adipose tissue. Both of these changes contribute to the secretion of pro-inflammatory adipocytokines including TNFα, IL-6, and IL-1β (Brenner, O'Hara et al. 1989). Altogether it is by now well established that the development of insulin resistance and to the pathogenesis of type 2 diabetes mellitus is closely linked to low-grade inflammatory state (Shoelson, Lee et al. 2006). However in the past decade the list for this possible causes of inflammation during obesity continuously extended and now includes numerous mechanisms mediating the release of acute phase proteins such as circulating free fatty acids (FFA) caused by enhanced adiposity, endoplasmic reticulum (ER) stress due to enhanced metabolic load, and mitochondrial reactive oxygen species (ROS) production due to elevated glucose metabolism (Wellen and Hotamisligil 2005).

## 1.6 Inflammatory pathways and insulin resistance

Although the link between obesity and inflammation is well established, the underlying mechanism(s) leading to enhanced inflammation are just beginning to emerge. The inhibition of insulin signaling under obesity is largely caused by the activation of two intracellular kinases: inhibitor of nuclear factor (NF) κB kinases (IKKs) and c-jun N-terminal kinases (JNKs) (Mercurio, Zhu et al. 1997; Taniguchi, Emanuelli et al. 2006). These kinases can phosphorylate IRS molecules at serine residues which prevents the activating tyrosine phosphorylation of IRS. As such efficient signal transduction from insulin receptor is abrogated (Fig. 2) (Hotamisligil, Peraldi et al. 1996; Gao, Hwang et al. 2002; Zick 2005). The role of these two kinases in inducing insulin resistance was further supported by genetic deletion of either the IKKβ subunit of the IKK complex or JNK, since these mice are protected from diet induced insulin resistance (Hirosumi, Tuncman et al. 2002; Arkan, Hevener et al. 2005). There are number of factors contributing to the activation of these two kinases, thereby leading to the activation of inflammatory pathways under obesity and thereby inducing insulin resistance.

### 1.6.1 Cytokine mediated inflammation

The pro-inflammatory cytokine TNFα is thought to be an important mediator of insulin resistance. As mentioned before, TNFα levels are increased in obesity, both in the serum as well as in tissues. Moreover, TNFα binds to tumor necrosis factor receptor (TNFR) and activates both IKKs and JNKs both implicated in insulin resistance (Mercurio, Zhu et al. 1997; Taniguchi, Emanuelli et al. 2006). Further evidence for the crucial role of TNFa in insulin resistance was provided by genetic ablation of TNF signaling. The deletion of TNFα or its receptor subunits (p55 and p75) was shown to protect from obesity induced insulin resistance in mice (Vozarova, Weyer et al. 2001). TNFα mediated inhibition of insulin signaling has also been reported to occur in obese individuals and that there it contributes to the development of metabolic disorders (Hotamisligil, Peraldi et al. 1996). Apart from TNFα, a variety of other cytokines and chemokines such as IL-6, IL-1β, monocyte chemoatractant protein-1 (MCP-1/CCL2) and C-reactive protein (CRP) have been shown to be unregulated under obesity in humans as well as in mice and have been shown to inhibit insulin action (Wellen and Hotamisligil 2005). The induction of IKK and JNK activation by cytokines activates nuclear factor kappa b (NFκB) and activator protein 1 (AP1) which thereby drive the expression of cytokines leading to feed forward inhibition of insulin signaling (Fig 2).

### 1.6.2 Lipid mediated inflammation

Elevated free fatty acid (FFA) are also an important modulator of obesity induced inflammation and participate in the induction of insulin resistance (Boden 1997; Shulman 2000). Two intracellular kinases, IKK and protein kinase (PKC) are known to be activated in response to lipid metabolites by two independent mechanisms. Similar to the bacterial cell wall component lipopolysaccharide (LPS), FFA are capable of binding to toll like receptor 4 (TLR4). This is a pathogen recognition receptor that activates pro-inflammatory signaling cascades in order to mount an immune response to infections. One of the main signal transduction pathways activated by TLR4 is the activation of NF-κB by the IKK complex. Because of their cross reactivity with TLR4, FFA are thought to promote the pro-inflammatory state by inducing cytokines as well as to induce insulin resistance via TLR4-dependent IKK activation (Fig 2) (Medzhitov 2001; Gao,

Wang et al. 2007). Beside LPS, saturated fatty acids such as palmitate are capable of stimulating TLR4 signaling at least in *vitro* and this effect is mediated by myeloid differentiation factor 88 ( (Myd88) a signal transducer for TLR4 signaling (Lee, Sohn et al. 2001; Lee, Ye et al. 2003; Shi, Kokoeva et al. 2006). Secondly, lipid infusion has been shown to lead to a rise in intracellular lipid metabolite levels namely, diacylglycerol (DAG) and fatty acyl coA, and this has been associated with the PKC activation which also contributes to serine phosphorylation of IRS molecule (Chin, Liu et al. 1994; Griffin, Marcucci et al. 1999; Yu, Chen et al. 2002).

### 1.6.3 Endoplasmic stress response mediated inflammation

Accumulation of unfolded proteins in the endoplasmic reticulum (ER), known as ER stress also plays a pivotal role in mediating inflammation under obesity. The ER has an important role in protein processing and lipid metabolism under normal conditions mediated by an assembly of chaperones (Hotamisligil and Erbay 2008). However the conditions of obesity, nutrient overload, hypoxia, or energy depreviation trigger the protective response known as unfolded protein response (UPR) which assists ER to fold and translocate proteins, decrease protein synthesis, coordinate stress responses ultimately resulting in cell cycle arrest. In addition to these protective responses under stress the UPR can also stimulate key inflammatory signals and apoptosis (Ozcan, Cao et al. 2004). ER stress is linked to inflammation through the activation of JNK and IKK pathways (Fig 2) (Urano, Wang et al. 2000; Ozcan, Cao et al. 2004; Hotamisligil and Erbay 2008). The relevance of this axis has been shown recently using deletion of X-box binding protein (XBP-1), a transcription factor that mediates expression of ER chaperones in mice. The mice lacking XBP-1 exhibit enhanced, ER stress, increased JNK activation and thereby leading to increased insulin resistance (Iwakoshi, Lee et al. 2003). In contrast induction of exogenous expression of active XBP-1 was shown to relieve ER stress, reduced JNK activity and to increase insulin sensitivity (Ozcan, Ozcan et al. 2008).

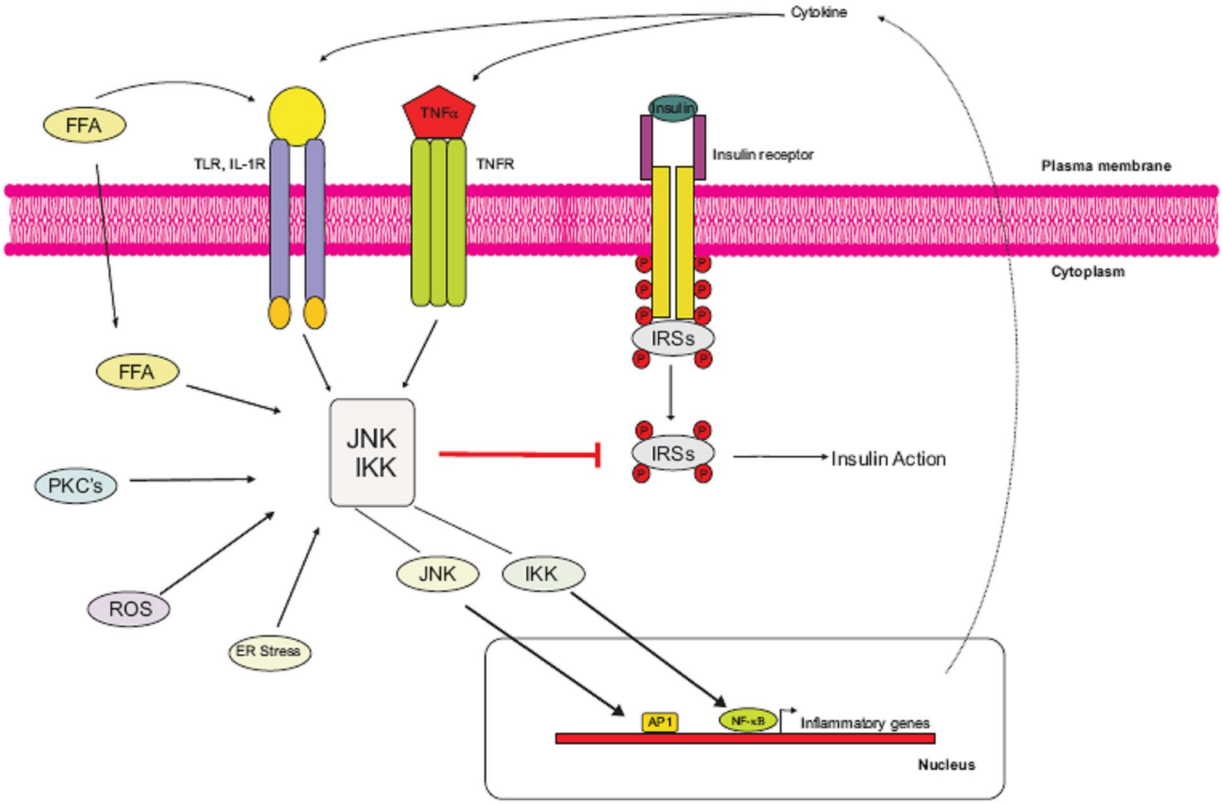



**Fig 2. Mechansims for inhibition of insulin signaling by inflammation under obesity**

Enhanced adiposity increases circulating inflammatory cytokine and free fatty acid concentration. This leads to activation of cell surface receptors which then induce serine kinases like c-jun N-terminal kinase (JNK), inhibitor of NFkB kinase (IKK) komplex and protein kinase c (PKC) isoforms. These kinases then mediate inhibitory serine (S307) phosphorylation events on insulin receptor substrates (IRS) thereby blocking insulin action. Additionally, transcription factors nuclear factor kB (NFkB), activator protein (AP) 1 and signal transducer and activator of transcription (Stat) activate inflammatory gene expression thereby enhancing production and secretion of inflammatory markers and mediators. Furthermore, endoplasmatic reticulum (ER) stress and intermediates of fatty acid metabolism may activate stress kinases. (Abbreviations: FFA: free fatty acid, FA: fatty acid, IL1R: interleukin-1 receptor, IR: insulin receptor, IRS: insulin receptor substrate, LPS: lipopolysaccharide, TLR: toll-like receptor, TNF: tumor necrosis factor, TNFR: TNF receptor)

## 1.7 Source of inflammation under obesity

Many of the inflammatory parameters contributing to the pro-inflammatory state in obesity are believed to originate from adipose tissue. Adipose tissue not only serves as a storage depot for excess calories in form of fat, but also actively secretes fatty acids, hormones, cytokines and chemokines which then act both in an endocrine and a paracrine fashion (Schenk, Saberi et al. 2008). The adipose tissue consists of different cell types comprising of preadipocytes, endothelial cells and immune cells. Weight gain is characterized by massive expansion in adipocyte size and adipose tissue mass. With this massive tissue expansion, there is enhanced secretion of FFA into the circulation, which in turn leads to decreased oxygen supply to adipocytes. This increased level of hypoxia (microhypoxia) activates IKK and JNK signaling cascade which therein enhance the expression of inflammatory cytokines and chemokines (Ye, Gao et al. 2007). Ultimately, activation of these stress pathways will make the adipocytes more prone to cell death. With the secretion of chemokine there is enhanced recruitment of bone marrow derived macrophages to the adipose tissue, where they form ring like structure around the adipocytes to remove dead adipocytes leading to tissue remodeling (Cinti, Mitchell et al. 2005). In addition, these newly recruited macrophages secrete pro-inflammatory cytokines and chemokines thereby further aggravating insulin resistance in the neighbouring adipocytes and recruiting even more macrophages. (Xu, Barnes et al. 2003). This ultimately results in the activation of hypoxia sensitive pathways thereby leading to enhanced proinflammatory tone which further aggravates systemic insulin resistance.

## 1.8 Role of macrophages in propagating inflammatory signal

The importance of macrophages in propagating inflammatory signals under conditions of obesity was first reported by Weisberg and collegues in 2003, wherein they reported that more than 40% of the total adipose tissue cell content from obese humans and rodents was comprised of macrophages in contrast to only 10% observed in lean counterparts (Weisberg, McCann et al. 2003). However the precise mechanisms involving macrophage recruitment to adipose tissue are just beginning to emerge. MCP-1, which is an important chemoattractant for immune cells, or its receptor CCR2 are believed to be key mediators for this effect, since disruption of MCP-1 or its

receptor CCR2 prevented the accumulation of macrophages in adipose tissue of obese mice and improved overall insulin sensitivity (Weisberg, Hunter et al. 2006, Kanda, Tateya et al. 2006).

The JNK and IKK pathways are considered to be of utmost importance in macrophages for propagating inflammatory tone due their central role in driving the expression of inflammatory markers and chemokines. Myeloid specific disruption of IKKβ and JNK1 protects mice against diet-induced insulin resistance. This effect is probably mediated by a reduction of the inflammatory tone and reduced macrophage accumulation in the adipose tissue (Arkan, Hevener et al. 2005; Solinas, Vilcu et al. 2007). In line with these results myeloid cell specific disruption of TNFα in mice also protects against obesity induced insulin resistance. These studies clearly indicated that systemic inflammation mediated by macrophages plays a key role in development of obesity induced insulin resistance.

## 1.9 Macrophages and activation of macrophages

Macrophages represent a heterogeneous population of ubiquitously distributed mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes. Initial macrophage development takes place in the bone marrow, where progenitors of myeloid cells differentiate into monocytes. These monocytes are transported to various organs throughout the body via the blood circulation to give rise to resident macrophages. Their wide tissue distribution makes these cells well suited to provide an immediate defense against foreign elements prior to leukocyte migration. Because macrophages participate in both specific immunity via antigen presentation and IL-1 production and nonspecific immunity against bacterial, viral, fungal, and neoplastic pathogens, it is not surprising that macrophages display a range of functional and morphological phenotypes.

Although it is now widely accepted that pro-inflammatory macrophages are recruited to adipose tissue upon obesity development, the factors associated with their activation are also emerging. Enhanced circulating levels of pro-inflammatory cytokines combined with lipid enrich environment are largely responsible for activating macrophages under obesity. On the other hand some new emerging data also suggest the role of gut derived LPS to be an additional factor in the

macrophage activation. Also increased amount of FFA can directly stimulate toll like receptors (TLR) a family of pattern recognition receptors. It has been shown that disruption of TLR2, TLR4 or both prevent the activation of macrophages by FFA (Lee, Ye et al. 2003; Nguyen, Favelyukis et al. 2007) and thus, these mice are protected against obesity-induced insulin resistance. In addition to FFA and LPS, cytokines derived from expanding adipose tissue, adipose tissue microhypoxia and ER stress can activate inflammatory pathways in these cell types.

## 1.10 TLR4 signaling in macrophages

The innate immune system comprising of macrophages, neutrophils and dendritic cells represents the first line of defense in the body against the invading pathogens and microorganisms (Fearon and Locksley 1996). These cells recognize a particular conserved motif in the invading pathogens using a group of transmembrane proteins called toll like receptors (TLR). This leads to the activation of TLR signaling resulting in an immediate defensive response by stimulating the production of cytokines and antimicrobial peptides (Akira 2003). There are 10 members of the TLR so far identified in mammals named TLR 1-10 (Takeda and Akira 2003); however it was TLR4 which was first shown to induce the battery of inflammatory responses (Medzhitov, Preston-Hurlburt et al. 1997).

TLR4 is an essential receptor that recognizes LPS, a component of the cell wall of gram negative bacteria. TLR4 stimulation leads to the activation of three different intracellular signaling cascades namely NFκB signaling, mitogen activated protein kinase (MAPK) and PI3K kinase signaling. LPS binding to TLR4, CD14 and Md2 on the cell surface leads to the recruitment of Myd88 through its binding to the Toll/IL-1 receptor (TIR) domain of TLR4 receptor. The binding of Myd88 to TLR4 leads the recruitment of interleukin-1 receptor associated kinase (IRAK) to Myd88. This leads to IRAK activation by phosphorylation upon which it is released from Myd88. Upon its release phosphorylated IRAK binds to TNF receptor -associated factor 6 (TRAF6), which is then activated by K63 linked polyubiquitination. The poly-ubiquitin chains on TRAF6 are recognized and bound by a kinase complex consisting of transforming-growth

factor- β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2. This complex can then both activate the IKK complex, leading to NFκB activation (Fig 3) or activates MAPK kinases (MEK) leading to activation MAPK cascade comprising of three components p38, JNK and ERK which ultimately leads to the activation of inflammatory response be activating transcription factor AP-1 and EGR1 (Li and Verma 2002; Akira 2003; Takeda and Akira 2003).

A third signaling cascade activated upon LPS binding to TLR4 is the phosphoinositide 3-kinases (PI3K) pathway. The activation of the PI3K pathway ultimately results in the activation of AKT which mediate the activation of NFκB via GSK3β through transcriptional co-activators CREB and CBP (Martin, Rehani et al. 2005). However the activation of PI3K pathway is argued to also negatively regulate the activation of MAPK and NFκB in dendritic cells (DC) and monocytes (Guha and Mackman 2002; Fukao and Koyasu 2003; Lee, Ye et al. 2003; Choi, Jang et al. 2006) (Fig 3).

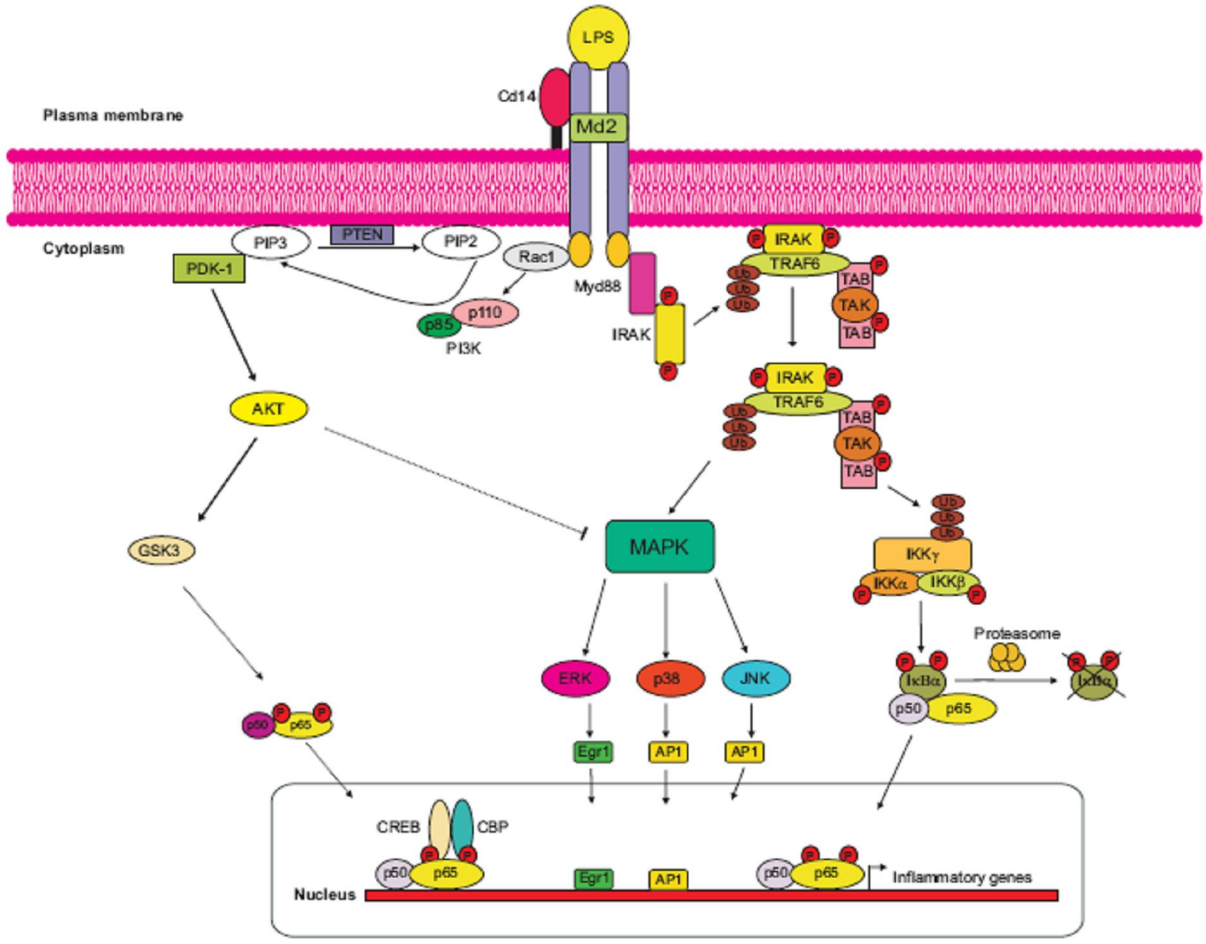



**Fig 3. TLR4 signaling pathways.**

LPS binding to Toll-like receptor 4 (TLR4)–CD14–MD-2 complexes activates an intracellular signalling cascade that involves the recruitment of Myd88 and IRAK (interleukin-1-receptorassociated kinase). Activation of IRAK results in the phosphorylation of TNF-receptorassociated factor 6 (TRAF6), which might relay signals through the TAK1–TAB1–TAB2 complex to IKK complexes to activate the NF-κB pathway or to MAPK pathway activation leading to activation of p38, JNK and ERK. LPS binding to TLR4 also activates PI3K casacade which results in the activation of NF-κB and it also potentially inhibit IKK and MAPK signaling cascades possibly mediated by PKB, and thereby limits the production of inflammatory cytokines (Abbreviations: ERK:Extracellular signal-regulated kinase, JNK: c-jun N terminal kinase, Egr1: Early growth response factor 1, AP1: activator protein-1, cAMP response element binding, CBP: CREB binding protein)

## 1.11 Role of Insulin signaling in macrophages

Insulin largely exerts metabolic effects in insulin responsive tissue. In addition, it plays a critical role in normal growth and development (Taniguchi, Emanuelli et al. 2006). Although the role of insulin signaling in macrophages is largely unknown, the activation of IR/IRS2/PI3k/AKT signaling in response to insulin is similar as that seen in other cell types. However it is believed that insulin signaling in macrophages might regulate protein synthesis and phagocytosis thus influencing macrophages function in innate immunity (Taniguchi, Emanuelli et al. 2006).

Early studies suggest that in contrast to other cell types insulin action in macrophages has no effect on glucose uptake (Liang, Han et al. 2004) but induces $H_2O_2$ production, thereby leading to enhanced phagocytosis (Fortuno, San Jose et al. 2006). Moreover, reduced expression of IR-signaling components as observed in macrophages isolated from diabetic subjects in various studies suggests their importance in maintaining macrophage function (Comi, Grunberger et al. 1987; Naidoo, Jialal et al. 1987; Liang, Han et al. 2004). In support of the role of insulin-mediated macrophage inflammatory responses, recent studies have shown that stimulation of monocytes with insulin leads to increased expression and secretion of TNFα (Iida, Shimano et al. 2001; Iida, Suzuki et al. 2002). In addition, conditional disruption of insulin receptor in macrophages has been shown to blunt the expression and secretion of IL-6 after stimulation with LPS indicating a reduced inflammatory response in these cells (Baumgartl, Baudler et al. 2006). Taken together the functional role of insulin signaling discussed, in macrophages remains controversial.

## 1.12 Aim

The activation of PI-3K pathway in response to various stimuli such as growth factors, leads to the activation of AKT and further downstream signaling cascade mediated by PDK1. The main aim of my project is to study what role does myeloid cell specific downstream kinase of insulin signaling PDK-1 has in

1. Inflammation and
2. Obesity induced insulin resistance.

# 2 Materials and Methods

## 2.1 Chemicals

Molecular weight markers for agarose gel electrophoresis (Gene Ruler DNA Ladder Mix) and for SDS-PAGE (Prestained Protein Ladder Mix) were obtained from MBI Fermentas, St. Leon-Rot, Germany. RedTaq DNA Polymerase and 10 x RedTaq buffer were purchased from Sigma-Aldrich, Seelze, Germany.

| **Chemicals** | **Company** |
|---|---|
| 2-Deoxy-D-[1-$^{14}$C]-Glucose | Amersham, Freiburg, Germany |
| α-[$^{32}$P]-dCTP | Amersham, Freiburg, Germany |
| ε-aminocaproic acid | Sigma-Aldrich, Seelze, Germany |
| Acetone | KMF Laborchemie, Lohmar, Germany |
| Acrylamide | Roth, Karlsruhe, Germany |
| Agarose (Ultra Pure) | Invitrogen, Karlsruhe, Germany |
| Amyloglucosidase | Roche, Mannheim, Germany |
| Aprotinin | Sigma-Aldrich, Seelze, Germany |
| Avertin | Sigma-Aldrich, Seelze, Germany |
| Benzamidine | Sigma-Aldrich, Seelze, Germany |
| β-Mercaptoethanol (β-ME) | AppliChem, Darmstadt, Germany |
| Bovine serum albumin (BSA) | Sigma-Aldrich, Seelze, Germany |
| Bradford reagent | Bio-Rad, München, Germany |
| Bromphenol blue | Merck, Darmstadt, Germany |
| Chloroform | Merck, Darmstadt, Germany |
| D-[3-$^{3}$H]-Glucose | Amersham, Freiburg, Germany |
| Desoxy-Ribonucleotid-Triphosphates (dNTPs) | Amersham, Freiburg, Germany |
| Dextran sulfate | AppliChem, Darmstadt, Germany |
| Dimethylsulfoxide (DMSO) | Merck, Darmstadt, Germany |
| Dithiothreitol (DTT) | Boehringer, Mannheim, Germany |
| Enhanced Chemiluminscence (ECL) Kit | Perbio Science, Bonn, Germany |
| Ethanol, absolute | AppliChem, Darmstadt, Germany |
| Ethidium bromide | Sigma-Aldrich, Seelze, Germany |
| Ethylendiamine tetraacetate (EDTA) | AppliChem, Darmstadt, Germany |
| Fetal Calf Serum (FCS) | Gibco BRL, Eggenstein, Germany |
| Glacial acetic acid | Roth, Karlsruhe, Germany |
| Glucose | DeltaSelect, Pfullingen, Germany |
| Glycerol | Serva, Heidelberg, Germany |
| Hydrochloric acid (37 %) | KMF Laborchemie, Lohmar, Germany |
| Insulin | Novo Nordisk, Bagsværd, Denmark |
| Isopropanol | Roth, Karlsruhe, Germany |
| Ladderman$^{TM}$ DNA Labeling Kit | Cambrex Bio Science, Verviers, Belgium |

| | |
|---|---|
| Leptin | Sigma-Aldrich, Seelze, Germany |
| Methanol | Roth, Karlsruhe, Germany |
| Non-essential amino acids | Gibco BRL, Eggenstein, Germany |
| Palmitate | Sigma-Aldrich, Seelze, Germany |
| Penicillin/Streptomycin Solution | Gibco BRL, Eggenstein, Germany |
| Phenol-Chloroform-Isoamyl alcohol | AppliChem, Darmstadt, Germany |
| Phenylmethylsulfonylfluoride (PMSF) | Sigma-Aldrich, Seelze, Germany |
| Phosphate buffered saline (PBS) | Gibco BRL, Eggenstein, Germany |
| Potassium hydroxide | Merck, Darmstadt, Germany |
| Proteinase K | Roche, Mannheim, Germany |
| RPMI 1640 w/out phenol red | Gibco BRL, Eggenstein, Germany |
| Sodium acetate | AppliChem, Darmstadt, Germany |
| Sodium chloride | AppliChem, Darmstadt, Germany |
| Sodium dodecyl sulfate | AppliChem, Darmstadt, Germany |
| Sodium hydroxide | AppliChem, Darmstadt, Germany |
| Sodium fluoride | Merck, Darmstadt, Germany |
| Sodium orthovanadate | Sigma-Aldrich, Seelze, Germany |
| Sodium pyruvate | Gibco BRL, Eggenstein, Germany |
| Sucrose | AppliChem, Darmstadt, Germany |
| Tetramethylethylenediamine | Sigma-Aldrich, Seelze, Germany |
| Thioglycollate | Sigma-Aldrich, Seelze, Germany |
| Trishydroxymethylaminomethane (Tris) | AppliChem, Darmstadt, Germany |
| Triton X-100 | Applichem, Darmstadt, Germany |
| Tween 20 | Applichem, Darmstadt, Germany |
| Western Blocking Reagent | Roche, Mannheim, Germany |

**Table 1.**

## 2.2 Molecular biology

Standard molecular biology methods were performed according to Sambrook and Russell (Sambrook and Russell 1989), unless otherwise stated.

### 2.2.1 Isolation of genomic DNA

Mouse tail biopsies were incubated 2-3 hours (h) in lysis buffer (100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% (w/v) SDS, 0.2 M NaCl, 500 µg/ml proteinase K) in a thermomixer (Eppendorf, Hamburg, Germany) at 56°C. Peritoneal macrophages were incubated in lysis buffer at 56°C over night. Precipitation was performed by addition of one equivalent of isopropanol. After centrifugation and a single washing step with 70% (v/v) ethanol, the DNA pellet was dried at room temperature (RT) for 30 minutes and resuspended in double distilled water (ddH2O).

### 2.2.2 Quantification of Nucleic Acids

DNA and RNA concentrations were assessed by measuring the sample absorption at 260 nm with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Peqlab, Erlangen, Germany). An optical density of 1 corresponds to approximately 50 µg/ml of double-stranded DNA and to 38 µg/ml of RNA.

### 2.2.3 Polymerase Chain Reaction (PCR)

The PCR method (Mullis and Faloona 1987; Saiki, Gelfand et al. 1988) was used to genotype mice for the presence of floxed alleles or transgenes with customized primers listed in Table 2. Reactions were performed in a Thermocycler iCycler PCR machine (Bio-Rad, München, Germany) or in a Peltier Thermal Cycler PTC-200 (MJ Research, Waltham, USA). All amplifications were performed in a total reaction volume of 25 µl, containing a minimum of 50 ng template DNA, 25 pmol of each primer, 25 µM dNTP Mix, 10 x RedTaq reaction buffer and 1 unit of RedTaq DNA Polymerase. Standard PCR programs started with 4 minutes denaturation at 95°C (Abel, Peroni et al. 2001), followed by 30 cycles consisting of denaturation at 95°C for 45 seconds (sec), annealing at oligonucleotide-specific temperatures for 30 sec and elongation at 72°C for 30 sec and a final elongation step at 72°C for 7 min. PCR-amplified DNA fragments were applied to 1% - 2% (w/v) agarose gels (1 x TAE, 0.5 mg/ml ethidium bromide) and electrophoresed at 120 V.

| **Primer** | **Sequence** | **Orientation** |
|---|---|---|
| *LysMCre5'* | CTC TAG TCA GCC AGC AGC TG | Sense |
| *LysMCre3'* | ATG TTT AGC TGG CCC AAA TGT | Antisense |
| *p99* | ATC CCA AGT TAC TGA GTT GTG TTG GAA G | Sense |
| *p100* | TGT GGA CAA ACA GCA ATG AAC ATA CAC GC | Antisense |
| *p70* | CAGTTCATCATTGCAAGAGTC | Sense |

**Table 2. Oligonucleotides used for genotyping.**

All primer sequences are displayed in 5'-3' order. Primer orientation is designated "sense" when coinciding with transcriptional direction. All primers were purchased from Eurogentec, Cologne, Germany.

### 2.2.4 RNA Extraction, RT-PCR and Quantitative Realtime PCR

Total RNA from murine cells and tissues was extracted using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany). 1 µg of each RNA sample was reversely transcribed using the Eurogentec RT Kit (Eurogentec, Cologne, Germany) according to manufacturer's instructions. The cDNA was subsequently amplified using an ABI Prism 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, USA).

| **Probe** | **Catalogue No.** |
|---|---|
| CCl2 | Mm00441242_m1 |
| CCl3 | Mm00441258_m1 |
| CCl4 | Mm0044311_m1 |
| CCl5 | Mm01302428_m1 |
| CCl7 | Mm0044113_m1 |
| CXCl2 | Mm00436450_m1 |
| CXCl10 | Mm00445235_m1 |
| F4/80 | Mm00802530_m1 |
| Gusb | Mm00446953_m1 |
| Hprt1 | Mm00446968_m1 |
| IL6 | Mm00446190_m1 |
| MacII | Mm00802901_m1 |
| TNFα | Mm00443258_m1 |

**Table 3. Taqman Gene Expression Assays**
All assays were purchased from Applied Biosystems, Foster City, USA.

Relative expression of mRNA was determined using standard curves based on cDNA isolated from macrophages or adipose tissue. Samples were adjusted for total cDNA content by Glucuronidase beta (Gusb) and hypoxanthine guanine phosphoribosyl transferase (Hprt-) 1 mRNA quantitative Realtime PCR. Calculations were performed by a comparative method (2-ΔΔCT). In brief, the amplification plot is the plot of fluorescence versus PCR number. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal

reached the detection threshold. Therefore, the input cDNA copy number and Ct are inversely related.Data were analyzed with the Sequence Detector System (SDS) software version 2.1 (ABI) and Ct value was automatically converted to fold change RQ value ((RQ) = 2− (ΔΔCT)). The RQ values from each gene were then used to compare the gene expression across all groups.

## 2.3 Biochemistry

### 2.3.1 Protein Extraction from cells

Cell pellets or snap-frozen tissues were disrupted in lysis buffer (50 mM HEPES (pH 7.4), 1% (v/v) Triton X-100, 0.1 M sodium fluoride, 10 mM EDTA, 50 mM sodium chloride, 10 mM sodium orthovanadate, 0,1% (w/v) SDS, 10 µg/ml aprotinin, 2 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride (PMSF)) by resuspension and gentle vortexing or by usage of a polytron homogenizer (IKA Werke, Staufen, Germany), respectively. Particulate matter was removed by centrifugation for 1 h at 4°C. The supernatant was transferred to a fresh vial and protein concentration was determined using a Bradford assay. Protein extracts were diluted to 5 mg/ml with lysis buffer and 4 x SDS sample buffer (125 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 43.5% (w/v) glycerol, 100 mM DTT, and 0.02% (v/v) bromophenol blue), incubated at 95°C over 5 min and stored at - 80°C.

### 2.3.2 Protein extraction from tissue

100-500 mg tissue were dissolved in 1 ml lysis buffer containing 50 mM HEPES [pH 7.4], 1% Triton X-100, 0.1% SDS, 100 mM NaF, 10 mM Na3O4V, 250 mM EDTA, 50 mM NaCl, 10 µg/ml aprotinin, 2 mM benzamidin, 348 µg/ml PMSF, and homogenized using a Ultra Thurrax homogenizer. Protein extracts were centrifuged for 45 min at 4°C to separate supernatants from debris. Protein concentration was measured using a photometer and the Christian-Warburg formula. 10 µg/µl protein stock solution was prepared in 1xSDS sample buffer and heated at 95°C for 5 min. 100µg protein were used for immunoblotting. Protein solutions were always stored at -80°C.

### 2.3.3 Nuclear and cytoplasmic protein extraction

Peritoneal macrophages were washed with PBS. 106 cells were resuspended in 15 µl hypotonic buffer A containing 10 mM HEPES [pH 7.6], 10 mM KCl, 2 mM MgCl2, 0.5 mM DTT, 0.1 mM EDTA, and 1 tablet of Proteinase Inhibitor (Complete mini, Roche, Germany) and incubated for 10 min at 4°C. NP40 were added to a final concentration of 1% and incubated at 4°C for 1 min. Cells were immediately collected by centrifugation at 13000rpm at 4°C for 1 min. The supernatant contained the cytoplasmic fraction. The pellet was washed with buffer A and resuspended in 10 µl high salt buffer B containing 50 mM HEPES [pH 7.8], 50 mM KCl, 300 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 10% glycerol, and 1 tablet of Proteinase Inhibitor (Complete mini, Roche, Germany). The pellet was incubated at 4°C on a full speed shaker for 1 h. After incubation, the suspension was centrifuged at 13000 rpm at 4°C for 1 h. The supernatant contained the nuclear fraction.

### 2.3.4 Immunoprecipitation

500 µg nuclear proteins were incubated with 2.5 µg of antibody at 4°C on a rotator overnight. Then, 100 µl of Protein A-Sepharose (100 mg/ml, Amersham, Freiburg, Germany) were added and incubated overnight. The suspension was washed 5 times with high salt buffer B, 50 µl 2xSDS loading buffer was added, and samples were incubated for 5 min at 95°C. The samples were separated on SDS-PAGE gels, and processed for western blot analysis.

### 2.3.5 IKK Kinase Assay

Freshly prepared whole cell lysates were immunoprecipitated with anti IKKγ antibody (SC-8330, Santa cruz). Kinase activity of NEMO dependent IKK complex was determined using biotinylated IκBα (Ser 32) peptide (#1146 cell signaling) as a substrate in a kinase reaction comprising of kinase buffer 20mM HEPES pH 7.7, 2mM $MgCl_2$, 2mM $MnCl_2$, 1mM DTT, γATP $^{32}P$ and phosphatase inhibitors at $30^0C$ for 30 minutes. The phosphorylated biotinylated IκBα (Ser 32) peptide was then purified from the kinase reaction buffer using streptavidin Agarose beads (invitrogen) and incubating the reaction cocktail with beads at $4^0C$ for 30 minutes, accompanied by periodical shaking. The purified biotinylated IκBα (Ser 32) peptide

coupled to streptavidin beads were the transferred to scintillation vials containing 4ml of scintillating counting media and counted in a BD Scintillator counter.

### 2.3.6 Electrophoretic mobility-shift Assay

Nuclear extracts prepared from 5 X $10^6$ peritoneal macrophages stimulated with 1µg/ml of LPS. 4µg of nuclear extracts was incubated for 30 min at room temperature with 2µg poly (dI-dC) and 0.5ng of probe for NFκB consensus site (5′-CGGGCTGGGGATTCCCCATCTCGGTAC-3′) labeled with $^{32}$P. the reaction was performed in the following reaction buffer 100mM Tris HCl pH 7.5, 1M NaCl, 50mM DTT, 10mM EDTA, 40% glycerol, and 1mg/ml of BSA. The samples were fractionated on a 5% PAGE and visualized using autoradiography.

### 2.3.7 Western Blot Analysis

Frozen protein extracts were thawed at 95°C for 5 min, then separated on 10-15% (v/v) SDS polyacrylamide gels (Laemmli 1970) and blotted onto PVDF membranes (Bio-Rad, München, Germany). Membranes were then incubated with 1% (v/v) blocking reagent (Roche, Mannheim, Germany) for 1 h at RT. Subsequently, primary antibodies (Table 4) diluted in 0.5% (v/v) blocking solution were applied over night at 4°C. PVDF membranes were then washed four times for 5 min with 1 x TBS/0.01 (v/v) Tween. After 1 h incubation at RT with the respective secondary antibodies, membranes were washed 4 times for 10 min with 1 x TBS/0.01 (v/v) Tween, rinsed in 1 x TBS, incubated for 1 min in Pierce ECL Western Blotting Substrate (Perbio Science, Bonn, Germany), sealed in a plastic bag and exposed to chemiluminescence film (Amersham, Braunschweig,
Germany). Films were developed in an automatic developer.

| **Antibody** | **Catalogue No.** | **Company** |
|---|---|---|
| Akt | 9272 | Cell Signaling |
| ß-Actin(clone AC-15) | A5441 | Sigma |
| GSKß | 9315 | Cell Signaling |
| IκBα | 9246 | Cell Signaling |
| IRAK 1 | 4362 | Cell Signaling |
| insulin receptor β subunit | sc-711 | Santa Cruz Biotechnology Inc |

| | | |
|---|---|---|
| PDK-1 | 611070 | BD bioscience |
| Phospho-AKT (Ser 473) | 9271 | Cell Signaling |
| Phospho p44/42 | 9106 | Cell Signaling |
| p44/42 | 9102 | Cell Signaling |
| Phospho SAPK/JNK | 9251 | Cell Signaling |
| Phospho p38 | 9216 | Cell Signaling |
| p38 | 9212 | Cell Signaling |
| Phosho IκBα(Ser 32/36) | 9246 | Cell Signaling |
| Phospho IRAK 1 | 4361 | Cell Signaling |
| SAPK/JNK | 9252 | Cell Signaling |
| TRAF 6 | sc-7221 | Santa Cruz Biotechnology Inc |
| Ub(n) | sc-8017 | Santa Cruz Biotechnology Inc |

**Table 4. List of antibodies used for western Blot Analysis.**

### 2.3.8 Enzyme linked Immunosorbent Assay (ELISA)

Mouse insulin (Mouse/Rat Insulin ELISA; Crystal Chem, Downers Grove, IL,USA), leptin (ACTIVE® Murine Leptin ELISA; Diagnostics Systems Laboratories, Webster, TX, USA), TNF-α (Quantikine Mouse TNF-alpha/TNFSF1A ELISA; R&D Systems, Wiesbaden, Germany), IL-6 (Quantikine Mouse IL-6 ELISA; R&D Systems, Wiesbaden, Germany), adiponectin (Quantikine Mouse Adiponectin/Acrp30 ELISA; R&D Systems, Wiesbaden, Germany).Concentration in serum or cell culture supernatant was determined using mouse standards according to manufacturer's guidelines and measured on a Precision Microplate Reader (Emax; Molecular Devices GmbH, München, Germany).

## 2.4 Cell Culture and Tissue Analysis

### 2.4.1 Culture of Primary Murine Macrophages

Cells were plated at a density of 1*106/ml on tissue culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany) in RPMI 1640 (supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) glutamine, 1% (v/v) penicillin-streptomycin) and were allowed to adhere overnight at 37°C, 5% CO2 and 95% humidity. On the next day, adherent cells were washed once with PBS followed by further incubation in RPMI 1640. Preceding all the experiments, cells were washed two times with sterile PBS and, if stimulated with LPS (1µg/ml), serum-starved for 16-20 h.

### 2.4.2 TUNEL Assay

TUNEL assay was performed using manufacturer's guidelines (Dead END™ Fluorometric TUNEL system, Promega). Briefly, dissected liver was snap-frozen in Jung Tissue Freezing Medium® (Leica Microsystems Nussloch GmbH, Nussloch, Germany), transferred to a cryostat (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and cut into 7µm thin sections. Specimens were collected on clean poly-L-lysine-coated glass slides (Menzel GmbH, Braunschweig, Germany), dried at room temperature. After two washing steps with PBS, cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min at RT. Following two additional washing steps with PBS, equilibration buffer was added to the slides and incubated for 10 min at RT. After removal of equilibration buffer, incorporation of fluorescein-12-dUTP by rTdT enzyme was carried out at 37°C in the dark for 60 min. The reaction was stopped by addition of 2x SSC and after three washing steps with PBS, slides were mounted with Vectashield DAPI medium (# H-1200, Vector Laboratories Inc, Burlingame, CA, USA) and analyzed under a fluorescence microscope. Quantification of DAPI- and FITC-positive cells was performed using AxioVision 4.2 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

### 2.4.3 Histological Analysis and Immunohistochemistry

White adipose tissue of diet-induced obese Control and PDK-1$^{\Delta myel}$ mice was dissected, fixed overnight in 4% (w/v) PFA and then embedded for paraffin sections. Subsequently, 7 µm thin

sections were deparaffinized and stained with hematoxylin and eosin (H&E) for general histology or with Mac-2/Galectin-3 antibody (#CL8942AP; Cedarlane Laboratories Ltd, Burlington, ON, Canada) for detection of adipose tissue macrophages. Immunohistochemistry was performed as previously described (Cinti, Mitchell et al. 2005). Quantification of adipocyte size and Mac-2-positive area was performed using AxioVision 4.2 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany).

### 2.4.4 Fluorescence–Activated Cell Sorting (FACS)

Peritoneal myeloid cells and resident myeloid cells were isolated from bone marrow, spleen and lymph node. Isolated myeloid cells were treated with Red cell lysis buffer (9X ammonium chloride, 1X PBS). Cells were then resuspended in 1X PBA (1X PBS /0.5% BSA/0.02%Na-Acid). This was followed with counting the cells in $F_c$-Block. After the Following staining, FITC- conjugated Mac1, R-PE-conjugated CD4 (L3T4) and APC-conjugated CD45R/B220, the cells were sorted according to the FITC, R-PE and APC signals. The percent of cell number was analyzed and absolute cell number was determined by relating with number of counted cells in $F_c$-Block (Becton Dickinson FACS Calibur. Cell Quest program.

### 2.4.5 Analysis of AST levels

Serum levels of AST were determined in the diagnostic laboratory using standard protocol**.**

## 2.5 Mouse Handling

All mouse handling was within institutional animal care committee guidelines. All animal procedures were conducted in compliance with protocols and approved by local government authorities (Bezirksregierung Köln, Cologne, Germany) and were in accordance with NIH guidelines. Mice were housed in groups of 3 to 5 at 22–24°C using a 12 h light/dark cycle.

### 2.5.1 Mouse Experiments

General animal handling was performed as described by Hogan (Hogan, Costantini et al. 1986) and Silver (Silver 1995).

### 2.5.2 Mice

Mice were housed in a virus-free facility at 22-24°C on a 12 h light/ 12 h dark cycle with the light on at 6 a.m. and were either fed a normal chow diet (NCD; Teklad Global Rodent 2018; Harlan Winkelmann GmbH, Borchen, Germany) containing 53.5% (w/v) carbohydrates, 18.5% (w/v) protein and 5.5% (w/v) fat or a high fat diet (HFD; C1057; Altromin GmbH, Lage, Germany) containing 32.7% (w/v) carbohydrates, 20% (w/v) protein, and 35.5% (w/v) fat (55.2% of calories from fat). All animals had access to water *ad libitum*. Food was only withdrawn if required for an experiment. At the end of the study period, animals were sacrificed by $CO_2$ anesthesia or cervical dislocation. All animal procedures and euthanasia were reviewed by the animal care committee of the University of Cologne, approved by local government authorities (Bezirksregierung Köln) and were in accordance with National Institutes of Health guidelines.

### 2.5.3 Generation of PDK-1$^{\Delta myel}$ mice

*LysMCre* mice were mated with *PDK-1*$^{lox/lox}$ mice, and a breeding colony was maintained by mating *PDK-1*$^{lox/lox}$ with *LysMCre-IR*$^{lox/lox}$ mice. *PDK-1*$^{lox}$ mice had been backcrossed for 3 generations on a C57BL/6 background, and *LysMCre* mice – initially established on a C57BL6/129sv background – had been backcrossed for 10 generations on a C57BL6 background before intercrossing them with *PDK-1*$^{lox}$ mice. Only animals from the same mixed background strain generation were compared to each other. *LysMCre* mice were genotyped by PCR as previously described (Clausen, Burkhardt et al. 1999), *PDK-1*$^{lox/lox}$ were genotyped by PCR with primers crossing the loxP site as previously described (Bruning, Michael et al. 1998).

### 2.5.4 Body weight and Blood Glucose level

Body weight and blood glucose levels were monitored weekly and at 20 weeks of age, respectively. Blood glucose values were determined from whole venous blood usingan automatic glucose monitor (GlucoMen® GlycÓ; A. Menarini Diagnostics, Neuss,Germany).

### 2.5.5 Glucose and Insulin Tolerance Test

Glucose tolerance tests (GTT) were performed on animals that had been fasted over night for 16 hours. Insulin tolerance test (ITT) was performed on random fed mice. Animals were injected with either 2 g/kg body weight of glucose or 0.75 U/kg body weight of human regular insulin into the peritoneal cavity. Glucose levels were determined in blood collected from the tail tip immediately before and 15, 30 and 60 minutes after the injection, with an additional value determined after 120 minutes for the GTT.

### 2.5.6 Isolation of Primary Peritoneal Macrophages

8-20 week old $IR_{\Delta myel}$ mice or control mice were injected intreaperitoneally with 2 ml thioglycollate medium (4% in PBS (w/v)) to induce a sterile peritonitis. On day 4 *post* injection, the animals were sacrificed by $CO_2$ anesthesia and cells were collected by peritoneal lavage with sterile PBS. Following centrifugation, cells were resuspended in erythrocyte lysis buffer for 3 min at RT. After one additional wash with PBS, cells were resuspended in RPMI 1640 (supplemented with 10% (v/v) heat inactivated FCS, 1% (v/v) glutamine, 1% (v/v) penicillin-streptomycin) and live cells were counted in 4% (w/v) trypan blue. Cell numbers were adjusted to $1*10^6$/ml and stored on ice for further experiments.

### 2.5.7 LPS induced septic Shock

Mice in the age group of 8-10 weeks were challenged by interperitoneal injection of a 50µg/gm bodyweight lethal dose of LPS (From E.coli Serotype 055:B5) in pyrogen free PBS. During all times animals were housed in sterile rooms, using autoclaved cages, food and water.

### 2.5.8 Hyperinsulinemic-euglycemic Clamp Studies

Catheter Implantation: At the age of 16-20 weeks, male mice were anesthetized by intraperitoneal injection of avertin and adequacy of the anesthesia was ensured by the loss of pedal reflexes. A Micro-Renathane catheter (MRE 025; Braintree Scientific Inc., MA, USA) was inserted into the right internal jugular vein, advanced to the level of the superior vena cava, and secured in its position in the proximal part of the vein with 4-0 silk; the distal part of the vein was occluded with 4-0 silk. After irrigation with physiological saline solution, the catheter was filled with heparin solution and sealed at its distal end. The catheter was subcutaneously tunneled, thereby forming a subcutaneous loop, and exteriorized at the back of the neck. Cutaneous incisions were closed with a 3-0 silk suture and the free end of the catheter was attached to the suture in the neck as to permit the retrieval of the catheter on the day of the experiment. Mice were intraperitoneally injected with 1 ml of saline containing 15µg/g body weight of tramadolhydrochloride and placed on a heating pad in order to facilitate recovery.

Clamp Experiment: Only mice that had regained at least 90% of their preoperative body weight after 6 days of recovery were included in the experimental groups. After starvation for 15 hours, awake animals were placed in restrainers for the duration of the clamp experiment. After a D-[3-$^{3}$H]Glucose (Amersham Biosciences, UK) tracer solution bolus infusion (5 µCi), the tracer was infused continuously (0.05 µCi/min) for the duration of the experiment. At the end of the 40-minute basal period, a blood sample (50 µl) was collected for determination of the basal parameters. To minimize blood loss, red blood cells were collected by centrifugation and reinfused after being resuspended in saline. Insulin (human regular insulin; NovoNordisc Pharmaceuticals, Inc., NJ, USA) solution containing 0.1% (w/v) BSA (Sigma-Aldrich, Germany) was infused at a fixed rate (4 µU/g/min) following a bolus infusion (40 µU/g). Blood glucose levels were determined every 10 minutes (B-Glucose Analyzer; Hemocue AB, Sweden) and physiological blood glucose levels (between 120 and 150 mg/dl) were maintained by adjusting a 20% glucose infusion (DeltaSelect, Germany). Approximately 60 minutes before steady state was achieved, a bolus of 2-Deoxy-D-[1-$^{14}$C]Glucose (10 µCi, Amersham) was infused. Steady state was ascertained when glucose measurements were constant for at least 30 min at a fixed glucose infusion rate and was achieved within 100 to 130 min. During the clamp experiment,

blood samples (5 µl) were collected after the infusion of the 2-Deoxy-D-[1- $^{14}$C]Glucose at the time points 0, 5, 15, 25, 35 min etc. until reaching the steady state. During the steady state, blood samples (50 µl) for the measurement of steady state parameters were collected. At the end of the experiment, mice were killed by cervical dislocation, and brain, liver, WAT and skeletal muscle tissue were dissected and stored at -20°C.

Assays: Plasma [3-$^{3}$H]Glucose radioactivity of basal and steady state samples was determined directly after deproteinization with 0.3 M $Ba(OH)_2$ and 0.3 M $ZnSO_4$ and also after removal of $^{3}H_2O$ by evaporation, using a liquid scintillation counter (Beckmann, Germany). Plasma Deoxy-[1-$^{14}$C] Glucose radioactivity was directly measured in the liquid scintillation counter. Tissue lysates were processed through Ion exchange chromatography columns (Poly-Prep$^{R}$ Prefilled Chromatography Columns, AG$^{R}$1-X8formate resin, 200-400 mesh dry; Bio Rad Laboratories, CA, USA) to separate 2-Deoxy- D-[1-$^{14}$C]Glucose (2DG) from 2-Deoxy-D-[1-$^{14}$C]Glucose-6-Phosphate (2DG6P).

Calculations: Glucose turnover rate ($mg \times kg^{-1} \times min^{-1}$) was calculated as the rate of tracer infusion (dpm/min) divided by the plasma glucose-specific activity (dpm/mg) corrected for body weight. HGP ($mg \times kg^{-1} \times min^{-1}$) was calculated as the difference between the rate of glucose appearance and glucose infusion rate. In vivo glucose uptake for each tissue ($nmol \times g^{-1} \times min^{-1}$) was calculated based on the accumulation of 2DG6P in the respective tissue and the disappearance rate of 2DG from plasma as described previously (Ferre, Leturque et al. 1985).

## 2.6 Computer Analysis

### 2.6.1 Densitometrical Analysis

Protein expression was assessed by western blot analysis and bands were measured in intensity per mm2 using the Quantity One Software (Bio-Rad, München, Germany).After background subtraction, each sample was normalized to an internal loading control.Average protein expression of control mice was set to 100% and compared to protein expression of knockout animals unless stated otherwise.

### 2.6.2 Statistical Methods

Data sets were analyzed for statistical significance using a two-tailed unpaired student's t test. All *p* values below 0.05 were considered significant.

# 3 Results

## 3.1 Generation of myeloid cell specific PDK-1 Knockout mice

Myloid cell specific disruption of gene in mouse is achieved by using lysozyme M cre mice (Clausen, Burkhardt et al. 1999)expressing cre recombinase under the lysozyme M promoter. Lysozyme M cre deletes a gene of interest specifically in myeloid cells with efficiency of 83-98% in mature macrophages and of 100% in granulocytes (Clausen, Burkhardt et al. 1999).

In order to generate PDK-1 deficient myeloid cell mice we crossed mice expressing the Cre-recombinase under control of the lysozyme M (lysM Cre $^{+/-}$) promoter which expresses Cre-recombinase specifically in myeloid cells with mice bearing PDK-1 floxed allele for exon 2 and 3 (Fig 4) (PDK-1$^{fl\Delta\ neo/fl\Delta\ neo}$) (Clausen, Burkhardt et al. 1999; Mora, Davies et al. 2003). Thus we generated mice lacking PDK1 specifically in myeloid cells PDK-1$^{\Delta myel}$. These mice were born at an expected Mendelian ratio, and the littermate lacking expression of Cre –recombinase PDK-1$^{fl\Delta\ neo/fl\Delta\ neo}$ mice were used as controls throughout the period of study.

To confirm the specificity of the lys M Cre mediated PDK-1 deletion peritoneal macrophages were isolated and a deletion PCR was performed. In control macrophages an amplification of 2kb band could be detected corresponding to the wild type allele (Fig 4). In DNA extracts obtained from myeloid cells of PDK-1$^{\Delta myel}$ mice the 2kb band was absent, whereas a 500bp band was amplified corresponding to the deleted allele. Hence we can conclude that PDK-1$^{\Delta myel}$ mice exhibit efficient deletion of PDK-1 deletion in myeloid cells.

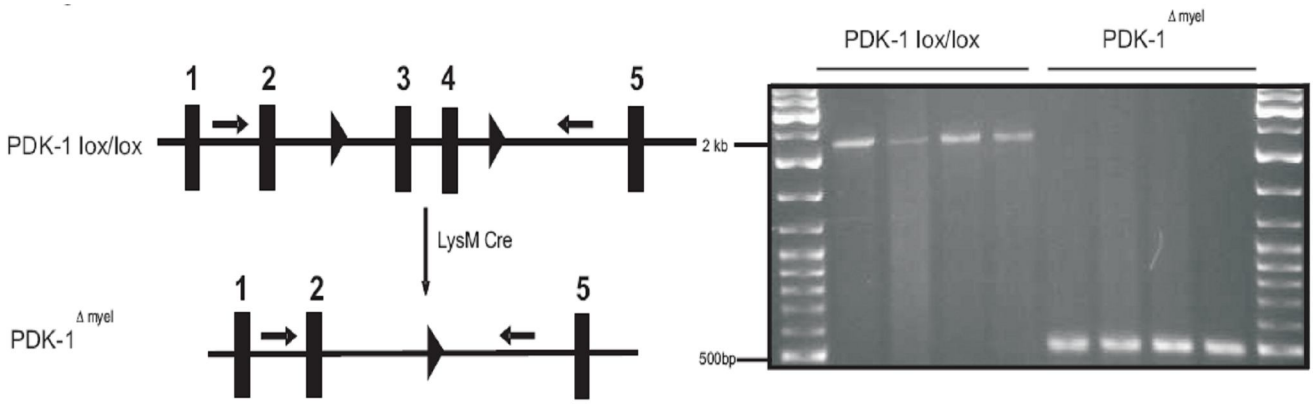



**Fig 4. Schematic representation of generation macrophage specific PDK-1 deletion and confirmation deleted exons in myeloid cells by deletion PCR.** Diagram illustrates the position of exons 1-5 and the *lox*P cre exsion sites. The arrow indicates the position of primers used for genotyping.

Deletion was further analyzed on the protein level by western blot analysis, and confirmed absence of PDK-1 expression in myeloid cells of PDK-$1^{\Delta myel}$ mice (Fig 5). However, the expression of PDK-1 was not affected in brain, lung, liver, spleen and skeletal muscle of PDK-$1^{\Delta myel}$ mice compared to control mice (Fig 5)

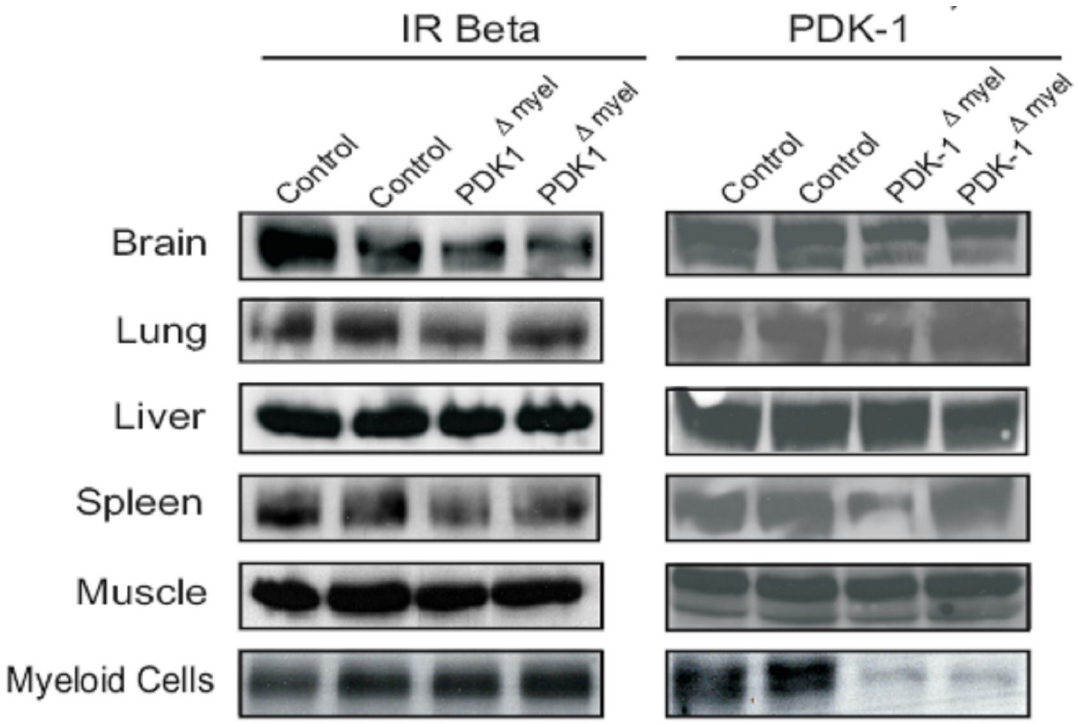



**Fig 5. Western blot analysis of PDK-1 expression.**

Immunoblot analysis of PDK-1 and insulin receptor (IR) (control) expression. Proteins were extracted from brain, lung, liver, spleen, muscle and myeloid cells of PDK-1$^{\Delta myel}$ mice and control mice.

Hence, by conditional gene targeting using lysozyme M promoter to drive cre expression we could efficiently and specifically delete PDK-1 in myeloid cells without disrupting PDK-1 protein expression in other tissues.

## 3.2 Effect of myeloid cell restricted PDK-1 deficiency in development and differentiation of myeloid cells

Since PDK-1 has been shown to be essential for development of mice and more recently has been shown to regulate cell proliferation and cell cycle progression (Mora, Davies et al. 2003; Hashimoto, Kido et al. 2006; Nakamura, Sakaue et al. 2008) we investigated the effect of PDK-1 deficiency in myeloid cells on their development and differentiation. To evaluate this we quantified the absolute number of myeloid cells (granulocytes and macrophages) in different

organs namely, bone marrow, spleen, lymph node and peritoneal cavity which are known to be either the site of generation or resident myeloid cells by Fluorescence activated cell sorting (FACS). This analysis revealed no difference in the absolute number of myeloid cells between the control and PDK-1$^{\Delta myel}$ mice (Fig 6). Thus indicating that myeloid lineage restricted PDK-1 deficiency does not affect development and differentiation of myeloid cells

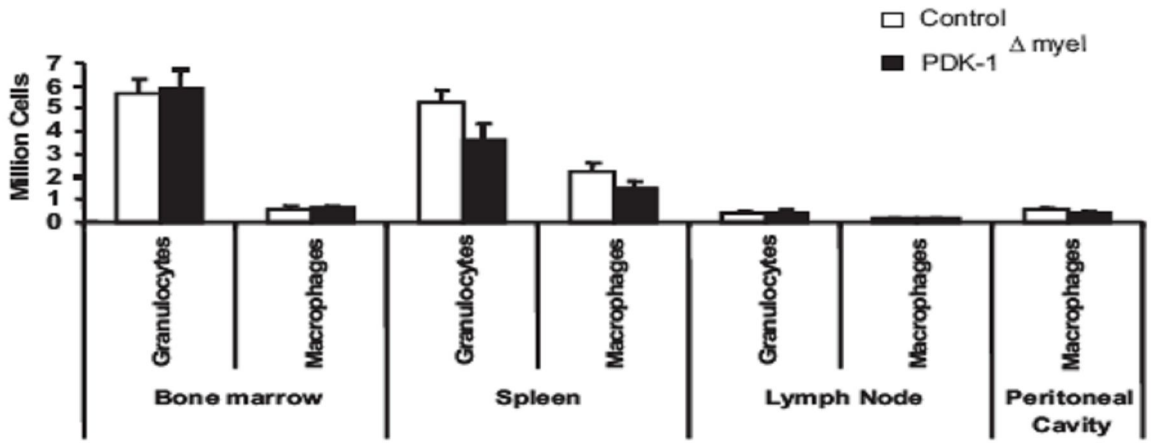



**Fig 6. FACS analysis to quantify absolute numbers of myeloid cells.**

Deletion of PDK-1 in myeloid cells does not alter its differentiation and development as marked by unaltered number of granulocyte and macrophages in Control (n=4) and PDK-1$^{\Delta myel-}$ (n=4) animals. All data are presented as mean ± SEM.

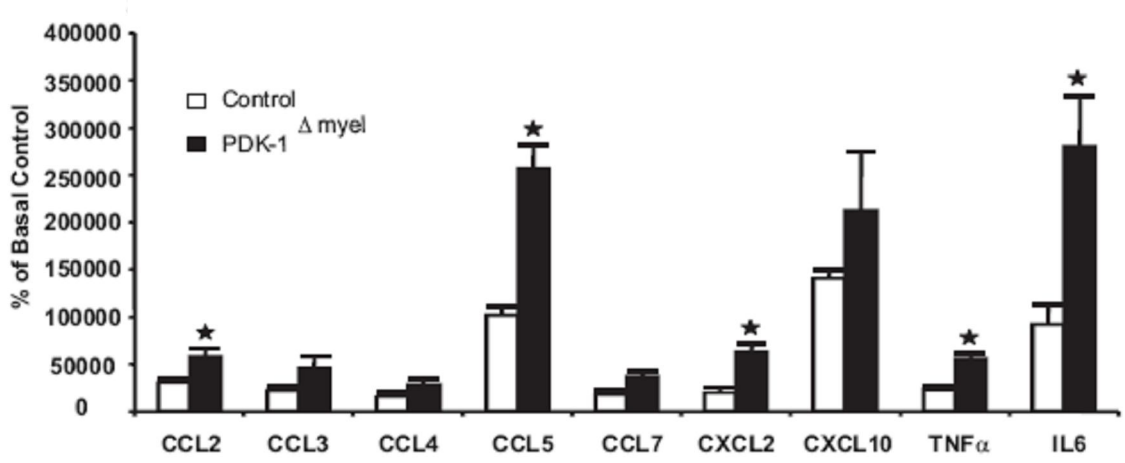
## 3.3 Role of myeloid cell restricted PDK-1 deficiency in Inflammation

### 3.3.1 Primary macrophages of PDK-1$^{\Delta myel}$ mice exhibit Increased Expression and Secretion of Inflammatory Cytokines upon LPS stimulation

To characterize the myeloid cell specific role of PDK-1 in inflammation, peritoneal macrophages were isolated and stimulated with LPS. Intrestingly, PDK-1$^{\Delta myel}$ macrophages exhibited enhanced expression of proinflammatory cytokines TNF α and IL6, and chemokines including

CCL2, CCL5 and CXCL2 after 24 hours of LPS stimulation (Fig 7. a). PDK-1$^{\Delta myel}$ macrophages also exhibited the enhanced secretion of TNF α and IL6 following 24 hours of LPS stimulation (Fig 7. b). These data indicate that PDK-1 negatively regulates the production of key inflammatory cytokines.

**a.**




**b.**

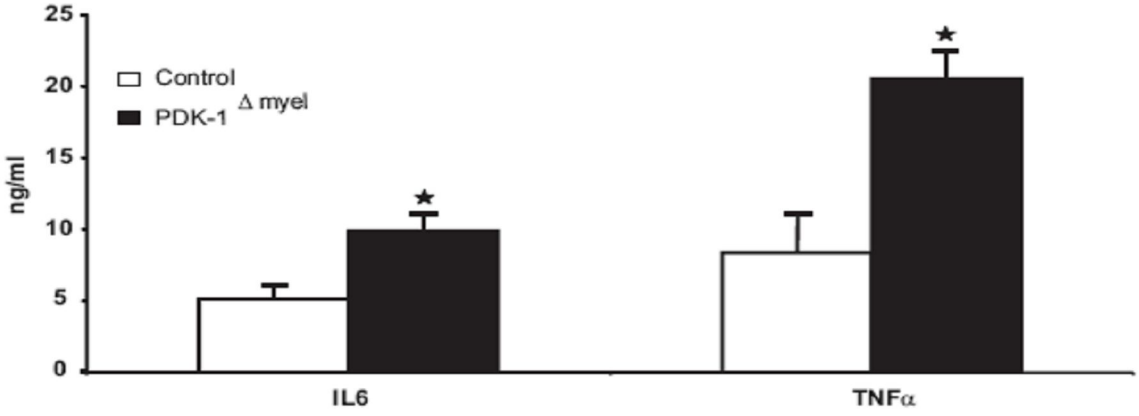



**Fig 7. Regulation of LPS induced inflammatory cytokines in PDK-1$^{\Delta myel}$ macrophages.**

(a) Increased expression of inflammatory cytokines in PDK-1$^{\Delta myel}$ primary macrophages stimulated with 1µg/ml of LPS for 24 hrs. Control (n=5), PDK-1$^{\Delta myel}$ (n=5). All data are presented as mean ± SEM and * p ≤ 0.05. (b) Elevated secretion of inflammatory cytokine IL6 and TNFα following 24 hrs LPS stimulation. Control (n=5), PDK-1$^{\Delta myel}$ (n=7), $p \leq 0.05$. All data are presented as mean ± SEM.

### 3.3.2 PDK-1$^{\Delta myel}$ mice exhibit increased Susceptibility to Septic Shock

In order to assess the systemic affect of PDK-1 deficiency in myeloid cells, the PDK-1$^{\Delta myel}$ and their littermate control mice were challenged with a sublethal dose of LPS. PDK-1$^{\Delta myel}$ mice show increased mortality (90%) compared to the control animals (10%) six days after LPS administration (Fig 8). Moreover following 24 hours of LPS challenge the PDK-1$^{\Delta myel}$ mice exhibit increased levels of TNF α in the blood serum (Fig.9).

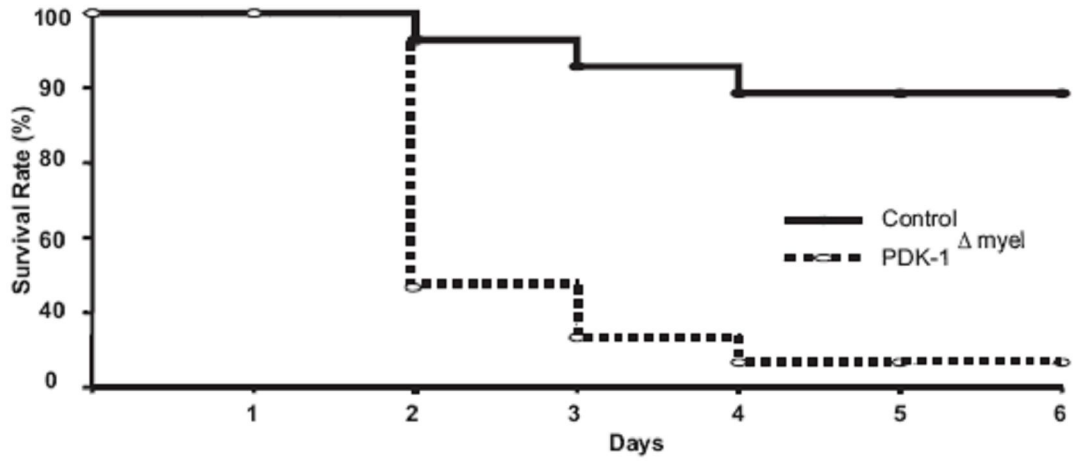



**Fig 8. Systemic Response to in *vivo* LPS injection**

Survival rate of control (Black line) (n=14) and PDK-1$^{\Delta myel}$ (dashed line) (n=14) mice following intraperitoneal injection of 50µg/gm body weight of LPS. All data are presented as mean ± SEM.

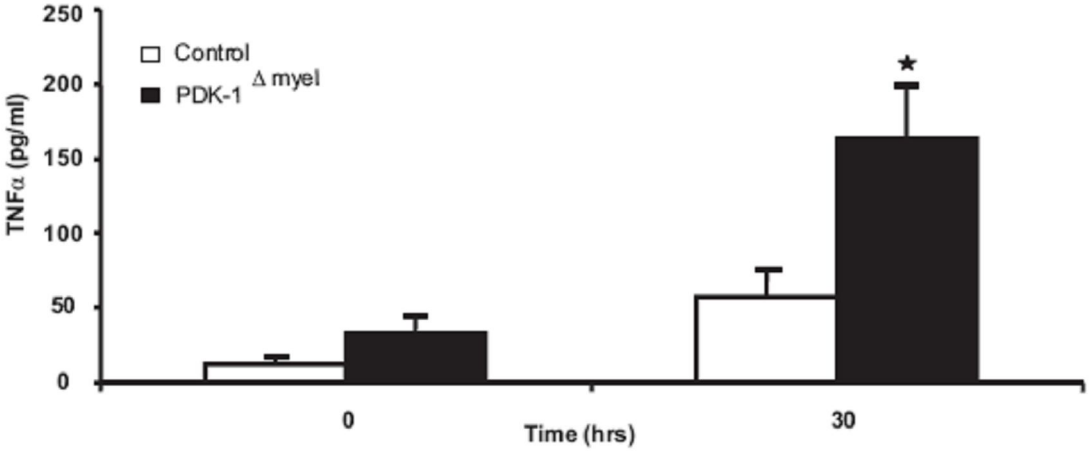


**Fig 9. Systemic Response to in *vivo* LPS injection**

Serum levels of TNFα Control and PDK-1$^{\Delta myel}$ mice following 30hrs of LPS injection with 50µg/gm body weight of LPS. Control (n=14), PDK-1$^{\Delta myel}$ (n=13), *p*= 0.044308. All data are presented as mean ± SEM and * p ≤ 0.05.

Although the inflammatory response is critical for host defense, enhanced level of proinflammatory cytokines can be harmful and even fatal (Beutler, Milsark et al. 1985; Danner, Elin et al. 1991). Thus excessive inflammation can lead to endotoxic shock (Ulevitch and Tobias 1995). Since the excessive inflammation during endotoxic shock is accompanied with multiple organ failure, we assessed the serum levels of AST following LPS challenge as a measure for acute liver damage. Although at basal level there is no difference in the serum levels of both AST, following 30 hours of LPS challenge PDK-1$^{\Delta myel}$ mice exhibited significantly increased level of serum AST (Fig 10).

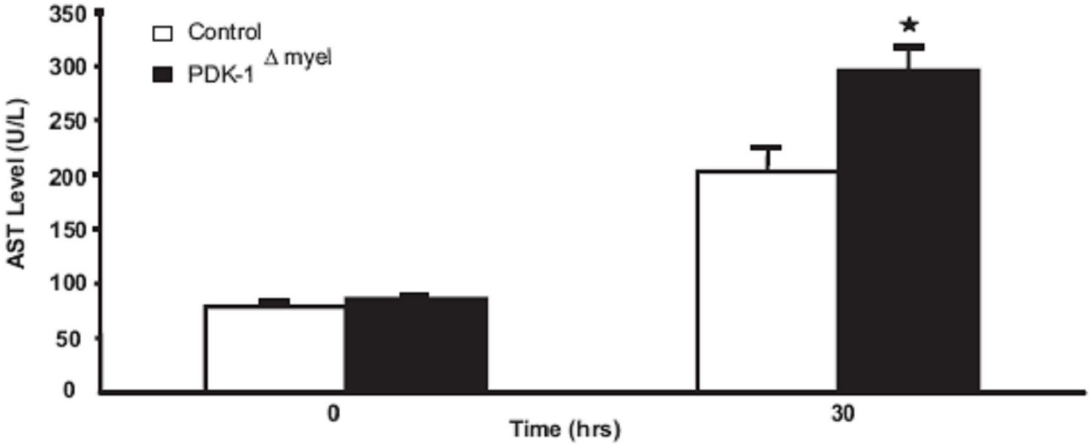



**Fig 10. Effect of peritoneal LPS injection on liver injury.**

Elevated levels of liver injury marker AST following 30 hrs of LPS. Control (n=15), PDK-1$^{\Delta myel}$ (n=13), *p*= 0.023225. All data are presented as mean ± SEM and * p ≤ 0.05.

The extent of liver injury was further analyzed by TUNEL assay to quantify the extent of apoptotic cells following 30 hours of LPS challenge. As expected, liver from PDK-1$^{\Delta myel}$ mice show significantly enhanced number of TUNEL positive cells (Fig 11). Thus collectively these data show that PDK-1$^{\Delta myel-}$ mice exhibit enhanced susceptibility to LPS induced septic shock

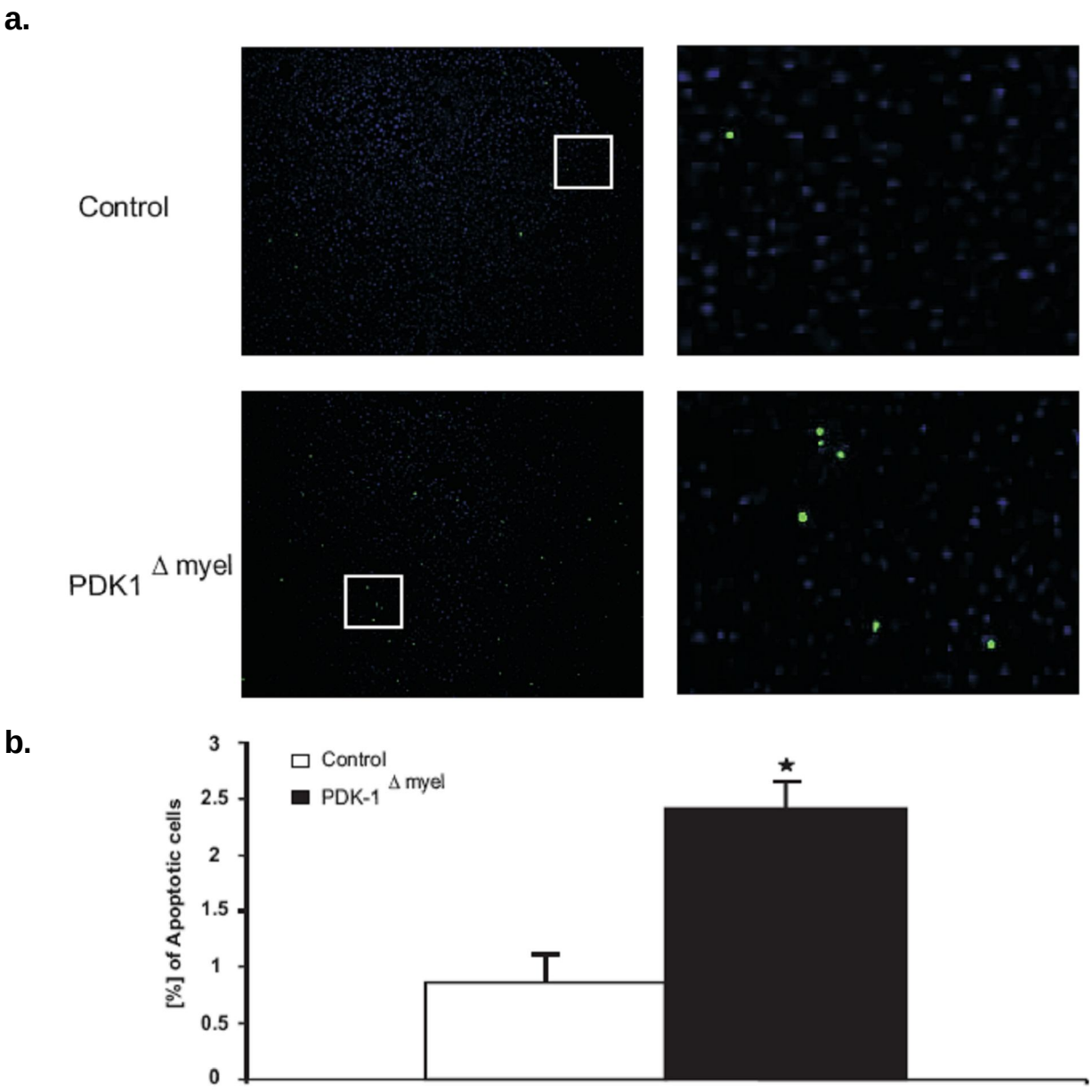



**Fig 11. Effect of peritoneal LPS injection on liver injury.**

(a) Liver sections from Control and PDK-1$^{\Delta myel}$ mice were examined by TUNEL Assay to measure apoptosis after 30 hrs of Peritoneal LPS injection. (b) Quantification of apoptosis from the TUNEL assay. Control (n=3), PDK-1$^{\Delta myel-}$ (n=3), *p*= 0.02809. All data are presented as mean ± SEM and * p ≤ 0.05.

### 3.3.3 Myeloid cell specific disruption of PDK-1 leads to inactivation of downstream PI3kinase signaling upon LPS stimulation

The stimulation of TLR4 signaling by LPS leads to activation of PI3 Kinase signaling, MAPK Kinase signaling cascade and a signaling cascade leading to the activation of NFκB (Han, Lee et al. 1994; Monick, Carter et al. 2001). PDK-1 phosphorylates AKT at Thr 308 enabling autophosphorylation of AKT at site Ser 473. This then lead to overall activation of AKT which then enhances the phosphorylation of its downstream target GSK3ß and thereby inhibiting its activity (Srivastava and Pandey 1998). Hence we looked at the overall activation of AKT and GSK3ß under LPS stimulation. As expected, PDK-1$^{\Delta myel}$ macrophages show reduced phosphorylation of AKT at Ser 473 and also reduced phosphorylation of its downstream target GSK3ß compared to control macrophages (Fig 12). These data suggest that deletion of PDK-1 in myeloid cells leads to complete inactivation of PDK-1 downstream targets in the presence of LPS.

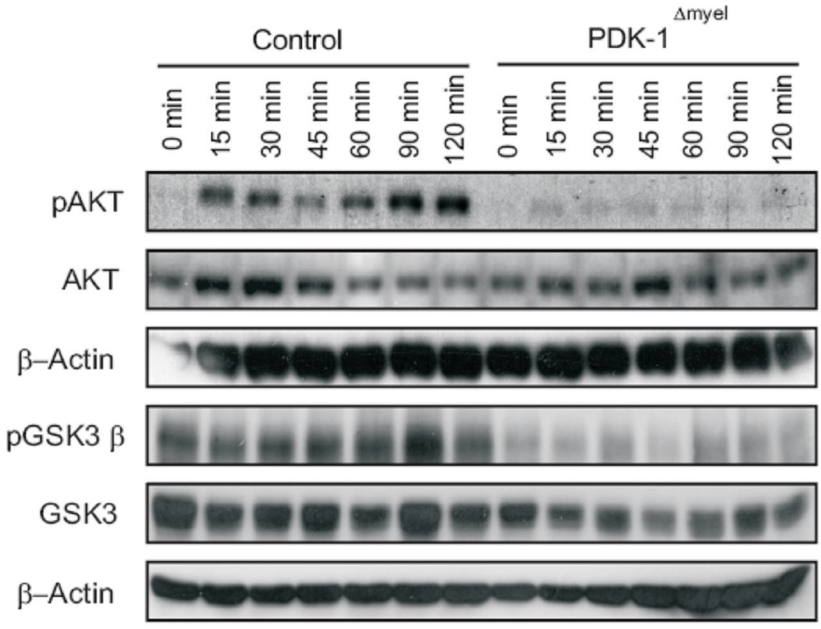



**Fig 12. Impaired PI3kinase mediated signaling in PDK-1 deficient myeloid cells.**

Macrophages were isolated and stimulated with 1µg/ml of LPS for various times as indicated and whole cell lysates were prepared. Levels of Phospho-AKT and Phospho-GSK3ß levels were measured by western blotting using anti-phospho-AKT (Ser473) and phospho- GSK3ß (Ser21, 9).

### 3.3.4 Myeloid restricted PDK-1 deficiency does not alter MAPK activity under LPS stimulation

The inflammatory response upon LPS administration is partially mediated by activation of components of the MAP kinase family namely: JNK, p38 and ERK1/2 (Liu, Herrera-Velit et al. 1994; Hambleton, Weinstein et al. 1996; Hall, Vos et al. 1999; Swantek, Christerson et al. 1999; Guha, O'Connell et al. 2001). We therefore examined whether myeloid specific absence of PDK-1 affects the activation of these three components of MAP kinase signaling. The peritoneal macrophages were isolated and stimulated with LPS and the activation status of JNK, p38 and ERK1/2 was assessed using their respective antibodies recognizing the phosphorylated or active forms. Surprisingly there was no difference in the activation of JNK, p38 and ERK1/2 between the control and PDK-1$^{\Delta myel}$ macrophages (Fig 13. a. b). This suggest that the activation of inflammatory response is independent of the traditional inflammatory regulator the MAPK kinase components.

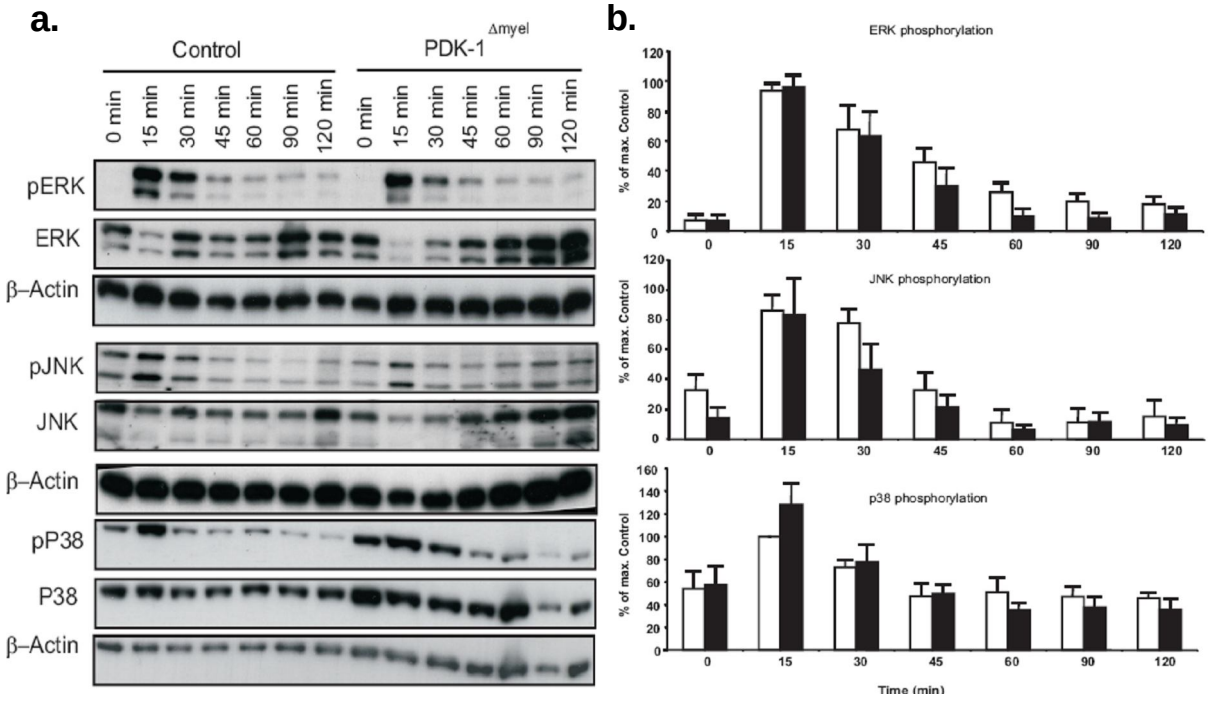



**Fig 13. Myeloid restricted PDK-1 deficiency does not alter MAPK activity under LPS stimulation.**

Macrophages were isolated and stimulated with 1µg/ml of LPS for the various times as indicated and whole cell lysates were prepared. (a) Western blot analysis of activation of MAPK pathway by western

blotting using Phospho-ERK, Phospho-SAPK/JNK and phospho-p38 antibodies. (b) Densitometrical quantification of the level of phosphorylation of individual protein band with respect to its unphosphorylated protein band. These data represent the mean ± sem. from four independent experiments.

### 3.3.5 Myeloid cells from PDK-1$^{\Delta myel}$ mice exhibit Increased NF-κB Activation

In unstimulated state NF-κB is sequestered in the cytoplasm by its inhibitor IκBα, whereas stimulation with proinflammatory cytokines causes phosphorylation of IκBα leading to its subsequent proteosomal degradation (Baeuerle and Baltimore 1988; Beg and Baldwin 1993; Finco, Beg et al. 1994). The degradation of IκBα leads to enhanced translocation of NF-κB to the nucleus where it binds to its consensus sequence, thereby driving the transcription of target genes. To analyse if the activation of NF-κB is somehow altered in myeloid cells from PDK-1$^{\Delta myel}$ mice, we performed an electromobility shift assay (EMSA). Stimulation of PDK-1$^{\Delta myel}$-macrophage showed enhanced translocation and binding of NF-κB to its consensus sequence within the nucleus (Fig 14 a). We further looked at the phosphorylation of IκBα over a time course of 2 hours after LPS stimulation. During the initial time course there was no difference in the phosphorylation of IκB α between the PDK-1$^{\Delta myel}$ macrophage and the control. Over the extended time course of stimulation with LPS, PDK-1$^{\Delta myel}$ macrophage exhibited enhanced phosphorylation and degradation of IκBα. Thus these data shows that although the PDK-1$^{\Delta myel}$-macrophages exhibit increased NF-κB activation upon LPS stimulation.

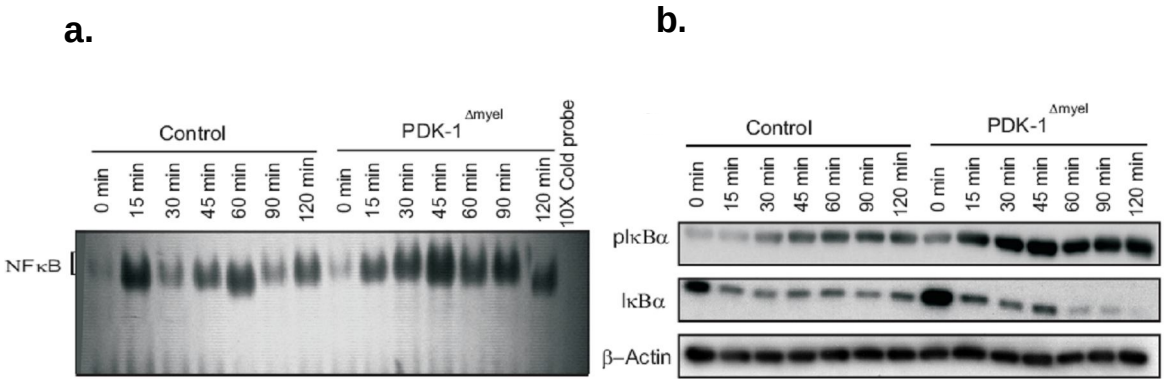



**Fig 14. LPS induced enhanced activation of NF-κB.**

PDK-1$^{\Delta myel}$ primary macrophages were stimulated with 1µg/ml of LPS for various times as indicated.

(a) PDK-1$^{\Delta myel}$ macrophages show enhanced LPS-induced DNA binding of NF-κB. Electrophoretic mobility shift assay was performed by incubating nuclear extracts with double-stranded radiolabeled oligonucleotide containing an NF-κB site. The autogadiogram is a representative of four independent experiments.(b) Phospho-IκBα and IκBα levels were measured by western blotting using anti-Phospho IκBα-Ser 32, 36 and anti-IκBα respectively.

### 3.3.6 Enhanced NF-κB Activation PDK-1$^{\Delta myel}$ is caused by increased activation of IKK complex

The critical regulator for NF-κB activation is the IKK complex. All signals leading to phosphorylation of IκBα are mediated by the IKK complex and more specifically by IKKß (Karin 1999). Thus we assessed the activation of IKK complex by NEMO dependent IKK kinase assay. And as expected the PDK-1$^{\Delta myel}$ macrophages exhibited enhanced activation of the IKK complex upon LPS stimulation. However of note was that unlike enhanced IκBα degradation during the extended time frame, the PDK-1$^{\Delta myel}$ macrophages exhibited enhanced activation of IKK complex only during the early time course (Fig 15).

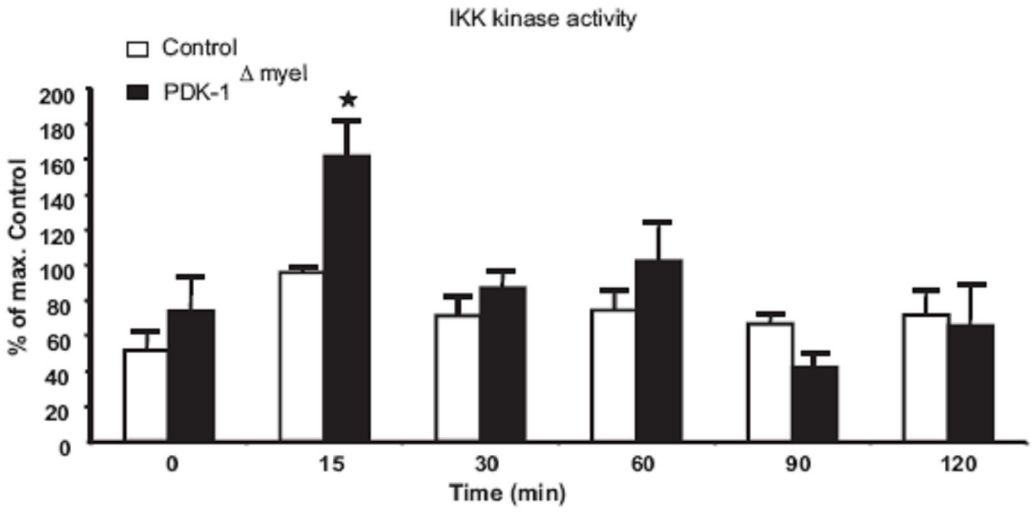



**Fig 15. PDK-1$^{\Delta myel}$ exhibit increased activation of IKK complex.**

NEMO dependent IKK kinase assay was performed with biotinylated IκBα (Ser 32). The result represents the mean ± sem from five independent experiments and * $p \leq 0.05$.

### 3.3.7 Myeloid cells from PDK-1$^{\Delta myel}$ mice exhibit unaltered activation of IRAK upon LPS stimulation

The stimulation of TLR4 ultimately leads to the activation of IKK complex. However, this occurs by different signaling molecules upstream of the IKK complex. The activation of TLR4 signaling leads to the phosphorylation and thereby activation of IRAK, which thereby transduces the signal downstream (Cao, Xiong et al. 1996; Muroi and Tanamoto 2008). Assuming that the difference in the activation of this adaptor molecule might be culminating into the activation of IKK complex seen under LPS stimulation, we looked into the activation status of IRAK under LPS stimulation in both the PDK-1$^{\Delta myel}$ macrophages and the control macrophages. Phosphorylation and levels of IRAK1 were not different upon stimulation with LPS among the PDK-1$^{\Delta myel}$ macrophages and control macrophages (Fig 16).

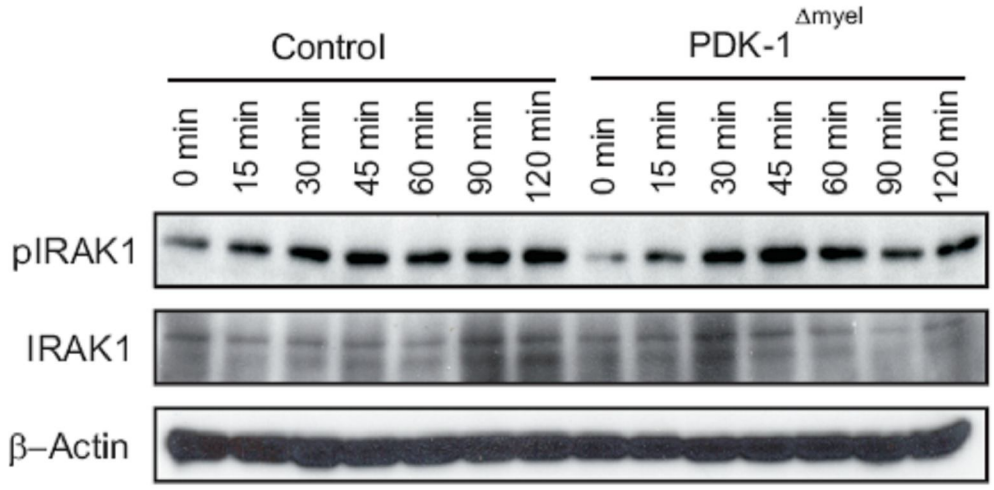



**Fig 16. Unaltered activation of IRAK1 upon LPS stimulation.**

PDK-1$^{\Delta myel}$ primary macrophages were stimulated with 1µg/ml of LPS for the various times as indicated. Phospho-IRAK1 and IRAK1 levels were measured by western blotting using anti-Phospho IRAK1 (Ser 376) and anti- IRAK1 respectively.

### 3.3.8 PDK-1 limits the ubiquitination of TRAF6

The activation of IRAK leads to recruitment of TRAF6 to IRAK where it is autoubiquitinated leading to its own activation. The activated TRAF6 in turn transduces the signal downstream and

hence leads to the activation of IKK complex. In order to investigate the ubiquitination of TRAF6, TRAF6 was immunoprecipitated from the whole cell lysate prepared from LPS stimulated macrophages and probed with ubiquitin antibody. The ubiquitination of TRAF 6 was specifically enhanced in PDK-1$^{\Delta myel}$ macrophages over an extended time frame under LPS stimulation. Hence suggesting that PDK-1 transduces the signal downstream of TLR4 receptor by limiting the ubiquitination of TRAF6 (Fig 17).

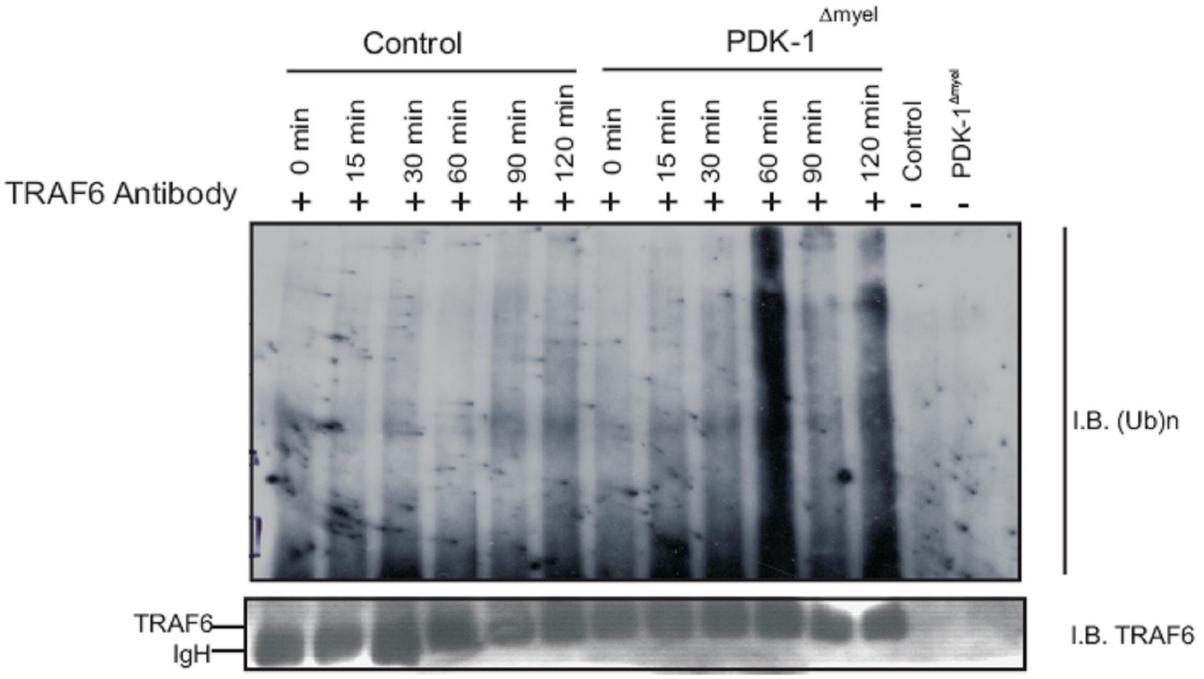



**Fig 17. Enhanced Ubiquitination of TRAF6 upon LPS stimulation.**

TRAF6 was immunoprecipitated from whole cell lysate using TRAF6 antibody. Ubiqutination of immunoprecipitated TRAF6 was then determined by ubiquitin antibody (Ub)n. The blot is a representative of four independent experiments.

## 3.4 Role of myeloid cell restricted PDK-1 deficiency diet induced obesity

### 3.4.1 PDK-1$^{\Delta myel}$ mice show steady increase in body weight upon High fat diet

In order to study the role of myeloid cell specific PDK-1 in diet induced obesity, control and PDK-1$^{\Delta myel}$ mice were fed either Normal chow diet (NCD) or High Fat diet (HFD) for a period of 12 weeks starting from 4 weeks of age. When fed NCD the control as well as PDK-1$^{\Delta myel}$ mice achieved a maximum weight gain of about 25 grams at the end of 16 weeks of age (Fig 18).

However under HFD the both the control and PDK-1$^{\Delta myel}$ mice showed steady increase in body weight which was significantly higher than their NCD counterparts. Although the mice showed steady increase in body weight under HFD reaching a maximum weight of about 40grams at the end of 16 weeks, there was no difference in the body weight between the control and PDK-1$^{\Delta myel}$ mice (Fig 18).

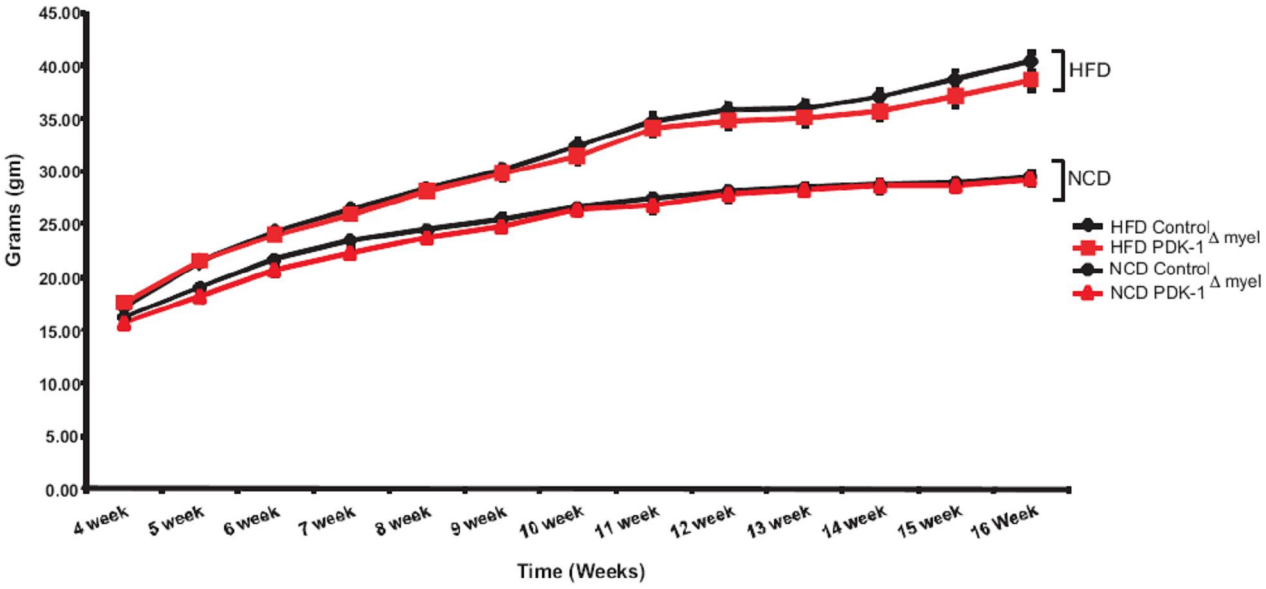



**Fig 18. Steady increase in body weight among control and PDK-1$^{\Delta myel}$ mice.**

Average body weight of Control and PDK-1$^{\Delta myel}$ mice when exposed to NCD and HFD. Body weight gain was measured from week 4 to week 16. The result represents the mean ± sem; NCD n= 17 Vs 15, HFD n= 22 Vs 24.

The increase in body weight under High fat diet results in increased adiposity, hence epididymal fat mass was measured in both the control and PDK-1$^{\Delta myel}$ mice both under NCD and HFD. As expected there was a significant increase in epididymal fat mass both in control and PDK-1$^{\Delta myel}$ mice under HFD compared NCD. However there was no difference in the epididymal fat mass content between control and PDK-1$^{\Delta myel}$ mice (Fig 19. a).

Circulating levels of leptin are an important indicator for an increase in obesity and is an important indicator for the degree of adiposity. Hence leptin concentration was measured in both the control and PDK-1$^{\Delta myel}$ mice fed either NCD or HFD (Fig 19.b). As expected leptin levels

were significantly increased in mice fed HFD compared to its counterparts on NCD. However there was no significant difference in the leptin levels between the control and PDK-1$^{\Delta myel}$ mice.

In summary these result suggest that myeloid cell specific PDK-1 deficiency does not affect the development of diet induced obesity.

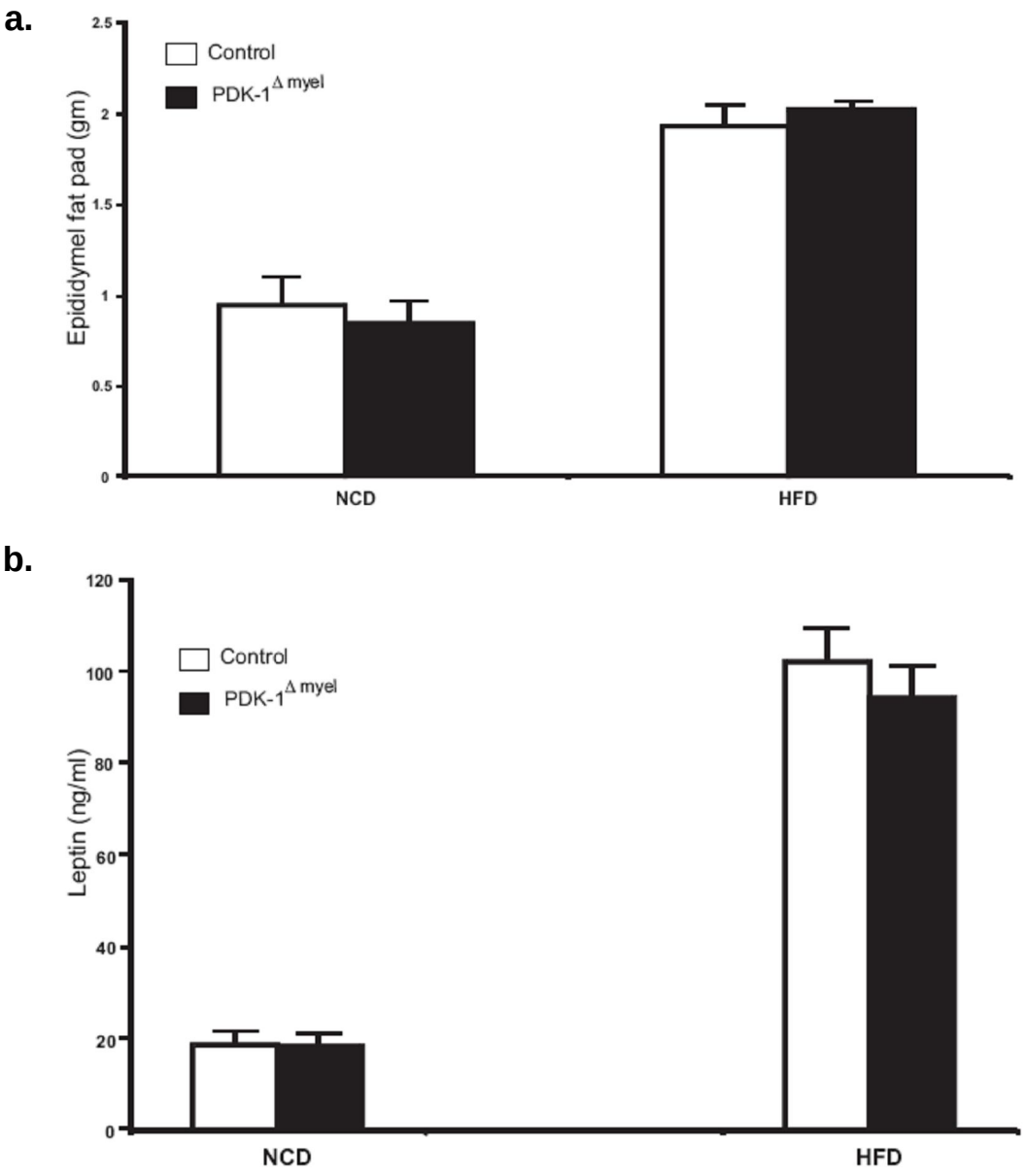



**Fig 19.Steady increase in epididymal fat mass and circulating levels of leptin in PDK-1$^{\Delta myel}$ mice.**

(a). Epididymal fat mass of control and PDK-1$^{\Delta myel}$ mice under NCD and HFD at the age of 16 weeks (NCD n=7 Vs 8, HFD n=6 Vs 6). (b). Serum leptin concentrations in control and PDK-1$^{\Delta myel}$ mice under

NCD and HFD at the age of 16 as determined by ELISA (NCD n=16 Vs 15, HFD 20 Vs 23). The result represents the mean ± sem.

### 3.4.2 PDK-1$^{\Delta myel}$ mice exhibit unaltered systemic inflammation under diet induced obesity

Obesity is characterized by chronic, low-grade tissue inflammation resulting from IKKβ/NF-κB and JNK pathways activation in adipocytes, hepatocytes, and associated macrophages. This low grade inflammation under obesity contributes to inhibition of insulin signaling in insulin target tissues, thereby leading to the development of insulin resistance. One of the characteristics of this low grade inflammation is increased infiltration of WAT with macrophages and increased levels of serum levels of inflammatory cytokines. In order to analyze the effect of HFD feeding on inflammatory cytokine, serum cytokine levels were measured in 16 weeks old mice (Fig 20 a, b). The serum levels of TNFα and MCP1 were increased, whereas that of adiponectin was decreased in control animals in response to HFD feeding. However PDK-1$^{\Delta myel}$ mice exhibited no difference in circulating levels of TNFα and MCP1 and adiponectin compared to its littermate controls under HFD feeding (Fig 20 a, b).

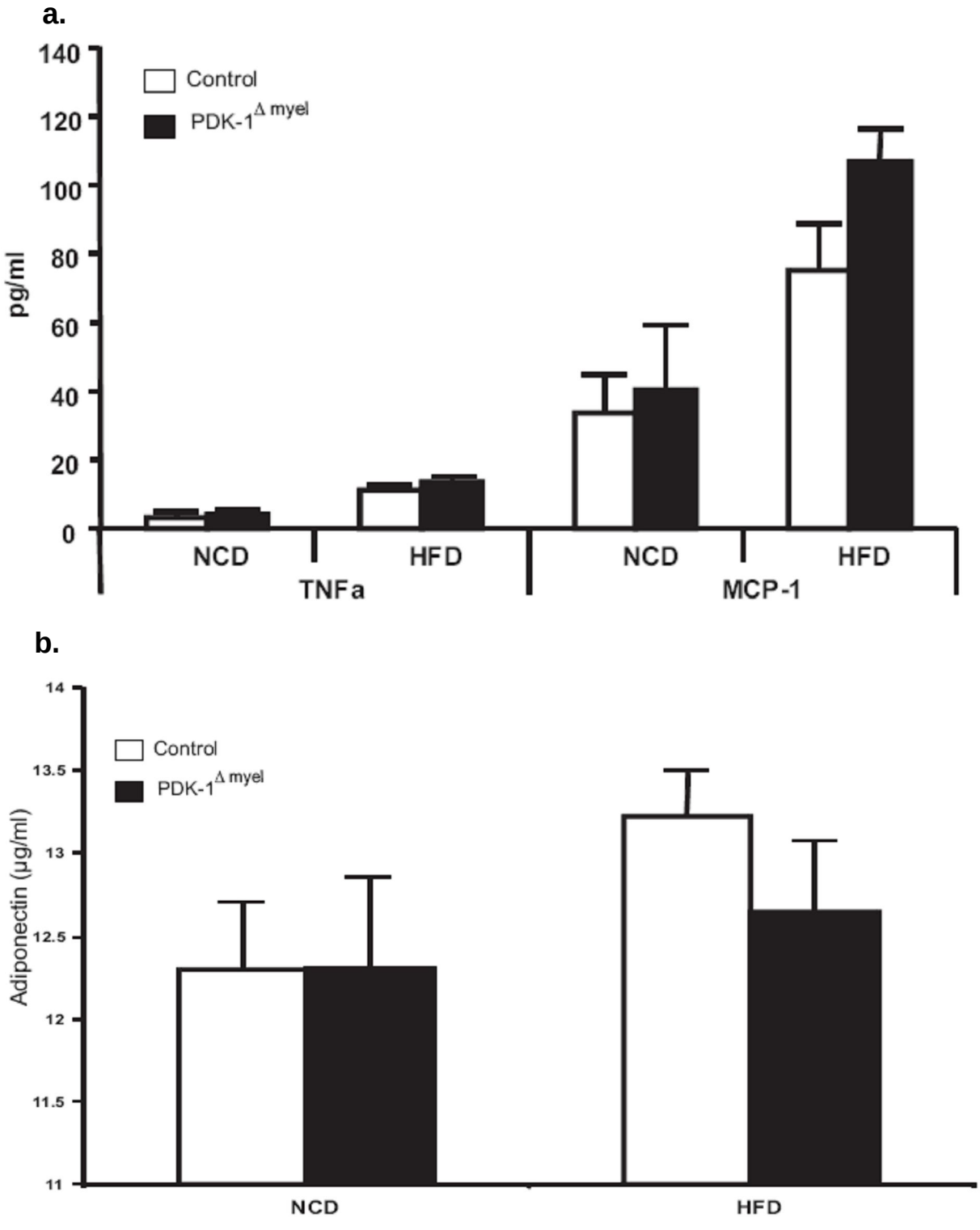



**Fig 20. Serum levels of inflammatory cytokines in response to HFD feeding.**

(a). Serum concentration of TNFα and MCP1 as determined by BD cytokine array® in control and PDK-$1^{\Delta myel}$ mice fed either NCD or HFD for 12 weeks. (b). Serum concentration of adiponectin as determined by ELISA in control and PDK-$1^{\Delta myel}$ mice fed either NCD or HFD for 12 weeks (NCD n=16 Vs 15, HFD n= 20 Vs 23). The result represents the mean ± sem.

White adipose tissue is believed to be the site of prime macrophage infiltration under obesity. Macrophages upon infiltration into the adipose tissue form crown-like structures (CLS) around dead adipocytes for removal of cell debris and tissue remodeling. Upon infiltration macrophages are activated and express a Mac2 a 30-35 kDa galactose-binding protein, a marker for active macrophages (Sato and Hughes 1994). Hence, to detect if there is enhanced infiltration and activation of macrophages, immunohistochemical analysis with an antibody directed against Mac-2 was performed on the white adipose tissue. This microscopical analysis revealed that PDK-$1^{\Delta myel}$ mice exhibit a modest increase in the infiltration of macrophages in the WAT as well as slight increase in their activation as analyzed by the quantification of CLS. However there was no significant difference between the PDK-$1^{\Delta myel}$ and it littermate control mice (Fig 21 a, b).

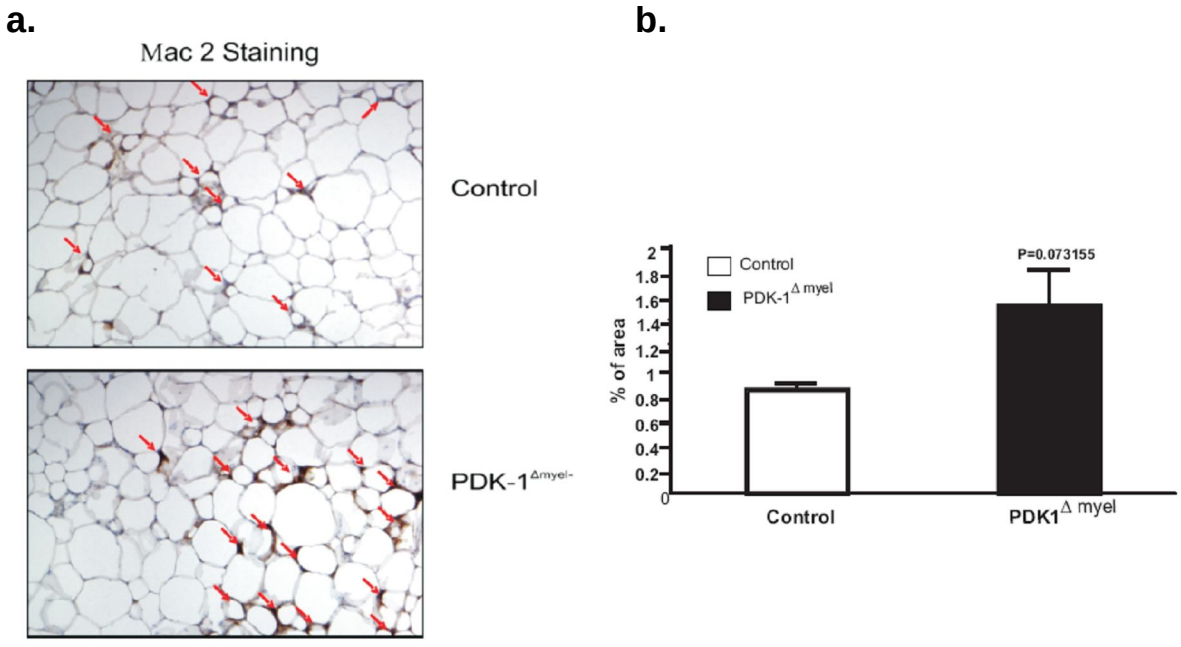



**Fig 21. Formation of crown like structures in WAT of obese mice.**

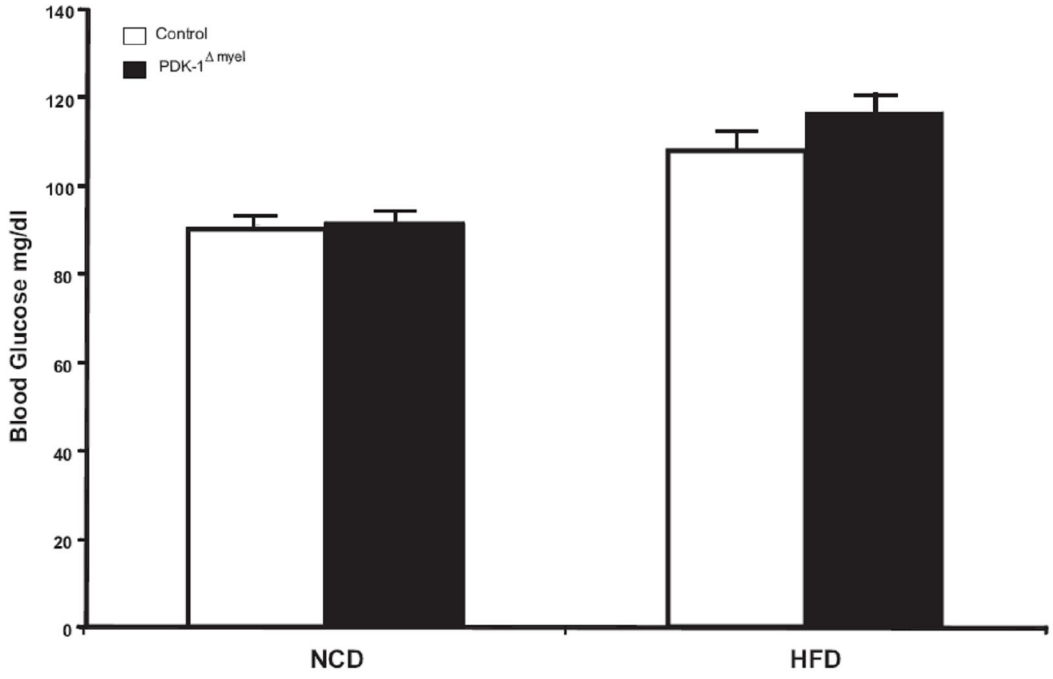
(a). Immunohistochemical staining of paraffin sections from WAT of Control and PDK-1$^{\Delta myel}$ mice with Mac2 antibody from 16 weeks old mice. (b). quantification of Mac2 positive crown like structure (CLS) area. Red arrow indicate the CLS. N=5 Vs 5, The result represents the mean ± sem.

Taken together these data indicate that although slightly more macrophages are recruited to the WAT, there is no systemic effect of myeloid cell specific PDK-1 deletion on the chronic inflammation induced by high fat feeding.

### 3.4.3 PDK-1$^{\Delta myel}$ mice exhibit impaired glucose homeostasis under diet induced obesity

Obesity results in insulin resistance which is characterized by enhanced circulating levels of glucose (hyperglycemia) and insulin (hyperinsulinemia) (Mittelman, Van Citters et al. 2002). To study the affect of myeloid cell specific PDK-1 on obesity induced insulin resistance, glucose metabolism of PDK-1$^{\Delta myel}$ mice was analyzed. Therefore we first measured the blood glucose concentration in 16 weeks old mice under random fed and fasting conditions both under NCD and HFD (Fig 22 a, b). These experiments revealed that PDK-1$^{\Delta myel}$ mice exhibit no alterations in blood glucose concentrations compared to control.

**a.**



**b.**

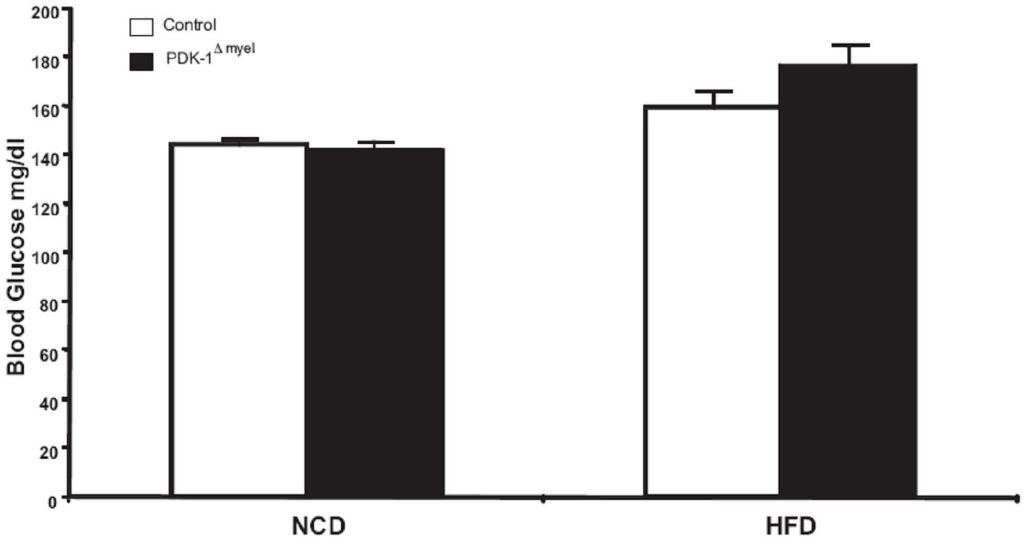



**Fig 22. PDK-1$^{\Delta myel}$ mice exhibit unaltered glucose levels in fasted and random fed state.**

(a). Fasted blood gluose concentrations of 16 weeks old control and PDK-1$^{\Delta myel}$ mice (NCD n=16 Vs 15n). (b).Random fed gluose concentrations of 16 weeks old control and PDK-1$^{\Delta myel}$ mice (HFD n= 20 Vs 23). The result represents the mean ± sem.

In consistency to the blood glucose levels, random fed and fasted insulin levels were also unaltered between the control and PDK-1$^{\Delta myel}$ mice fed either NCD or HFD (Fig 23 a, b).

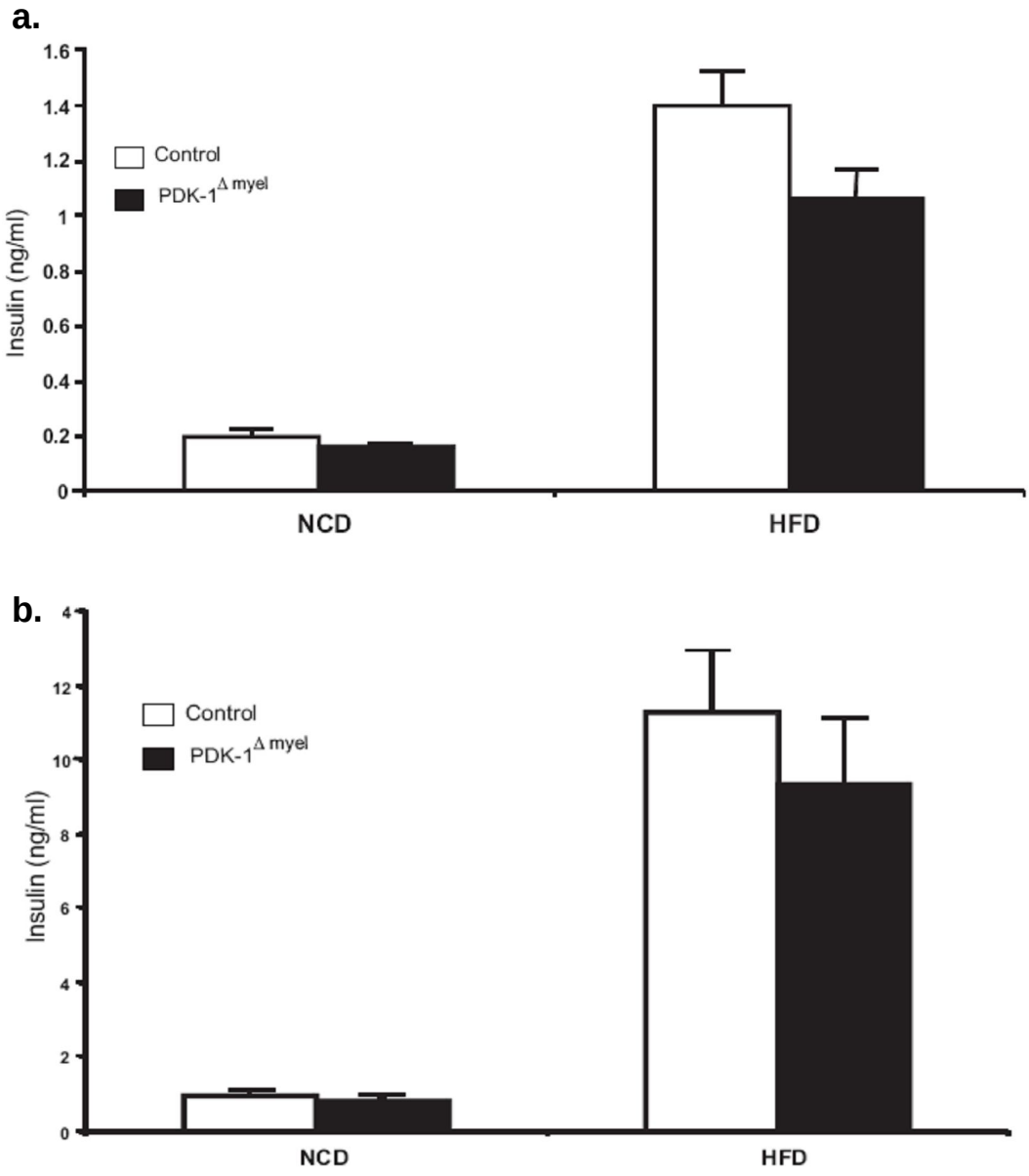


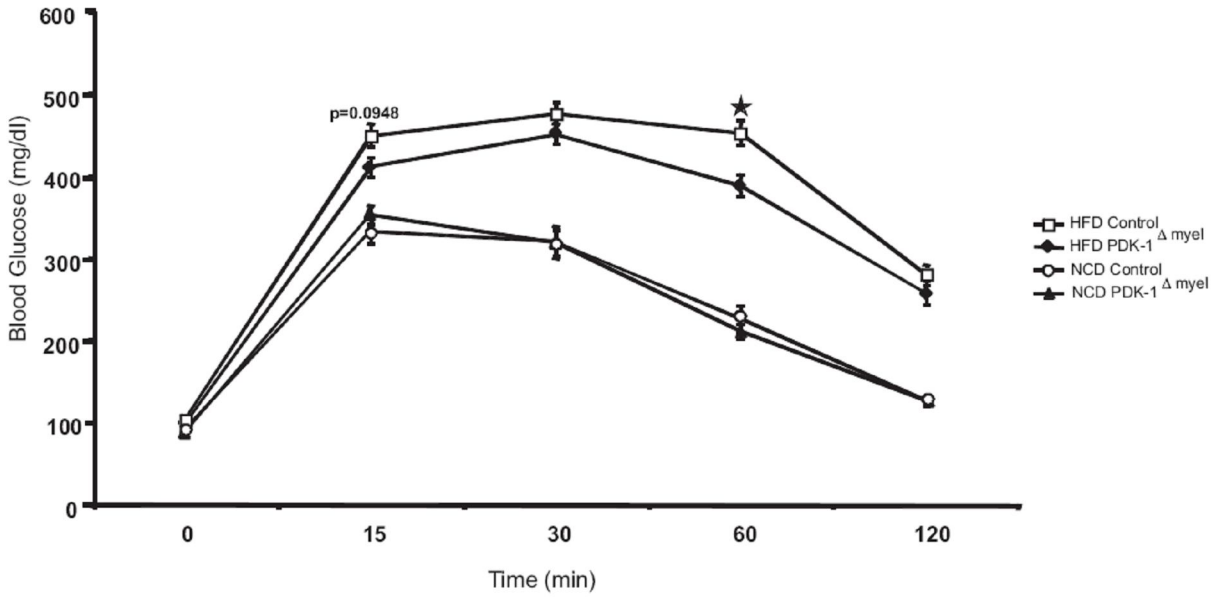

**Fig 23. PDK-1$^{\Delta myel}$ mice exhibit unaltered serum insulin levels in fasted and random fed state.**

(a). Fasted blood insulin concentrations of 16 weeks old control and PDK-1$^{\Delta myel}$ mice as determined by ELISA (NCD n=16 Vs 15n). (b).Random fed insulin concentrations of 16 weeks old control and PDK-1$^{\Delta myel}$ mice as determined by ELISA (HFD n= 20 Vs 23). The result represents the mean ± sem.

Moreover, glucose homeostasis in NCD and HFD fed control and PDK-1$^{\Delta myel}$ mice was further investigated by glucose tolerance test (GTT) and Insulin tolerance test (ITT) at the age of 12 and

14 weeks respectively. There was no difference in the rate of glucose clearance in control and PDK-1$^{\Delta myel}$ mice after intraperitoneal glucose injection under NCD. However upon HFD feeding PDK-1$^{\Delta myel}$ mice exhibited modest impairment in glucose tolerance compared to control on HFD (Fig 24 a), indicating that diet induced obesity alters insulin sensitivity in PDK-1$^{\Delta myel}$ mice. However when challenged with intraperitoneal injection of insulin under ITT no difference was observed in insulin sensitivity of control and PDK-1$^{\Delta myel}$ mice either under NCD or HFD (Fig 24 b).

**a.**



**b.**

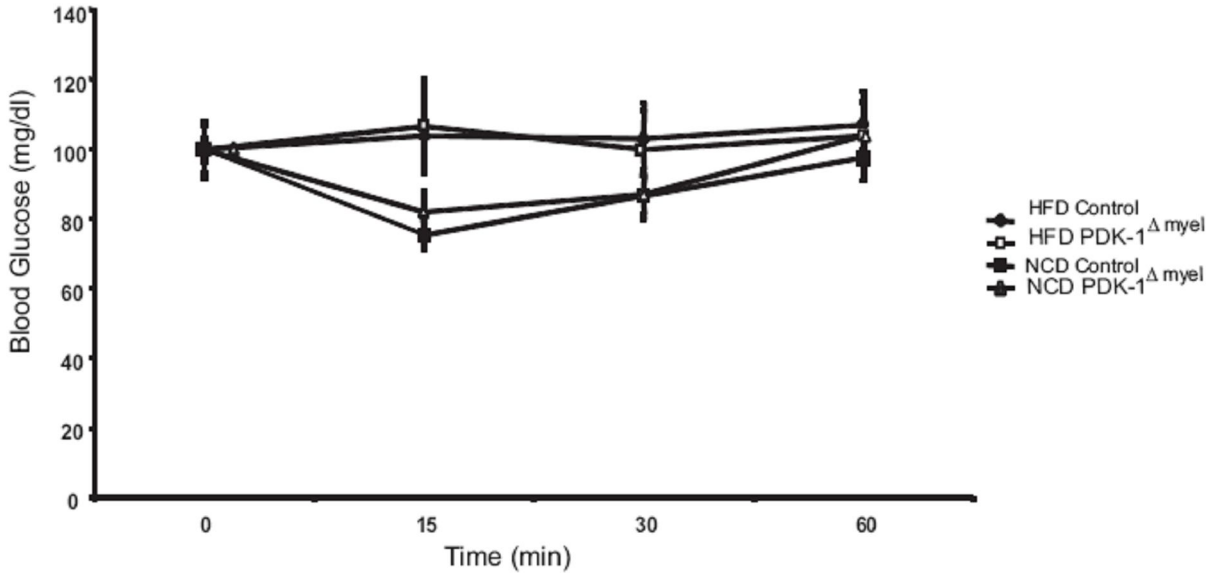



**Fig 24. Unaltered insulin sensitivity and impaired glucose tolerance in PDK-1$^{\Delta myel}$ mice.**

(a). Glucose Tolerance Test performed on control and PDK-1$^{\Delta myel}$ mice at the age of 12 weeks (NCD n= 16 Vs 15n, HFD n=38 Vs 36). (b). Insulin Tolerance test performed on control and PDK-1$^{\Delta myel}$ mice at the age of 14 weeks (NCD n= 16 Vs 15n, HFD n=38 Vs 36). The result represents the mean ± sem.

Since there was a modest impairment of glucose metabolism under HFD we employed euglycemic-hyperinsulinemic clamps to understand if the systemic effect of insulin is ablated and if so which organs are affected by the decreased glucose clearance from the blood stream. During the euglycemic-hyperinsulinemic clamps on animals fed HFD the glucose infusion rate (GIR) required to maintain the glucose homeostasis under the influence of increasing concentration of insulin in PDK-1$^{\Delta myel}$ mice was significantly lower under the steady state compared to its littermate controls (Fig 25. a). However there was no difference observed in the basal hepatic glucose production (HGP) and insulin mediated suppression of hepatic glucose production in control and PDK-1$^{\Delta myel}$ mice (Fig 25. b).

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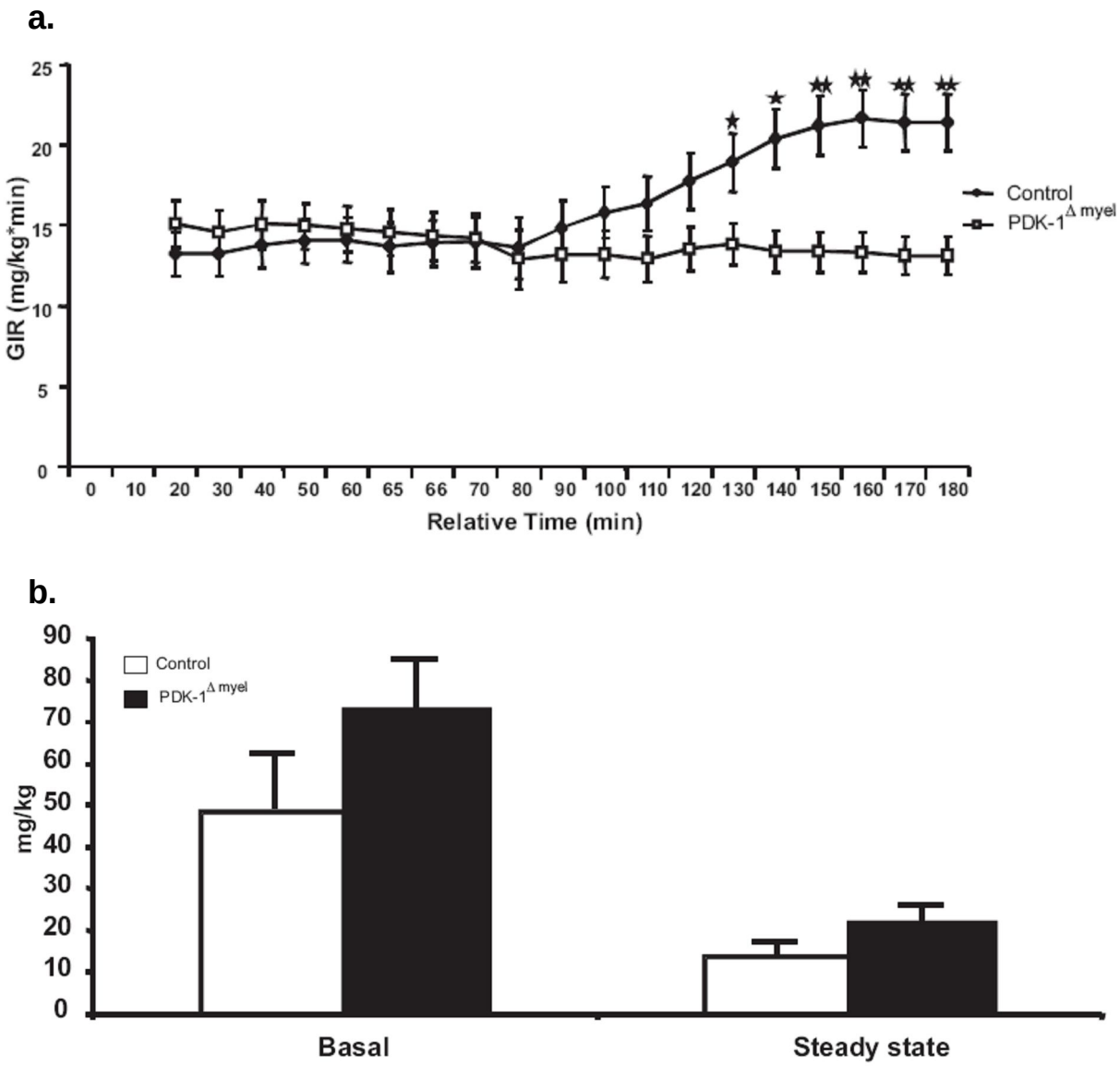


**Fig 25. PDK-1$^{\Delta myel}$ mice exhibit impaired glucose homeostasis under High fat diet.**

(a). glucose infusion rate of 16-18 weeks old control and PDK-1$^{\Delta myel}$ mice during euglycemic-hyperinsulinemic clamps analysis. (b). Hepatic glucose production in 16-18 weeks old control and PDK-1$^{\Delta myel}$ mice during euglycemic-hyperinsulinemic clamps analysis. n= 15 vs 12. The result represents the mean $\pm$ sem *$p \leq 0.05$).

In summary these result suggest that PDK-1$^{\Delta myel}$ mice exhibit impaired glucose homeostasis which is reflected by the enhanced glucose intolerance under GTT and reduced glucose infusion rate essential for maintaining glucose homeostasis under clamps.

# 4 Discussion

Increasing evidence has established correlative and causative links between chronic inflammation and development of insulin resistance. However the way inflammation contributes to the development of insulin resistance is just beginning to emerge. In past decade it has been shown that under conditions of obesity there is increased infiltration of monocytes and macrophages in to the white adipose tissue, which in turn release inflammatory cytokines, which interferes with insulin action in classical insulin target tissues. Although the relevance of the key inflammatory signaling kinases JNK and IKK in myeloid cells have been demonstrated to induce insulin resistance (Hirosumi, Tuncman et al. 2002; Arkan, Hevener et al. 2005; Solinas, Vilcu et al. 2007), the role of myeloid cell specific insulin signaling components in inflammation and its relative contribution to systemic insulin action is yet to be understood.

Since the recent findings support the role of IR signaling in macrophages, and its importance in mediating inflammation (unpublished observation). Most of the signaling mediated by insulin converges at PDK-1, which then phosphorylates and activates downstream kinase including AKT. To mimic insulin resistance in macrophages at the level of this important signaling node, we generated myeloid cell specific PDK-1 deficent mice to study its role in inflammation and obesity induced insulin resistance.

## 4.1 Myeloid cell specific PDK-1 does not affect development and differentiation of myeloid cells

In order to attain myeloid cell specific disruption of PDK-1, mice expressing the Cre-recombinase under control of the lysozymeM (lysM Cre $^{+/-}$) promoter which expresses Cre-recombinase specifically in myeloid cells were crossed with mice bearing PDK-1 floxed allele (PDK-1$^{fl\Delta\ neo/fl\Delta\ neo}$) (Mora, Davies et al. 2003; Hashimoto, Kido et al. 2006). Figure 4 and 5 show the specificity and efficiency of myeloid cell specific PDK-1 disruption achieved using this strategy. Mice lacking PDK-1 die *in utero* at embryonic day 9.5 (Williams, Arthur et al. 2000). Moreover mouse lacking PDK-1 in pancreatic β cells were recently shown to have reduced numbers of beta cells as a result of enhanced apoptosis (Hashimoto, Kido et al. 2006). In line

with these observations PDK-1 was found to inhibit cell proliferation but did not promote apoptosis in mouse embryonic fibroblasts (Nakamura, Sakaue et al. 2008). However, in contrast mouse embryonic stem cells in which both copies of PDK-1 were disrupted were viable and proliferated at a rate similar to that of control cells hence suggesting the importance of PDK-1 in regulating development and differentiation of MEF (Williams, Arthur et al. 2000). Moreover it's been debated if the PDK-1 exhibit these effect as a result of reduced cell numbers or cell size. The reason for this argument comes from the fact that mice expressing only 10% of the PDK-1 in whole body exhibit unaltered cell numbers or proliferation but reduced cell volume by about 30-60%. This reduced cell size is reported to occur because of the lack of plekistrin homology (PH) domain of PDK-1 (Hashimoto, Kido et al. 2006; Bayascas, Wullschleger et al. 2008). Hence it might be that either although deletion of PDK-1 in myeloid cells does not affect the development and differentiation of myeloid cells as shown in figure 6, there might still be a possibility of reduced myeloid cell volume. However it might also be that the observed phenotype of unchanged cell numbers in myeloid cells could be because of the cell type specific function of PDK-1 in regulating development and differentiation.

## 4.2 Role of myeloid cell specific PDK-1 in inflammation

The activation of innate immune system is important for an efficient first line of defense. The activation of TLR4 in regulating innate immune response and thereby stimulating an efficient adaptive immune response is well established (Medzhitov 2001; Takeda and Akira 2003). Although, tight regulation of TLR4 is essential for an effective immune response, excessive and prolonged activation of TLR4 leads to endotoxin shock. Large numbers of pathologies to this date have been associated with disregulated immune response (Van Heel, McGovern et al. 2001). Hence, the regulation of TLR4 is checked by a number of mechanisms, largely unknown, to limit its hyperactivation. IKK α, IRAK M, SOCS-1, TRIM30 α, CYLD and PI3 kinase signaling cascade are some of the known negative regulators of TLR activation (Fukao, Tanabe et al. 2002; Kinjyo, Hanada et al. 2002; Kobayashi, Hernandez et al. 2002; Trompouki, Hatzivassiliou et al. 2003; Shi, Deng et al. 2008).

The negative regulation of TLR4 signaling by PI3K signaling cascade in innate immune cells has been well documented (Fukao and Koyasu 2003). In previous studies using inhibitors for PI3K inhibitors wortaminn and LY294002 it was shown that upon LPS there is enhanced inflammatory response in both monocytes and dendritic cells (Kim, Khursigara et al. 2001; Guha and Mackman 2002). In follow up studies Fukao and his collegue could show that p85α knockout mice exhibit impaired enterobacteria clearance upon its injection into the peritoneal cavity. Taken together these suggested the importance of PI3K signaling cascade in limiting inflammatory response and thereby is crucial for maintaining the integrity of the immune system (Fukao, Yamada et al. 2002). Apart from p85α, the role of individual components of PI3K signaling in regulation of inflammatory cytokine has not been widely studied due to the limitation of multiple isoforms of the majority of the components within this cascade. However, PDK-1 is thought to be encoded by a single gene and no isoforms of this protein has been detected till now. Hence in order to elucidate the role of PDK-1 in regulation of TLR activation we generated PDK-1 deficient myeloid cell mice.

We show here that macrophages from PDK-1$^{\Delta myel}$ mice show enhanced expression and secretion of proinflammatory cytokines when challenged with LPS a TLR4 agonist (Fig 7 a, b). Moreover PDK-1$^{\Delta myel}$ mice are more prone to LPS induced septic shock (Fig 8-11). This aggravated response of PDK-1$^{\Delta myel-}$ mice is likely to result from enhanced activation of either TLR signaling or MAPK kinase cascade. In previous studies it's been shown that AKT, one of the downstream substrate of PDK-1, blocks the activation of p38 by two mechanisms. Firstly, by phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) which thereon inhibit the activation of MKK3 or 6, the upstream regulators of p38 (Kim, Khursigara et al. 2001). Secondly, AKT blocks the activity of another kinase MEKK3, kinase upstream of p38 which thereon leads to inhibition of p38 activity (Gratton, Morales-Ruiz et al. 2001). It has also been described that the PI3K-Akt pathway also negatively regulates JNK and ERK activity (Guha and Mackman 2002; Park, Kim et al. 2002). However we show here that LPS stimulation of peritoneal macrophages isolated from control and PDK-1$^{\Delta myel}$ mice did not alter the activation of MAPK Kinase signaling involving p38, JNK and ERK (Fig 13). Thus the negative regulation of inflammatory cytokine by PDK-1 in macrophages is independent of the MAPK kinase cascade activation. This is contrary to previously reported observations in human monocyte cell line using pharmacological

inhibition of PI3kinase pathway (Guha and Mackman 2002). The probable explanation for this difference in the regulation of MAPK kinase cascade between immortalized cell line and primary cells could be that most of the immortalized cells carry a modulation of PI3 kinase signaling largely involving the lack of PTEN (negative regulator of PI3K) and hence show enhanced AKT activity.

Activation of TLR4 signaling cascade can also result in the enhanced expression and secretion of inflammatory cytokines. The activated TLR4 signaling leads to activation of ''canonical'' and ''non-canonical'' pathways leading to the activation of NFκB. Although component of non-canonical pathway largely mediated by IKKα do exist in macrophages it is largely activated by growth stimulus such as lymphotoxin and is implicated in growth and development of the cell. Although in some recent reports, it's been shown that macrophage specific IKKα negatively regulate NFκB, but the mechanism of its negative regulation is still a matter of debate (Lawrence, Bebien et al. 2005; Li, Lu et al. 2005). However it is the classical or canonical pathway which largely mediates inflammatory response in macrophage and consists of IKKβ. Upon activation of IKKβ, it causes enhanced activation of NFκB by promoting the degradation of IκBα (inhibitor for NFκB). Here we show that upon LPS stimulation of peritoneal macrophages isolated from control and PDK-$1^{\Delta myel}$ mice, there is enhanced recruitment and binding of NFκB to its consensus sequence and that this is preceded by the enhanced degradation of IκBα (Fig 14 a, b). This enhanced degradation of IκBα is accounted for by increased activation of IKK complex involving IKKβ upon LPS stimulation. Although it has been shown that PDK-1 is essential for NFκB activation in overexpression studies (Tanaka, Fujita et al. 2005). And that PDK-1 can moderately activate NFκB through IKKß under non stimulating conditions, whereas under TNF alpha stimulation this activation is mediated by IKKα (Hu, Lee et al. 2004). These studies overall indeed supports the notion of a crosstalk between PI3Kinase pathway and IKK/ NFκB pathway. However, results presented here suggest that PDK-1 limits the activation of IKK complex.

The induction of inflammatory cytokines has also been shown to be regulated by two downstream kinases of AKT namely GSK3β and mTOR via two independent mechanisms. Firstly, using inhibitors for GSK3β it was shown that GSK3β differentially regulate the expression of pro and anti-inflammatory cytokines. GSK3β regulated this inflammatory response

by differentially affecting the nuclear amounts of transcription factors NF-κB subunit p65 and CREB interacting with the coactivator CBP (Martin, Rehani et al. 2005). Secondly, using rapamycin an inhibitor for mTOR it was shown that mTOR negatively regulate IL12 production in dendretic cells (DC) after LPS stimulation, however the mechanism for this regulation is still to be determined but is independent of GSK3 β (Ohtani, Nagai et al. 2008). In the current study we observed an enhanced activation of IKK complex and its downstream target IκBα upon disruption of PDK-1 in myeloid cells, implicating that it is very unlikely that this effect is mediated by either GSK3β or mTOR. On the other hand, it cannot be ruled out that the absence of GSK3β activation in the PDK-1 deficent myeloid cells might be synergistically aggravates the inflammatory response along with the direct effect of PDK-1 on the canonical NFκB activation pathway.

How PDK-1 does regulate the activation of IKK complex? We show here that PDK-1 exerts its negative regulatory effect on IKK complex activation by specifically limiting the activation of TRAF6 and not that of IRAK1 (Fig 16, 17). This activation of TRAF6 could possibly be mediated either directly by PDK-1 or any downstream targets of PDK-1such as AKT, PKC, SGK, GSK3β and p70S6K. Since PDK-1 and all the downstream targets of PDK-1 are kinases it very tempting to speculate the existence of a probable phosphorylation sites on TRAF6 which might be regulated by previously mentioned kinases. And that these kinase might limit the activation of TRAF6 by modulating the phosphorylation of TRAF6. However it is also likely that since ubiquitination of TRAF6 regulated by deubiquitinting enzymes CYLD (unpulished observation) and A20, it might be that PDK-1 or downstream kinases modulate the activity of these two deubiquitinating enzymes. It is also worth a mention that although A20 has been implicated in removing K63 linked ubiquitin chain in TRAF6, they are largely involved in K48 linked polyubiquitination and thereby mediating the degradation of protein (Boone, Turer et al. 2004; Wertz, O'Rourke et al. 2004). Since there is no observed difference in the level of TRAF6 following LPS stimulation (Fig 17), it's very unlikely if the effect seen here is mediated by A20. CYLD is a second deuiquitinating enzyme which has recently been shown to limit the ubiquitination of TRAF6 (Zhang, Stirling et al. 2006). Moreover using genetic deletion of CYLD in the whole body it was shown that macrophage isolated from these mice exhibit enhanced activation of NF-κB upon LPS stimulation. The data from CYLD study suggest that CYLD

regulate NF-κB and that loss of CYLD causes enhanced inflammation (Zhang, Stirling et al. 2006). However future investigation will be directed to study the effect of PDK-1 on CYLD and TRAF6, thereby possibly allowing us to understand the regulation of TRAF 6 by PDK-1 and hence unraveling their functional interaction.

However in summary we have identified a key negative regulatory function of PDK-1 for TLR4 signaling in myeloid cells. Moreover we show here that, PDK-1 activation is essential for endotoxin shock and serves as a key regulatory feedback unit of TLR4 signaling. PDK-1 therefore is essential for maintaing immune homeostasis.

## 4.3 Role of myeloid cell specific PDK-1 in obesity induced insulin resistance

### 4.3.1 Role of myeloid cell specific PDK-1 in obesity

High fat diet (HFD) induced is a common model for studying obesity induced insulin resistance. In this study in order to investigate the role of PDK-$1^{\Delta myel}$ mice in obesity induced insulin resistance, both control and PDK-$1^{\Delta myel}$ mice were put on HFD for 12 weeks and their weight gain monitored over the 12 weeks time. Although both the control and PDK-$1^{\Delta myel}$ mice significantly gained weight over the period of time monitored, there was no significant difference observed in weight gain, adiposity and circulating leptin levels between the control and PDK-$1^{\Delta myel}$ mice. This is in line with previous observations wherein no difference was observed either in body weight, adiposity and circulating leptin levels with myeloid specific disruption of kinases IKK and JNK known to regulate inflammation (Arkan, Hevener et al. 2005; Solinas, Vilcu et al. 2007). Taken together these observations suggest that myeloid cell specific disruption of PDK-1 does not affect the development of obesity.

### 4.3.2 Role of myeloid cell specific PDK-1 in obesity induced inflammation

Exposure to HFD stimulates the infiltration of macrophages to the WAT where these cells are activated and results in induction of inflammatory cytokines and chemokines ultimately leading to a chronic inflammatory state. An enhanced circulating level of TNFα is a characteristic marker for this chronic inflammatory state (Hotamisligil, Shargill et al. 1993; Hotamisligil, Arner et al. 1995). Although adipocytes do contribute considerably to this enhanced level of TNFα,

macrophages are believed to be the key source of this cytokine especially under obesity. This enhanced level of TNFα inhibits systemic insulin action by modulating serine phosphorylation in IRS molecule (Gao, Hwang et al. 2002; Zick 2005). Although the peritoneal macrophages isolated from PDK-1$^{\Delta myel}$ mice *exvivo* exhibited enhanced induction of inflammatory cytokines under LPS stimulation, there was no difference was observed in the circulating levels of TNFα between the control and PDK-1$^{\Delta myel}$ mice under HFD.

Second characteristic of HFD induced obesity is the enhanced infiltration of macrophages to adipose tissue wherein they are activated (Sartipy and Loskutoff 2003; Xu, Barnes et al. 2003). These activated macrophages apart from secreting TNFα also induce the secretion of chemokines such as MCP-1 which in turn facilitate the increased recruitment of macrophages to the adipose tissue (Xu, Barnes et al. 2003). Although there was no difference observed in the circulating levels of MCP-1 in PDK-1$^{\Delta myel}$ mice compared to its control counterparts under HFD, there was relatively enhanced recruitment and activation of macrophages to the adipose tissue as indicated by Mac2 staining of the macrophages in the adipose tissue. However expression analysis of the key inflammatory markers and chemokines in WAT will enable to address if these active macrophages do in turn contribute to the local inflammation in adipose tissue in PDK-1$^{\Delta myel}$ mice under HFD. However it could still be possible that there is no observable difference in the local inflammation in the adipose tissue despite the enhanced recruitment and activation of macrophages because the maximum threshold of inflammation achieved in response HFD in both control and PDK-1$^{\Delta myel}$ mice.

However, taken together these data indicate that although there is relatively modest enhanced infiltration and activation of macrophages in PDK-1$^{\Delta myel}$ mice to the WAT compared to control, this enhanced activation does not translates into the enhanced systemic inflammation in PDK-1$^{\Delta myel}$ mice.

### 4.3.3 Role of myeloid cell specific PDK-1 in glucose homeostasis under diet induced obesity

Obesity not only results in enhanced inflammatory tone but is also characterized by the enhanced circulating levels of glucose and insulin (Mittelman, Van Citters et al. 2002). Although both control and PDK-1$^{\Delta myel}$ mice animals exhibited enhanced circulating levels of glucose and

insulin under HFD compared to NCD, there was no difference observed between the two genotypes. However in glucose tolerance test (GTT) under HFD, PDK-$1^{\Delta myel}$ mice exhibited impairment in glucose tolerance compared to control animals under HFD. Indicating that at least partially PDK-1 deletion in myeloid cells contributes to maintain glucose homeostasis. The impaired glucose homeostasis observed PDK-$1^{\Delta myel}$ mice was further confirmed by the more sensitive assay euglycemic-hyperinsulinemic clamp where in the amount of glucose needed to maintain the glucose homeostasis was significantly reduced in the PDK-$1^{\Delta myel}$ mice compared to control animals. The increasing concentration of insulin under clamps not only promote glucose uptake in classical target tissues, but also helps in clearing the circulating glucose by inhibiting the glucose production from the liver. Hence there is enhanced glucose production from the liver under the basal levels and this is reduced under increasing concentration of insulin and depending on the ability of insulin to inhibit the extent of glucose production one can characterize if the mouse is insulin resistant or sensitive. Although both the control and PDK-$1^{\Delta myel}$ mice respond to increasing concentration of insulin by inhibiting the glucose production there was no difference observed in hepatic glucose production between the control and PDK-$1^{\Delta myel}$ mice under HFD.

Taken together these results indicate that deletion of PDK-1 specifically in myeloid cells impairs glucose homeostasis under obesity, thereby underlining the crucial role of myeloid cell specific PDK-1 in maintaing glucose homeostasis.

## 4.4 Conclusion

Collectively the data presented in this study indicate that PDK-1 in macrophages serves as a negative regulator of inflammatory signaling and that disruption of PDK-1 in macrophages leads to exaggerated inflammation and impaired glucose homeostasis.

## 4.5 Outlook

Although the data presented here highlight the importance of myeloid cell specific PDK-1 in 1. negative regulation of inflammation and 2. maintaining glucose homeostasis, future studies should be directed to study how firstly, PDK-1 regulates the activation of TRAF6 for its negative regulation. Secondly, the importance of myeloid specific PDK-1 in regulating glucose

homeostasis should further be in direction to characterize which organs were affected by the reduced glucose infusion rate during clamps and also to identify the possible reason behind the observed impaired glucose homeostasis in PDK-1$^{\Delta myel}$ mice under HFD induced obesity.

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# 6 Summary

Type 2 diabetes mellitus is a major disease affecting approximately 6% of the western population. Type 2 diabetes mellitus results from insulin resistance in organs such as liver, skeletal muscle and brain. Obesity is one of the underlying causes for increased insulin resistance and is associated with the development of type 2 diabetes mellitus. Obesity is also associated with a systemic chronic inflammatory state reflected by increased levels of inflammatory cytokines. Since macrophages are the major source for their secretion, we planned to determine the role of macrophages in the development of insulin resistance. Phosphoinositide-dependent kinase (PDK-1) is an important component of PI3 kinase pathway activated by the insulin receptor. Upon activation, PDK-1 phosphorylates and activates PKB (AKT) and p70 ribosomal protein S6 kinase. To mimic insulin resistance in macrophages at the level of this important signaling node, we generated mice with specific disruption of PDK-1 in macrophages. Here we show that deletion of PDK-1 specifically in myeloid cells (PDK-1$^{\Delta myel}$) leads to increased expression and secretion of TLR4 induced inflammatory cytokines under Lipopolysaccharide (LPS) stimulation. In addition macrophages derived from PDK-1$^{\Delta myel}$ show increased phosphorylation of Iκbα, negative regulator of NF-κB and increased translocation and activation of NF-κB in the nucleus. This enhanced phosphorylation of Iκbα is caused by the increased activation of the upstream IKK complex. Futher studies revealed that, this activation of IKK complex is caused by enhanced ubiquitination of signal transducer TRAF6. Thus PDK-1 in macrophages serves as a negative regulator of inflammatory signaling. When exposed to high fat diet (HFD) in order to induce obesity, PDK-1$^{\Delta myel}$ mice gain significant body weight compared to its counterpart on normal chow diet (NCD), however there is no difference in the body weight between the control and PDK-1$^{\Delta myel}$ mice under HFD. In addition, PDK-1$^{\Delta myel}$ mice exhibit impaired glucose tolerance and decreased glucose infusion rate during euglycemic and hyperinsulinemic clamps. Taken together the disruption of PDK-1 in macrophages leads to exaggerated inflammation and impaired glucose homeostasis.

# 7 Zusammenfassung

Typ 2 Diabetes mellitus ist eine weit verbreitete Krankheit, von der ungefähr 6 % der Bevölkerung der Industrieländer betroffen ist. Die Ursache für Typ 2 Diabetes ist Insulinresistenz in Organen, wie der Leber, Skelettmuskel und Hirn. Adipositas ist eine der grundlegenden Ursachen für eine gesteigerte Insulinresistenz und mit der Entwicklung von Typ 2 Diabetes assoziiert. Außerdem geht Adipositas mit einer systemischen chronischen Entzündungsreaktion einher, die sich durch ein erhöhtes inflammatorisches Cytokinniveau im Blut auszeichnet. Da die Sekretion dieser Cytokine in erster Linie von Makrophagen gewährleistet wird, wurde im Rahmen dieser Arbeit die Rolle von Makrophagen in der Entwicklung der Insulinresistenz untersucht. Die Phosphoinositol-abhängige Kinase 1 (PDK-1) als wichtiger Bestandteil des PI3-Kinase Signaltransduktionsweges wird durch Insulin aktiviert. Nach Aktivierung phosphoryliert PDK-1 die Kinase PKB(AKT) und die p70 ribosomale Proteinkinase S6. Um die Insulinresistenz in Makrophagen an einem wichtigen Signalknotenpunkt zu untersuchen, generierten wir Mäuse mit einer spezifischen Deletion von PDK-1 in myeloiden Zellen (PDK-1$^{\Delta myel}$). PDK-1$^{\Delta myel}$ Mäuse zeichnen sich durch eine erhöhte Expression und Sekretion von TLR4 induzierten inflammatorischen Cytokinen bei Lipopolysaccharid (LPS) Stimulation aus. Außerdem ist die Phosphorylierung von Iκbα in Makrophagen erhöht, was mit erhöhter Translokation in den Nukleus und Aktivierung von NF-κB verbunden ist. Die vermehrte Phosphorylierung von Iκb resultiert aus der verstärkten Aktivität des stromaufwärts agierenden IKK Komplexes. Weitere Studien ergaben, dass diese verstärkte Aktivität des IKK Komplexes durch eine erhöhte Ubiquitinierung des Signaltransduktionsproteins TRAF6 verursacht ist. Somit konnte gezeigt werden, dass PDK-1 in Makrophagen als negativer Regulator der inflammatorischen Signaltransduktion fungiert. Des Weiteren, ist das Körpergewicht von PDK$^{\Delta myel}$ Mäusen, die eine fettreiche Diät erhalten (HFD), gegenüber regulär gefütterten Tieren (NCD) erhöht, was bei einer Diät induzierten Adipositas zu erwarten ist. Kein Unterschied ist jedoch bei PDK$^{\Delta myel}$ Tieren (HFD) im Vergleich zu Kontrolltieren (HFD) zu beobachten. Allerdings weisen PDK$^{\Delta myel}$ Mäuse auf HFD eine beeinträchtigte Glukosetoleranz und eine verminderte Infusionsrate während des euglycämischen und hyperinsulinämischen Clamps auf. Zusammengefasst führt die Deletion von PDK-1 in

Makrophagen zu einer verstärkten Inflammationsreaktion und unter Diät induzierten Adipositas Bedingungen zu einer veränderten Glukosehomöostase.

# 8 Curriculum Vitae

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**2005-2008** PhD Fellowship from International Graduate School for Genetics and Functional Genomics, University of Cologne, Germany.

**Publication**

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**Chaurasia, B**, Mauer, J; Koch, L; Blaumgartel, J; Alessi, D & Brüning, J. "PDK-1 negatively regulates Liposaccharide induced activation of TLR4 signaling in myeloid cells". *(Manuscript in Preparation)*.

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# 10 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzen Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich der Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Jens C. Brüning betreut worden.

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