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Effect of hyperglycaemia on the activation and epigenetic programming of primary human macrophages

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Abbreviations

Ab	antibody
acLDL	acetylated low density lipoproteins
ADCC	antibody dependent cellular cytotoxicity
AGEs	Advanced glycosylated end products
Amp	ampicillin
APC	allophycocyanin
APS	ammonium persulfate
AT	adipose tissue
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony-forming unit
CO_2	carbon dioxide
CSF-1	colony-stimulating factor-1
DAG	diacylglycerol
DAPI	4', 6-Diamidino-2-phenylindole
ddNTP	dideoxynucleotide triphosphate
Dex	dexamethasone
DMEM	Dulbecco´s Modified Eagle's Medium
DMRs	differentially methylated regions
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
EEA-1	early endosomal antigen 1
EGF	epidermal growth factor
ERK	extracellular-signal-regulated kinase
EtBr	ethidium bromide
FACS	fluorescent activated cell sorting
FC	fragment crystallizable region
FCS	foetal calf serum
FITC	fluorescein-5-isothiocyanate
FIZZ1	found in inflammatory zone 1
FSC	forward scatter
g	centrifugal force/gravity
GAPDH	glutaraldehyde phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour
HG	high glucose
HIF	hypoxia-inducible factor
HLA	histocompatibility complex locus
HRP	horse radish peroxidase
HSP	heat shock protein
IF	immunofluorescence
IFNg	interferon gamma
Ig	immunoglobulin
IGF-1	insulin growth factor 1
IL	interleukin
IL-1Ra	interleukin 1 receptor antagonist
iNOS	inducible nitric oxide synthases
kb	kilobases
kDa	kilodalton
LB	Luria-Bertani
LPS	lipopolysaccharides

LYVE-1	lymphatic vessel endothelial receptor-1
M	molar
mA	milliampere
MAC-1	macrophage-1 antigen
MAC-3	macrophage-3 antigen
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MDSC	myeloid-derived suppressor cells
MEM	modified Eagle's medium
mg	milligrams
MGL1	macrophage galactose N-acetylgalactosamine (GalNAc) specific lectin 1
MHC	major histocompatibility complex
mi	minute(s)
MIP-1alpha	macrophage inflammatory protein-1 alpha
ml	millilitre
miRNA	microRNA
mM	millimolar
MMP	matrix metallopeptidase
MMR	macrophage mannose receptor
MPRs	mannose-6 phosphate receptors
MRC1	mannose receptor, C type 1
NaOH	sodium hydroxide
ncRNAs	non-coding RNAs
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanograms
NG	normal glucose
nm	nanometre
NO	nitric oxide
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PE	phycoerythrin
PEG	polyethylene glycol
PFA	paraformaldehyde
PGE2	prostaglandin E2
PI3K	phosphatidylinositide 3-kinase
PKC	protein kinase C
pM	picomolar
PS	phosphatidylserine
PyMT	polyoma middle T antigen
RBC	red blood cells
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SI-CLP	stabilin-1 interacting chitinase-like protein
siRNA	small interfering ribonucleic acid
SPARC	secreted protein acidic and reach in cysteine
SR-A	scavenger receptor A
SSC	side scatter
STAT	signal transducers and activators of transcription
T1D	type 1 diabetes
T2D	type 2 diabetes
TAE	Tris/Acetate/EDTA-buffer

TEMED	N,N,N´,N´-Tetramethylethylenediamide
TGFbeta	transforming growth factor-beta
TGN	trans-Golgi network
TGS	tris-glycine-SDS
TLR	toll-like receptor
TNF	tumour necrosis factor
TP	thymidine phosphorylase
t-PA	tissue-type plasminogen activator
TRAMP	transgenic adenocarcinoma of the mouse prostate
UKPDS	U.K. Prospective Diabetes Study
uPA	urokinase-type plasminogen activator
uPAR	urokinase plasminogen activator receptor
UV	ultra violet
V	volt
VEGF	vascular endothelial growth factor
WB	Western blotting
Wnt	wg (wingless) and Int
Wt	wild type

1. <u>Introduction</u>

1.1 Diabetes and hyperglycaemia

Diabetes became a major epidemic of this century with rapidly increasing incidence worldwide (Forbes and Cooper, 2013). Diabetes is clinically characterised by chronic elevation of blood sugar, termed "hyperglycaemia". There are two major types of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D), although diabetes can also manifest during pregnancy (gestational diabetes) (Forbes and Cooper, 2013). In type 1 diabetes, hyperglycaemia is the result of autoimmune reactions destroying pancreatic beta cells producing insulin. In type 2 diabetes, which is much more common, hyperglycaemia is the result of progressively impaired glucose regulation due to a combination of insulin resistance in certain organs and dysfunction of pancreatic beta cells (Lewis et al., 2014). Diabetes is associated with a number of macro- and microvascular complications leading to the failure of organs such as the heart, kidneys and eyes (Beckman and Creager, 2016; Cade, 2008). It is widely accepted that achieving optimal glycaemic control as early as possible in the course of the disease is the most effective way to reduce the risk of vascular complications in both type 1 and type 2 diabetes (Forbes and Cooper, 2013).

1.1.1 Type 1 diabetes: autoimmune disease

In type 1 diabetes, a complex interplay between genetic and environmental factors leads to the autoimmune reactions resulting in the destruction of insulin-producing beta cells in pancreatic Langerhans islets (Forbes and Cooper, 2013; Simmons et al., 2016). The incidence of T1D is increasing in westernised societies, and it is mostly diagnosed in young children (Roep and Tree, 2014; Simmons et al., 2016). The genetic basis of T1D includes specific determinants in the major histocompatibility complex locus (HLA), however the specific HLA determinants can explain only about 50% of the familial clustering of T1D (Forbes and Cooper, 2013). The development of T1D involves many immune cells; CD4+ and CD8+ T cells, as well as macrophages are major immune cell types that mediate beta cell destruction by autoantigen-specific inflammatory mechanisms (Lehuen et al., 2010). Glutamic acid decarboxylase (GAD/GAA), and protein tyrosine phosphatase (IA-2AA) are the most common autoantigens involved in the process of beta cell destruction. A study by Pardini et al. demonstrated that GAD frequency was higher compared to IA-2 in Brazilian T1D patients (Pardini et al., 1999).

1.1.2 Type 2 diabetes: metabolic disease

Type 2 diabetes is caused by a complex interplay between insulin resistance, beta cell dysfunction, and a relative lack of insulin (Jeffery and Harries, 2016). Insulin resistance is most frequently detected in muscle, liver and adipose tissue due to the intensive glucose uptake for metabolism in these tissues (Forbes and Cooper, 2013). Obesity is one of the causes of insulin resistance leading to the development of T2D (Saltiel and Olefsky, 2017). Adipose tissue (AT) is composed of adipocytes, pre-adipocytes and a high number of macrophages, that can develop pro-inflammatory phenotypes (M1-like phenotype) in response to metabolic factors (Espinoza-Jiménez et al., 2012). As a result, obesity leads to increased production of inflammatory cytokines such as TNF-alpha, IL-1beta, IL6 and CCL2, as well as reactive oxygen species (ROS) in fat tissue, resulting in obesity-associated low-grade inflammation (Lackey and Olefsky, 2016). In turn, low-grade inflammation can lead to insulin resistance and development of T2D as a consequence (Espinoza-Jiménez et al., 2012; Lackey and Olefsky, 2016). It was demonstrated that in both *in-vivo* and *in-vitro* models that exposure of adipocytes to hyperglycaemia leads to insulin resistance (McArdle et al., 2013). Furthermore, blocking of pro-inflammatory signalling pathways involving JNK and TNF-alpha contribute to obesity-related insulin resistance improvement (Eguchi and Manabe, 2013; Hotamisligil, 2006). Insulin resistance can be accompanied by compensatory hypersecretion of insulin by pancreatic islets that may precede a decline in beta cell functions. However, it appears that at later stages, insulin secretory defects are a critical parameter in the ultimate development of T2D (Forbes and Cooper, 2013).

1.2 Vascular complications of diabetes

Diabetes mellitus is associated with the development of long-term vascular complications that include microvascular and macrovascular complications. Microvascular complications include retinopathy, nephropathy and neuropathy, while major macrovascular complications include cardiovascular and cerebrovascular diseases (Forbes and Cooper, 2013).

Several studies have shown that hyperglycaemia is a critical factor for the development of microvascular and macrovascular complications in type 1 and type 2 diabetes (Schalkwijk and Stehouwer, 2005; van Diepen et al., 2016). However, other factors such as age, smoking, obesity, hypertension, hyperhomocysteinaemia, hypercholesterolaemia and dyslipidemia have a significant contribution to the damage to the vascular wall and lead to endothelial dysfunction (Caballero, 2003; Martin-Timon et al., 2014; Schalkwijk and Stehouwer, 2005). Moreover, development of diabetic vascular complications is a result of a complex interplay

between metabolic and inflammatory pathways affecting not only endothelial cells, but also various somatic and immune cells.

1.2.1 Microvascular complications

Microvascular complications of diabetes affect small blood vessels. Hyperglycaemia is considered to be the main causative agent for microvascular complications. The risk of microvascular complications depends on the level and duration of the hyperglycaemic condition (Forbes and Cooper, 2013). Major microvascular complications include diabetic retinopathy, nephropathy and neuropathy and are described in following paragraphs.

Diabetic retinopathy

One of the most commonly affected organs in microvascular complications are the eyes. According to the U.K. Prospective Diabetes Study (UKPDS), development of diabetic retinopathy in type 2 diabetic patients was found to correlate with both severity of hyperglycaemia and the presence of hypertension (Davis et al., 1999). In most cases, type 1 diabetic patients developed retinopathy within 20 years of diagnosis (Keenan et al., 2007). Degeneration or occlusion of retinal capillaries is indicative of a worsening prognosis of retinopathy (Forbes and Cooper, 2013). There are many pathological mechanisms by which diabetes leads to the development of retinopathy.

Aldose reductase can contribute to the progression of diabetic complications (Tang et al., 2012). Aldose reductase is the enzyme involved in the polypol pathway that regulates the conversion of glucose into sorbitol. Hyperglycaemic states lead to increased accumulation of sorbitol in the cells. Accumulation of sorbitol leads to osmotic stress resulting in the progression of diabetic microvascular complications, including diabetic retinopathy (Fowler, 2008; Keenan et al., 2007; Sorrentino et al., 2016; Vedantham et al., 2012).

Oxidative stress plays an important role in cellular injury in response to hyperglycaemia. Hyperglycaemia stimulates free radical production and ROS formation that leads to the injury of endothelial cells. In mouse models, treatment with antioxidants, such as vitamin E, is shown to reduce some vascular dysfunctions linked with diabetes (Keenan et al., 2007; Sorrentino et al., 2016).

Hyperglycaemia can induce the formation of advanced glycosylated end products (AGEs), which interact with RAGE receptor leading to the activation of NF-kB-mediated inflammatory responses in endothelial cells and macrophages (Bierhaus and Nawroth, 2009). It was suggested that hyperglycaemia-induced activation of inflammatory responses in retinal endothelial cells, as well as in Müller cells, astrocytes, ganglion cells and microglial cells, is a central process that integrates vasculopathy and neuro-inflammation at the level of neurovascular damage leading to diabetic retinopathy (Yu et al., 2015). Major inflammatory molecular players produced as a consequence of hyperglycaemic conditions include cytokines and growth factors (VEGF, IL-1beta, CCL2, TNF-alpha, IL6) (Yu et al., 2015). However, especially for microglial cells, which represent resident macrophages in the retina, the identified pro-inflammatory effects were rather mediated by AGE, and the direct effect of hyperglycaemia on cell activation remains to be identified.

Diabetic nephropathy

Diabetic nephropathy is the leading cause of kidney failure in the United States. It is defined as proteinuria > 500 mg/24 hours or albumin excretion of 30-299 mg/24 hours in the diabetic condition (Josipovic et al., 2013). Diabetic patients with microalbuminuria normally progress to diabetic nephropathy. Diabetic nephropathy can occur in both type 1 and type 2 diabetes (Beckman and Creager, 2016; Josipovic et al., 2013). The pathological changes in the kidney include increased glomerular basement membrane thickness, microaneurysm formation and mesangial nodule formation. Hyperglycaemia affects several resident cell types in the kidney, including endothelial cells, smooth muscle cells, mesangial cells, podocytes, and cells of the tubular and collecting ducts system, immune cells and myofibroblasts. The hyperglycaemia-induced pathological processes in diabetic nephropathy can be similar to that in diabetic retinopathy including losing control of energy production, activation of the aldose reductase pathway and induction of pro-inflammatory reactions (Forbes and Cooper, 2013; Wada and Makino, 2016). It was also recently recognised that hyperglycaemia is an essential factor in the activation of the NLRP3 inflammasome during the development of diabetic nephropathy (Qiu and Tang, 2016). The NLRP3 inflammasome is responsible for the conversion of the pro-inflammatory cytokines IL-1beta and IL18 to their active forms, and targeting of the NLRP3 inflammasome was suggested as a promising therapeutic strategy (Hutton et al., 2016; Qiu and Tang, 2016).

Diabetic neuropathy

The risk of developing diabetic neuropathy depends on both the magnitude and duration of hyperglycaemia, and genetic factors can contribute to a predisposition (Fowler, 2008). The possible mechanisms involved in hyperglycaemic progression of neuropathy include polyol accumulation, injury from AGEs and oxidative stress. In diabetic neuropathy, more than 80% of amputations occur after injury or foot ulceration (Fowler, 2008). Diabetic neuropathy causes major sickness and mortality in patients with diabetes. The primary treatment of diabetic neuropathy is to control the hyperglycaemic conditions (Beckman and Creager, 2016). Persistent hyperglycaemia is believed to be an essential factor leading to neuroinflammation and nerve damage (Sandireddy et al., 2014b). Polyol, PKC, the MAPK pathway, as well as an increase in the formation of AGE, can all have direct or indirect effects on the activation of inflammatory processes in diabetic neuropathy. Persistent hyperglycaemia affects structural features on neurones leading to the recruitment of monocytes and macrophages. Accumulating AGE acts on the spectrum receptors present on microglia and newly recruited macrophages, and stimulates the production of pro-inflammatory cytokines (including IL1, IL6, TNF-alpha, CCL2) that exert pathological effects on endothelial cells and neurones (Sandireddy et al., 2014b; Wada and Yagihashi, 2005).

1.2.2   Macrovascular complications

Macrovascular complication affects major arteries leading to the development of atherosclerosis; a complex chronic inflammatory disease involving the pathological action of numerous cell types. The dysfunction within endothelium in diabetic conditions seems to be a key early factor contributing to the initiation of atherosclerosis (Bertoluci et al., 2015). Development of atherosclerosis is considered to be a consequence of low-grade inflammation of the arterial walls where both endothelial cells and monocytes/macrophages are pathologically programmed by metabolic factors including hyperglycaemia (van Diepen et al., 2016). Macrophages are key innate immune cells that contribute to the formation and progression of atherosclerotic plaque. Several processes are essential for the progression of atherosclerosis including intensive infiltration of monocytes into subendothelial space, differentiation of monocytes into pathological macrophage phenotypes, deficiency in degradation of the internalised lipoproteins, and defects in cholesterol efflux resulting in foam cell formation (Kzhyshkowska et al., 2012). Macrophage scavenger receptors play a role in the pathological processing of modified lipoproteins leading to the formation of foam cells (Kzhyshkowska et al., 2012). Recently, it was shown that loss of IGF-1 (insulin growth

factor) signalling drives pro-inflammatory macrophages and increases lipid accumulation in macrophages (Higashi et al., 2016). Foam cells stimulate the attraction of T-lymphocytes which in turn stimulate smooth muscle cell proliferation in the arterial walls leading to the accumulation of collagen and the formation of a lipid-rich atherosclerotic lesions; atherosclerotic plaques, covered with a fibrous cap. The breaking of these lesions direct to acute vascular infarction (Boyle, 2007; Fowler, 2008). Hyperglycaemia is an essential factor in the development of macrovascular complications, since a significant long-term controlled study demonstrated that improved hyperglycaemic control in type 2 diabetic patients leads to a decreased incidence of macrovascular diseases (Fowler, 2008). Hyperglycaemia was identified as an independent risk factor for cardiovascular diseases (CDV). Even transient hyperglycaemia upon return to normoglycemia increases the risks of CVD, a phenomena named "hyperglycaemic memory". Long-term epigenetic programming of monocytes and macrophages was suggested to be critical for hyperglycaemic memory resulting in diabetes-associated CVD (van Diepen et al., 2016).

1.3 Hyperglycaemia links oxidative stress and inflammation

Hyperglycaemia activates both oxidative stress and inflammatory pathways resulting in the pathological activation of a number of cell types that mediate progression of diabetes and vascular complications (Sandireddy et al., 2014a). Hyperglycaemia induces the generation of superoxide anions by increasing metabolic flux through the mitochondrial electron transport chain that in turn results in the production of ROS (Brownlee, 2005; Sandireddy et al., 2014a). ROS has the ability to damage DNA, proteins and lipids facilitating endothelial cell injury leading to vascular complications. ROS can also activate the NF-kB and JNK mediated inflammatory pathways that are crucial for inducing insulin resistance and dysfunction of beta cells (Evans et al., 2003).

Oxidative stress produced by hyperglycaemia activates Nrf2, NF-kB and MAPK mediated signalling pathways (Sandireddy et al., 2014a) (Figure 1). Activation of the Nrf2 pathway is a natural homeostatic mechanism that protects the cells from oxidative stress and inhibits NF-kB activation (Sandireddy et al., 2014a; Tan et al., 2011) (Figure 1). However, Nrf2 activation in hyperglycaemic conditions is attenuated by the activated ERK pathway. ROS activates the inhibitory kappa-B kinase (IKK), which mediates phosphorylation of IkB and causes proteasomal cell death (Tan et al., 2011). Released from IkB, free NF-kB heterodimers translocate to the nucleus and activate transcription by binding the kappa region of the

genome, which leads to the production of inflammatory cytokines such as TNF-alpha, IL6, iNOS and COX2 (Figure 1) (Cameron and Cotter, 2008; Sandireddy et al., 2014a). Hyperglycaemia can also directly or indirectly activate p38 MAPK which facilitates the activation of NF-kB in the cytoplasm (Figure 1) (Sandireddy et al., 2014a). Moreover, oxidative stress activates c-Jun N-terminal kinases (JNK) leading to increased production of MCP-1, TGF-beta and collagenase, which mediate pro-inflammatory and pro-fibrotic reactions (Figure 1) (Karin, 1995; Sandireddy et al., 2014a; Sutariya et al., 2016; Takaishi et al., 2003). Hyperglycaemia-mediated activation of the JNK, NF-kB and p38 MAPK pathways leads to the development of chronic inflammation and cellular damage, and is responsible for the development of late diabetic complications (Matough et al., 2012). Additionally, hyperglycaemia-dependent ROS induces the activity of methyltransferase Set7/9 in endothelial cells which is responsible for monomethylation of histone 3 lysine 4 (H3K4me1) (Paneni et al., 2013). The presence of H3K4me1 on the promoter region of NF-kB subunit p65 associates with the development of vascular complications (Paneni et al., 2013). Despite intensive investigation of the pro-inflammatory effects of hyperglycaemia, most mechanistic studies are limited to various types of endothelial cells. However, both the initiation and resolution of inflammation in the organism are under the precise control of the innate immune system, where macrophages are key immune cells in the local tissue microenvironment in virtually all organs and tissues that orchestrate inflammatory responses.

1.4 Origin and differentiation of macrophages

Macrophages are mature functional cells of the mononuclear phagocyte system which includes bone marrow monoblasts, pro-monocytes, peripheral blood monocytes and tissue macrophages (Embleton, 2003). The differentiation of bone marrow precursors to macrophages includes the following steps: committed progenitor cell – monoblast - pro-monocyte - monocyte (bone marrow) - monocyte (peripheral blood) - macrophage (tissues). Resident macrophages have tissue-specific phenotypes and historically can also have tissue-specific names, like microglial cells in the neuronal system and eyes, histiocytes in the connective tissue, osteoclasts in bones and Kupfer cells in the liver. Mature tissue macrophages are characterised by the expression of different proteins including CD14, CD11b, F4/80 (mice)/EMR1(human), CD68, lysozyme M, CSF-1R and MAC-1/MAC-3, (Hume, 2006; Taylor et al., 2005). However, it was later recognised that resident tissue macrophages can also derive from yolk-sac during early developmental stages (Gordon and Taylor, 2005). Later in development, the foetal liver can generate a next wave of macrophage

precursors populating the tissues during development of the embryo (Gordon and Taylor, 2005). Therefore, monocyte-derived macrophages are not the primary macrophages in the tissue, but rather constitutively replace resident macrophages in adult organisms (Gordon and Taylor, 2005). A summary of the origin and major differentiation steps of macrophages is presented in Figure 2 (Gordon and Taylor, 2005).
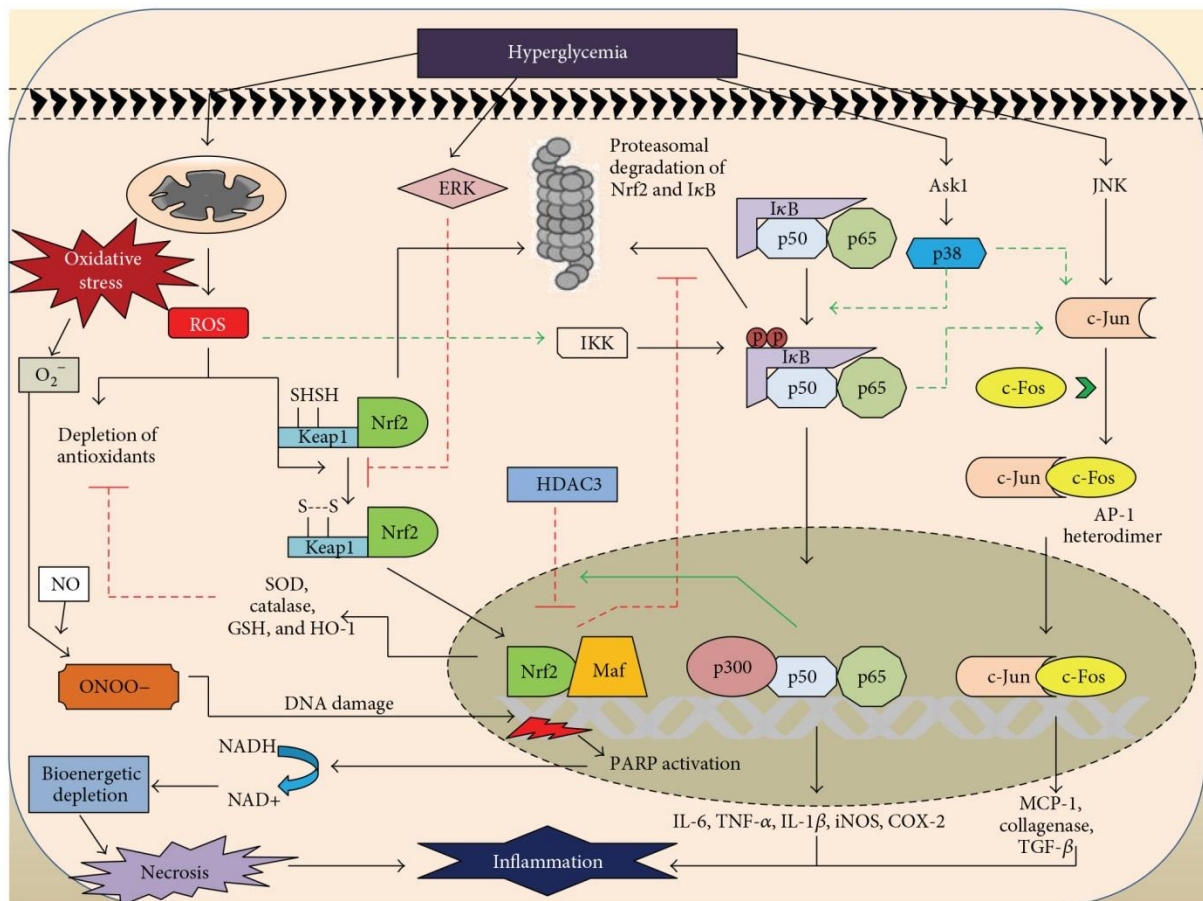
Figure 1. Mechanisms that link hyperglycaemia and inflammation. Reprinted from distributed under the Creative Commons Attribution-Non-Commercial License (Sandireddy et al., 2014a).

Most recently the details of macrophage origin during the developmental stage were identified. Primitive yolk sac macrophages originate from early EMPs (erythroid-myeloid progenitors, E7.5) and, after blood in circulation is initiated, primitive macrophages populate various peripheral organs (Hoeffel et al., 2015; Schneider and Kopf, 2015) (Figure 3). The next wave of EMPs (around E8.5) migrate into the liver, where part of them differentiate into foetal monocytes. From E13.5, foetal monocytes colonise the majority of organs where, under stimulation with specific microenvironment factors, they differentiate into resident tissue-specific macrophages. However, microglia directly derive from early primitive macrophages, but not from foetal monocytes (Hoeffel et al., 2015; Schneider and Kopf, 2015). There is also

evidence that tissue-specific macrophages can proliferate and self-renew, however, the contribution of this self-renewal process compared to the contribution of newly infiltrating adult bone marrow derived monocytes is an open question that is under intensive investigation (Rosas et al., 2014).

Specific transcription factors are responsible for determining function and adoption of tissue-specific macrophages. For example, microglial cells (brain resident macrophages) express Sall1 and mef2c (Schlitzer and Schultze, 2016). PPARγ and BACH2 have been recognised to control alveolar macrophage population function and fate (Nakamura et al., 2013; Schlitzer and Schultze, 2016; Schneider et al., 2014). In adipose tissue, macrophages play a major role in crosstalk between white adipose tissue cells and the immune system (Castoldi et al., 2015; Schlitzer and Schultze, 2016). During obesity, more bone marrow derived monocytes are recruited to the fat that programs them towards a pro-inflammatory M1 phenotype (Lumeng et al., 2007).
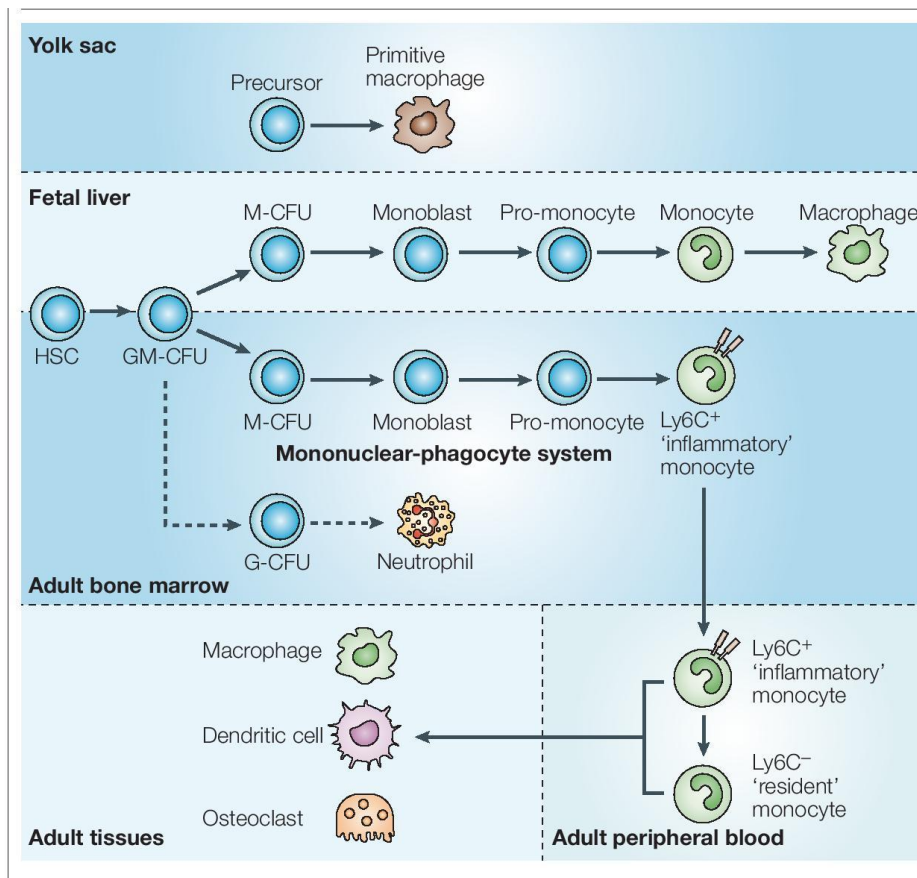
Figure 2. Origin and major differentiation steps of cells of the mononuclear phagocyte system in a mouse. Permission obtained from Copyright Nature Publishing Group (Gordon and Taylor, 2005).

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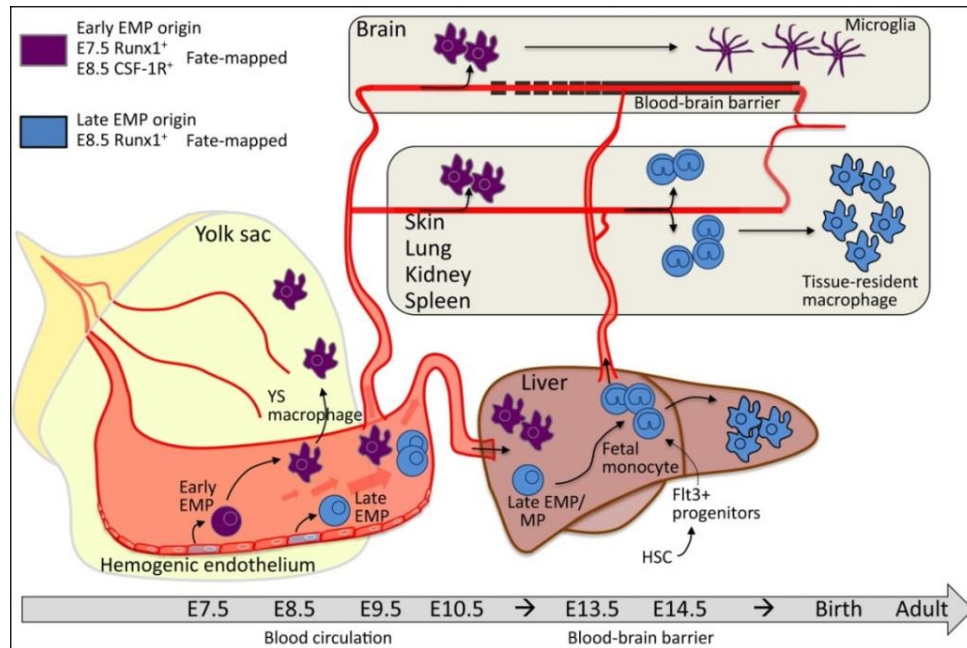
Figure 3. Development of primitive macrophages and foetal monocytes from erythro-myeloid progenitors (EMP) in the Yolk Sac. Permission obtained from Copyright Elsevier (Schneider and Kopf, 2015).

1.4.1 Classification of macrophages and activation

Depending on the wide range of microenvironmental factors, macrophages can differentiate into different types of macrophages. The original concept of macrophage activation considered macrophages as effectors of Th1 and Th2 cells, and defined major macrophage types as classically (M1) and alternatively (M2) activated. Correspondingly, two major cytokines, IFNg produced by Th1 cells, and IL4 produced by Th2 cell, were found as major drivers for the M1 and M2 types of macrophages activation (Goerdt and Orfanos, 1999; Gordon and Martinez, 2010; Gratchev et al., 2001). M1 macrophages play a key role in the defence mechanism against bacterial pathogens by producing lysosomal enzymes, reactive oxygen species (ROS) and nitrogen species (NO) (Ding et al., 1988). Cytokines released by classically activated macrophages are TNF-alpha, IL-1beta, IL6 and IL12 (Van Ginderachter et al., 2006). These cytokines can induce inflammatory and cytotoxic responses, and can activate NK cells, CD8+ cytotoxic lymphocytes and the recruitment of neutrophils.

M2 macrophages comprise a different subpopulation that functions in tissue homoeostasis, resolution of inflammation and healing. M2 macrophages have increased clearance potential, express high levels of phagocytic and endocytic receptors, secrete a wide range of cytokines, growth factors, structural components of extracellular matrix and matrix-remodelling enzymes (Kzhyshkowska et al., 2016; Martinez and Gordon, 2014a). Major stimuli that

induce M2 polarisation, besides IL4, include glucocorticoids and TGF-beta (Figure 4). M2 macrophages can also have detrimental functions by stimulating allergic reactions, pro-fibrotic processes and tumour progression (Kzhyshkowska et al., 2016). More recently the concept of macrophage activation underwent further revision, and macrophages were classified according to a variety of stimuli resulting in the development of distinct phenotypes (Murray et al., 2014). However, the M1 and M2 classification remains to be a frequently used and convenient tool for distinguishing major types of macrophage functional polarisation, especially in different types of pathologies, including obesity and atherosclerosis. Mature macrophages were identified to be also highly plastic cells that can change their molecular profiles and functional phenotypes in response to secondary stimuli and a changing microenvironment (Kzhyshkowska et al., 2016; Mantovani et al., 2013). This functional plasticity of macrophages makes them an attractive target for therapy of chronic inflammatory diseases and cancer (Mantovani et al., 2013).
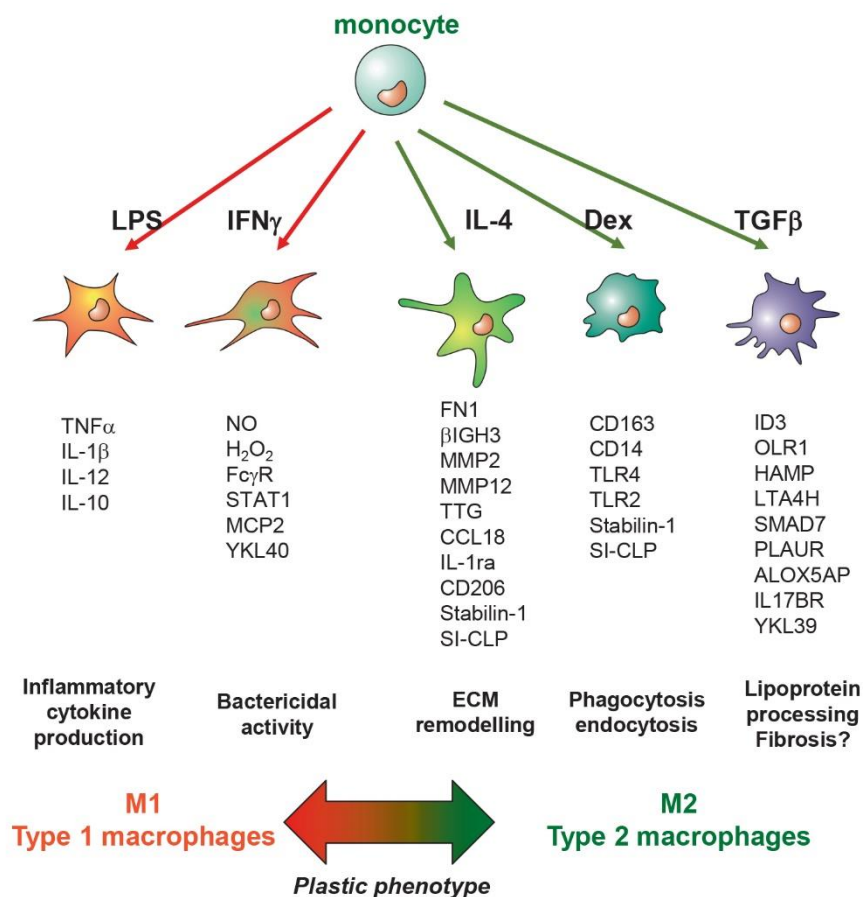
Figure 4. Molecular markers and major functions identified for human monocyte-derived macrophages *ex vivo*. Each specific stimuli induces a specific molecular profile during monocyte to macrophages differentiation in culture conditions. The molecular profiles are indicative of the major functional activities of macrophage subtypes. Permission obtained from Copyright © 2016, Karger Publishers (Kzhyshkowska et al., 2016).

1.4.2 Functions of macrophages during acute and chronic inflammation

Macrophages are key tissue resident cells that sense trauma or pathogen attack and initiate acute inflammation, which is a natural host defence mechanisms. During acute inflammation, macrophage actively kills pathogens and attracts other immune cells, including neutrophils and monocytes, to amplify the inflammatory reaction and eliminate pathogens. Macrophages also attract cells of the adaptive immune system that mediate antigen-specific responses to the pathogens. In response to bacterial infection, activated macrophages secrete a broad range of pro-inflammatory cytokines and chemokines (IL1, TNF-alpha, CC and CXC chemokines), a number of reactive oxygen intermediates and reactive nitrogen intermediates that have potent antimicrobial activity. These biologically active substances initiate the process known as inflammation (Duffield, 2003; Szekanecz and Koch, 2007).

After the acute phase where the exogenous danger is eliminated and inflammatory reactions are not needed anymore, macrophages initiate resolution of inflammation and a healing phase that leads to the restoration of homeostatic tissue-specific balance. However, not completely eliminated pathogens (for example, residual virus infection), foreign bodies (like an implant) or endogenous factors (cytokines, fatty acids, AGEs) can drive local chronic inflammatory reactions that are characterised by imbalances in immune cell infiltration and activation (Murakami and Hirano, 2012).

1.4.2.1 Chemotaxis

Chemotaxis is an essential process in the course of both acute and chronic inflammatory reactions. Chemotaxis of different types of immune cells is strongly activated by chemokines produced locally by the inflamed tissue (Embleton, 2003). Resident monocytes continuously patrol the intravascular space (Kamei and Carman, 2010a). In the case of acute inflammation, inflammatory stimuli, including chemical irritants, aseptic wounding, and infection agents, induce rapid recruitment of patrolling monocytes. Initial monocyte infiltration precedes neutrophil accumulation by at least 1 hour (Kamei and Carman, 2010a). During acute inflammation, circulating neutrophils are actively recruited to the sites of tissue damage by several types of factors including leukotrienes (LTB4), complement factors (C5a), bacterial products and chemokines (Jones, 2000). During chronic inflammation (including atherosclerosis), there is a constant requirement of monocytes and T-cells that produce unbalanced amounts of pro-inflammatory mediators that interfere with the resolution of inflammation (Lindholt and Shi, 2006) (Zernecke et al., 2008). When leukocytes sense a chemoattractant, such as CCL2, CCL5 etc., and move directionally by changing the

cytoskeleton rearrangements, they form pseudopods and change cell shape. Chemoattractants activate leukocytes to bind to the endothelial cell surface and move across the cell layer towards the site of inflammation (Embleton, 2003). During obesity, adipose tissue secretes several adipokines (chemokines, cytokines and hormones) that include CCL2, TNF-alpha, IL1, IL6 and IL8 (Hotamisligil et al., 1993; Jung and Choi, 2014). These inflammatory cytokines and chemokines attract monocytes into the fat tissue and stimulate the formation of the pro-inflammatory M1 phenotype, which in turn contributes to the development of insulin resistance (Jung and Choi, 2014; Xu et al., 2003).
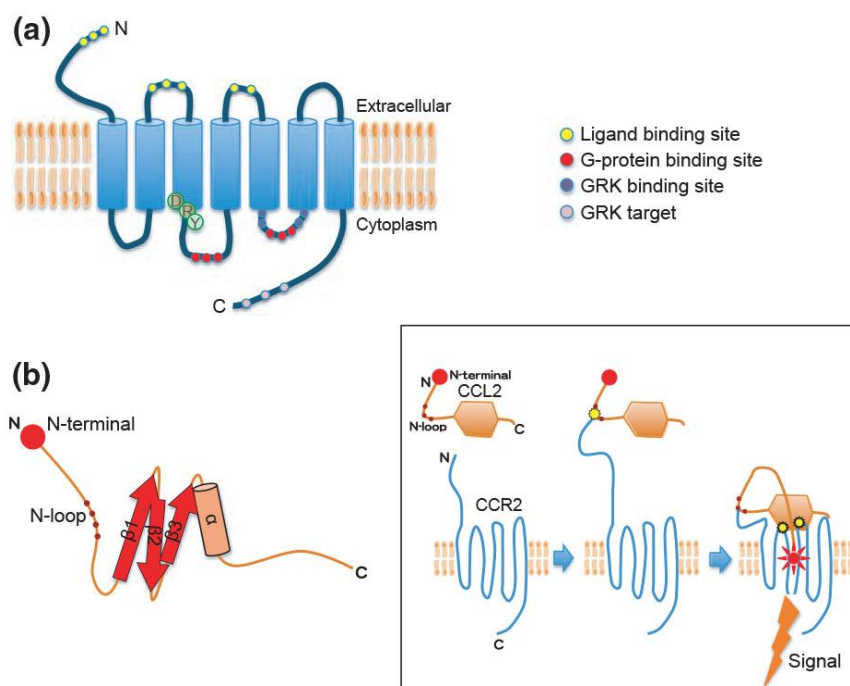
Figure 5. Structures of CCR2 and CCL2 (monocyte chemoattractant protein [MCP]-1).
Permission obtained from Copyright John Wiley and Sons (Yamasaki et al., 2012).

Major receptors that respond to chemotactic factors on monocytes include CCR1, CCR2, CCR5, CCR6, CCR7, CCR8, CX_3CR1 and CXCR2 (Shi and Pamer, 2011). CCR2 is the best-investigated receptor that is expressed not only on monocytes, but also on endothelial cells, T lymphocytes, B lymphocytes, natural killer cells, basophils and dendritic cells (Salcedo et al., 2000) (Yamasaki et al., 2012). CCR2 ligands include CCL2 (MCP1), CCL7 (MCP3), CCL8 (MCP2), CCL13 (MCP4) and CCL16 (Yamasaki et al., 2012). Studies demonstrated that hyperglycaemia induces the production of CCL2, CCL5 and CCL11 in type 1 diabetic patients (Jamali et al., 2013; van der Torren et al., 2016). CCR2 is a seven transmembrane protein consisting of 3 extracellular and 3 intracellular loops, an extracellular

N-terminus, and a C-terminal cytoplasmic tail (Figure 5). The ligands bind to the extracellular N-terminal region. The 3 extracellular regions are also important for triggering intracellular reactions (Yamasaki et al., 2012). However, little is known about the effect of diabetic conditions on the expression of chemokine receptors on immune cells.

1.4.2.2 Transmigration in inflammatory conditions

In inflammatory conditions, endothelial cells overexpress P and E selectins that mediate cell rolling, signalling and chemotaxis of leukocytes (Barthel et al., 2007; McEver, 2015). Selectin-mediated adhesion and signalling were shown to contribute to various vascular pathologies including atherosclerosis, arterial and deep vein thrombosis, ischaemia-reperfusion injury, and other cardiovascular diseases (McEver, 2015). Selectins interact with glycoprotein ligands on the surface of recruited immune cells allowing leukocytes to bind weakly to vascular endothelium and initiate the adhesion cascade (Somers et al., 2000). In inflammatory conditions (both acute and chronic), stromal cells, leukocytes and endothelial cells produce various chemokines (CC-and CXC-chemokines) and chemotactic compounds, such as leukotriene B_4 (LTB$_4$), platelet-activated factor (PAF) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), which stimulate rolling of leukocytes (Murdoch and Finn, 2000). Chemokines bind to seven-transmembrane spanning G-protein-coupled receptors (GPCRs) expressed by leukocytes and induce rapid integrin activation (Ley et al., 2007; Murdoch and Finn, 2000). Activated surface molecules integrin-β_1 and integrin-β_2 promote tight adhesion of monocytes to the vessels via vascular cell-adhesion molecule 1 (VCAM-1) and intercellular adhesion molecules (ICAMs), and induce migration (Kim et al., 2003; Thelen, 2001). Monocytes are polarised via an atypical protein kinase C signalling pathway and form lamellipodium (Kamei and Carman, 2010b). They move along the luminal side of vascular endothelial cells and transmigrate in a paracellular or transcellular manner (Kamei and Carman, 2010b). During paracellular trans-endothelial migration of polarised leukocytes, platelet/endothelial cell-adhesion molecule-1 (PECAM-1) mediates the interaction between migrating cells and endothelial cells. PECAM is expressed on the surfaces of transmigrating monocytes and concentrated on the borders of endothelial cells. Integrins expressed on transmigrating leukocytes interact with ICAMs, VCAM-1 or junctional adhesion molecules (JAMs) on the surface of endothelial cells. In response to cytokines, TNF-alpha and INFg inflamed endothelium redistributes JAM-A molecules from the intercellular junctions to the luminal surface of the cells (Ozaki et al., 1999). Downregulation of tight junction molecule VE-cadherin contributes to the resealing of the junctions between the endothelial cells (Ley et

al., 2007). Endothelial junctional molecules can guide liminal leukocytes to the junctions and also actively mediate trans-endothelial migration (Ley et al., 2007). Interference with the transmigrating process has been considered as a therapeutic strategy in vascular diseases. For example, P-selectin is a critical molecule for the interaction of monocytes with activated endothelium during the progression of atherosclerosis, and targeting of P-selectin was suggested for treatment of atherosclerosis (Ley et al., 2007).

1.4.2.3 Inflammatory mediators and endothelial cell activation

During acute and chronic inflammation several classes of secreted mediators affect activation of macro- and microvasculature. The mediators of acute inflammation include vasoactive amines, plasma protein systems, prostaglandins and leukotrienes, acetyl glycerol ether phosphocholine, cytokines and nitric oxide (Rankin, 2004). These inflammatory mediators activate endothelial cells and attract leukocytes. Monocytes attracted to the site of local inflammation differentiate into macrophages under the influence of local pro-inflammatory factors and release a broad spectrum of biologically active substances. These include enzymes, chemokines, reactive oxygen species, cytokines and structural components of extracellular matrix (Duffield, 2003; Embleton, 2003; Zeremski et al., 2007). During chronic inflammation, macrophages secrete cytokines such as TNF-alpha, IL1, IL6, IL8 and IL12, chemokines, leukotrienes, prostaglandins, and complement components that induce increased vascular permeability and further attraction of inflammatory cells (Arango Duque and Descoteaux, 2014). During inflammation, angiogenesis is initiated by pro-angiogenic factors such as VEGF, PDGF and FGF released by macrophages and other cell types (Granger and Senchenkova, 2010). These pro-angiogenic factors allow endothelial cells to migrate and proliferate to form a cord-like structure in tissues which later can develop into functional vessels. Angiogenesis is a critical process for cancer progression, cardiovascular diseases and diabetic retinopathy (Granger and Senchenkova, 2010).

1.5   Role of macrophages in the regulation of metabolic conditions

Recent evidence at the cellular and molecular level proved that obesity can be a chronic low-grade inflammatory disease, where macrophages play a role in the regulation of glucose and lipid metabolism (Vandanmagsar et al., 2011b; Weisberg et al., 2003). In lean conditions, adipocytes release factors such as IL13, promoting alternative activation of macrophages (Olefsky and Glass, 2010).

Activated alternatively activated macrophages (M2) secrete anti-inflammatory cytokines, such as IL10, and lead to the release of insulin-sensitising factors (Figure 6) (Olefsky and Glass, 2010). In obese conditions, monocytes are actively recruited to the adipose tissue by adipokines, including CCL2, and differentiate into adipose tissue macrophages (ATM). During obese conditions, several factors effect the change of adipocyte metabolism and expression of genes leading to enhanced lipolysis and production of free fatty acids and factors (CCL2, TNF-alpha), attracting monocytes and driving the M1-type of macrophage activation (Figure 6) (Olefsky and Glass, 2010; Ray et al., 2016). These activated M1 macrophages secret several pro-inflammatory mediators, such as IL-1beta, TNF-alpha, IL6, leukotriene B4, nitric oxide and resistin, which induce insulin resistance in adipocytes (Figure 6) (Henao-Mejia et al., 2014; Hotamisligil et al., 1996; Olefsky and Glass, 2010).
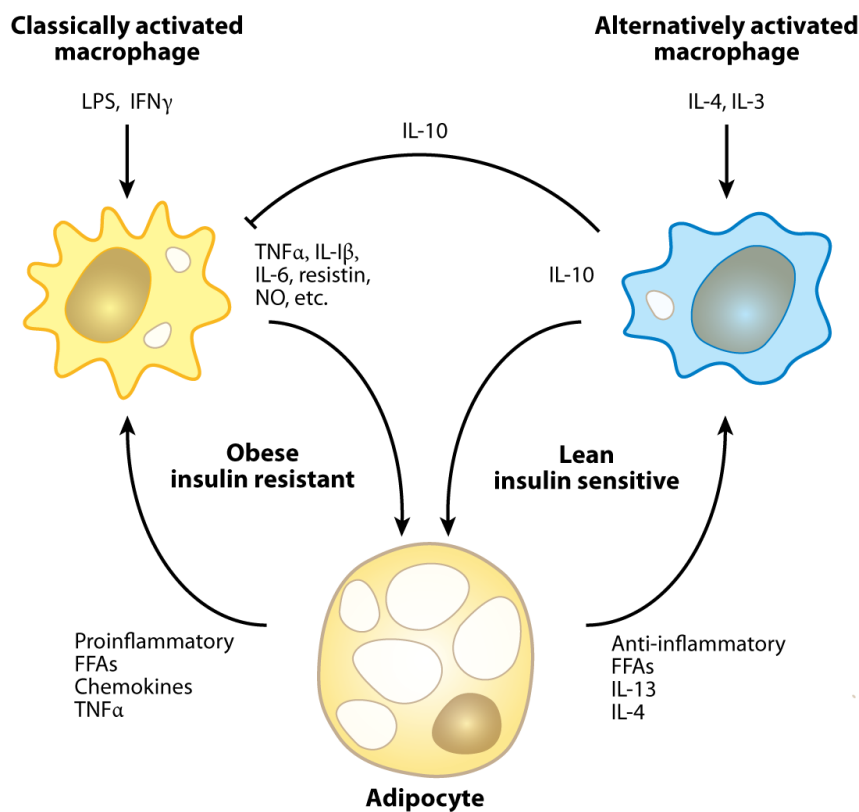
Figure 6.   Factors affecting macrophage polarity and insulin resistance in adipose tissue. Permission obtained from Copyright Clearance Center (Olefsky and Glass, 2010).

Similar mechanisms are involved in inflammation-dependent insulin resistance in other cells, including pancreatic beta cells (Emanuelli et al., 2004). In obesity, inflammatory and metabolic stress conditions are created, not only in fat tissue, but also in other organs, including pancreatic islets, the liver and muscle, that leads to increased local production of cytokines and chemokines such as IL-1beta, TNF-alpha, CCL2, CCL3 and CXCL8/IL8

(Figure 7) (Ray et al., 2016). These cytokines mediate the recruitment of immune cells in insulin-sensitive tissues, amplifying inflammatory reactions that in turn interfere with insulin signalling by activation of NF-kB and JNK-mediated pathways and augment transcription of genes involved in lipid processing (Ray et al., 2016).
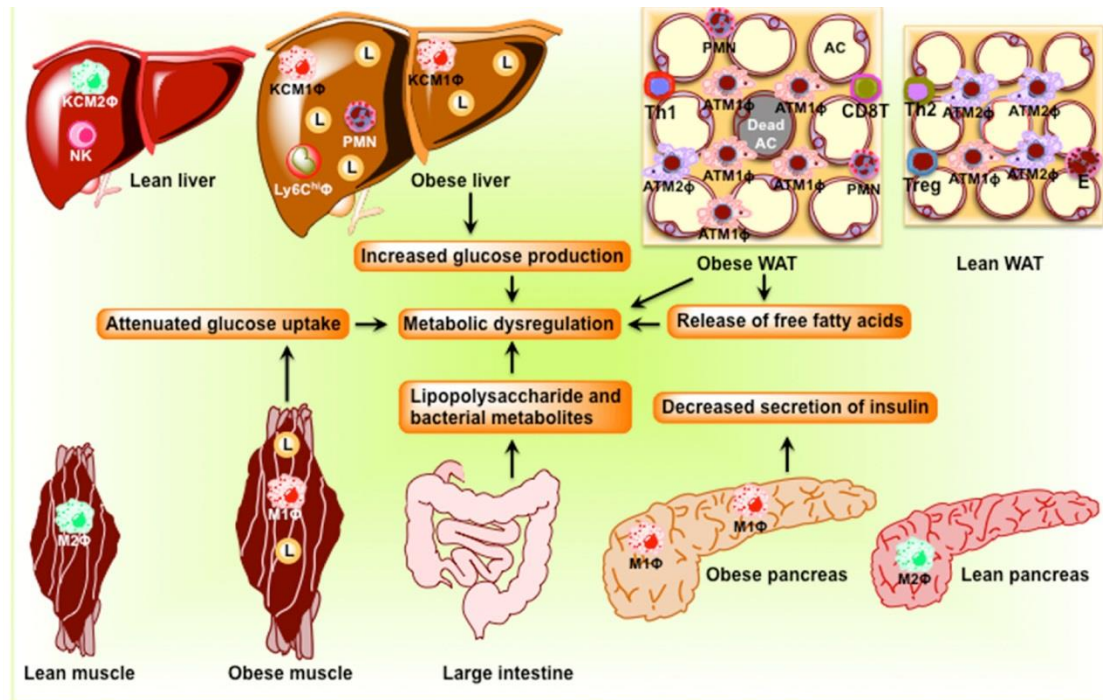
Figure 7. Schematic presentation of obesity-induced inflammation in the peripheral organs. During obesity, inflammatory reactions are mediated by tissue-specific macrophages in adipose tissue, the liver, skeletal muscle and the pancreas. During obesity, tissue-specific macrophages change their phenotype from M2ϕ (alternatively activated macrophages) to M1ϕ (classically activated macrophages). Obesity-induced inflammation leads to the development of dysbiosis in the intestine. AC (adipocyte); KC (Kupffer cell); L (lipid droplets); AT (adipose tissue); NK (natural killer cell); PMN (polymorphonuclear neutrophil); WAT (white adipose tissue). Reprinted from distributed under the Creative Commons Attribution-Non-Commercial License (Ray et al., 2016).

1.6   Effect of hyperglycaemia on monocytes and macrophages

Despite recognising the critical role of macrophages in the regulation of metabolic processes and vascular compilations in diabetes, information about the direct effect of hyperglycaemia on the activation of human primary macrophages is still limited. M1 macrophages utilise the glycolytic pathway for the generation of energy, while M2 macrophages utilise fatty acids as an energy source, whereas PPAR δ and PPAR γ transcription factors, as well as co-activator PGC1β, are important for the stimulation mitochondrial biogenesis and fatty acid β-oxidation (Johnson et al., 2012; Tannahill et al., 2013; Vats et al., 2006). Experimental animal models and as well as in vitro models provided evidence demonstrating the effect of hyperglycaemia on the biology of monocytes and macrophages. The stimulating effect of hyperglycaemia on

the leukocytosis resulting in an increased amount of circulating monocytes (predominantly the Ly6-C[hi] pro-inflammatory subset) and neutrophils, was demonstrated in mouse models of insulin-deficient diabetes (Nagareddy et al., 2013).

High-fat diet (HFD)-induced obesity leads to an accumulation of adipose tissue macrophages (ATMs), critical for the pathogenesis of T2D (Fujisaka et al., 2009). In a STZ-induced diabetic mice model it was found that hyperglycaemia induces the appearance of proinsulin (PI)-producing pro-inflammatory bone marrow (BM)-derived cells (PI-BMDCs) (Kojima et al., 2004). Furthermore, using a high-fat diet (HFD)-induced obesity mouse model, it was found that amelioration of hyperglycaemia by different hypoglycaemic agents prevented accumulation of PI-producing ATMs as well as adipose inflammation, the critical factors for the development of systemic insulin resistance (Buras et al., 2015).

The direct effect of hyperglycaemia on the production of increased amounts of TNF-alpha, IL-1beta and CCL2 was also identified using the human monocytic cell line THP-1 as a model, where the central role of NF-kB in response to THP-1 cells in hyperglycemic conditions was demonstrated (Dasu et al., 2007; Guha et al., 2000; Shanmugam et al., 2003a). In addition to activation of inflammatory cytokine release, a stimulating effect of hyperglycaemia adhesion of THP-1 cells to HUVEC monolayer was also shown; transmigration and stress fiber response was demonstrated (Nandy et al., 2011). These effects correlated with activation of the Akt, and glycogen synthase kinase (GSK3β) and PI-3 kinase pathways (Nandy et al., 2011). Hyperglycaemia was found to enhance the response of THP-1 cells to LPS. Thus, cultivation of THP-1 cells for 24 hours in the presence of high (25mM) versus normal (5.5mM) glucose concentrations led to a significant increase of LPS-induced release of TNF-alpha and CCL2 (Iwata et al., 2007). The effect of hyperglycaemia on cytokine release was also analysed in vitro using human primary PBMC-derived macrophages (Lachmandas et al., 2015). It was found that stimulation of M0 and M2 macrophages on day 6 with LPS and M. Tuberculosis lysate (H37Rv) slightly increased the release of both pro-inflammatory (TNF-alpha, IL6) and anti-inflammatory cytokines (IL10, IL-1Ra). However, the strongest effect was found upon the release of IL10 in M0, while effects of hyperglycaemia on the inducible production of TNF-alpha and IL6 were not more than 30%. The biological significance of the LPS and M. Tuberculosis – induced production of TNF-alpha and IL6 in hyperglycaemic conditions was questionable and was not associated with an increase in bacterial killing of phagocytic activity of macrophages. The limited biological

significance of the data obtained in this experiential system was also related to the differentiation of primary macrophages in RPMI medium, but not in macrophage-specific serum free medium (Lachmandas et al., 2015). Therefore, the information about the mechanisms of hyperglycaemia-mediated activation of macrophages is mostly limited to the data obtained in mouse models and in human THP-1 cells, which significantly differ in their biological activities from primary human macrophages. The current interpretation of the increased responsivity of macrophages to bacterial inflammatory stimuli is that hyperglycaemia can "prime" macrophages to inflammatory reactions (van Diepen et al., 2016).

1.7   Epigenetic mechanisms

Epigenetic mechanisms are responsible for the meiotically or mitotically heritable changes that regulate gene expression independent of changes in nucleotide sequence (Allis and Jenuwein, 2016; Egger et al., 2004). Epigenetic changes can be constitutive and cell-type specific or can be induced by several factors such as age, environment, lifestyle and disease state. These epigenetic modifications are reversible by modulation of environmental, dietary or pharmacological interventions (Allis and Jenuwein, 2016; Egger et al., 2004). There are three major epigenetic mechanisms that regulate gene expression: DNA methylation, histone modifications and non-coding RNAs (ncRNAs) (Allis and Jenuwein, 2016; Collins et al., 2011; Egger et al., 2004).

1.7.1   DNA methylation

DNA methylation is the first epigenetic mechanism that was identified. DNA methylation is an addition of methyl groups to the cytosine residue by enzymes named DNA methyltransferases (Du et al., 2015). DNA methylation most frequently occurs at CpG islands, which are associated with the promoter regions of genes (Deaton and Bird, 2011). DNA methylation results in the suppression of transcription (Dantas Machado et al., 2015; Riggs, 1975). DNA methylation is a critical component in many cellular processes, including embryonic development, genomic imprinting, X-chromosome inactivation and preservation of chromosomal stability (Horvath, 2013; Phillips, 2008). In cancer, DNA methylation is well characterised. In transformed cells, tumour suppressor genes can be downregulated by hypermethylation, while hypomethylation promotes activation of proto-oncogenes (Klutstein et al., 2016; Phillips, 2008). The process of DNA methylation occurs in dividing cells and is an essential mechanism that controls cell differentiation. DNA demethylation can be a passive of active process, where passive demethylation is a consequence of the absence of active

methylation during DNA replication in dividing cells. In contrast, active removal of methyl groups can be independent of DNA replication and can occur in non-dividing cells.

1.7.2 Histone modifications

Histones are major structural proteins of chromatin that interact with DNA and define chromatin structure. There are five major histones: H1, H2a, H2b, H3 and H4 (Bhasin et al., 2006). H2a, H2b, H3 and H4 are referred to as "core histones", while histone H1 is referred to as linker histone. The core histones form dimers, and these four distinct dimers come together to form one octameric nucleosome core. Histones are subjected to post-translational modifications including, among others, methylation, acetylation, phosphorylation, SUMOylation, ubiquitination, biotinylation, citrullination, ADP-ribosylation and cis-trans isomerization (Figure 8) (Egger et al., 2004; Latham and Dent, 2007). The histone code concept defines which post-translational modifications of histones alone or in combination modulate chromatin structure and function (Prakash and Fournier, 2016). Activated histone modifications are associated with the formation of transcriptionally active euchromatin. Repressing histone modifications leads to the formation of transcriptionally inactive heterochromatin. Major histone modifications that are involved in the regulation of gene expression include acetylation and methylation (Egger et al., 2004; Latham and Dent, 2007). Acetylation of histones at any amino acid and any position acts as an activating mechanism for transcription. Histone methylation can act both as an activating and repressing mechanism depending on the type and position of amino acid, and the amount of methyl groups added. For example, addition of single, double or triple methyl groups to the lysine (K4) on a histone 3 (H3) acts as an activating histone code, while the addition of single, double or triple methyl groups to lysine 9 (K9) on histone 3 (H3) acts as a repressing histone code (H3K9me3). However, mono-methylation of lysine 27 (K27) on histone 3 (H3) is an activating histone mark (H3K27me1), and tri-methylation of lysine 27 (K27) on histone 3 (H3) is a repressing histone mark (H3K27me3) (Barski et al., 2007; Egger et al., 2004).
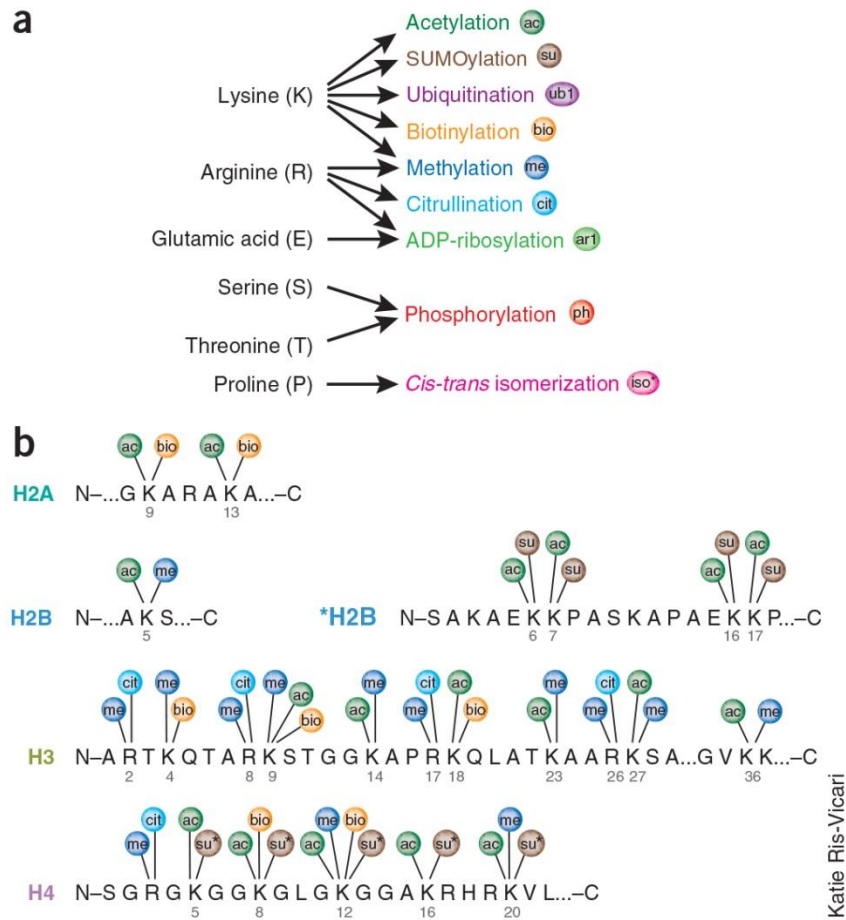
Figure 8. Post-translational modifications of histones. (A) Types of post-translational modifications at the amino acid residues (B) Amino acid residues that can undergo different forms of post-translational modification or can cross-talk. Each modification inhibits subsequent modification. Numbers indicate the position of amino acid. Permission obtained from Copyright Nature Publishing Group (Latham and Dent, 2007).

Patterns of histone modifications correlate with the dynamics of chromatin (Peterson and Laniel). Histone modifications control structure and chromatin fibres, where site-specific combinations define particular biological functions. Each type of histone modification is catalysed by a specific class of enzymes (histone acetyltransferases/HATs, histone methyltransferases/HMTs). These modifications are reversible and can be removed by histone deacetylases/HDATs and histone demethylases/HDMs correspondingly (Figure 9) (Arrowsmith et al., 2012). The histone code is a highly flexible system and provides the possibility of the cell to respond to a range of extracellular stimuli, including inflammatory and metabolic stimuli, without the event of cell division. Therefore, the histone code is the major epigenetic mechanism that controls activation of phenotypically and functionally plastic cells--macrophages (Ahmed et al., 2016). The histone code can act in cooperation with

DNA methylation to define the chromatic structure. Moreover, histone modification can be recognised by a number of proteins that control transcription of specific genes ("readers" of the histone code).
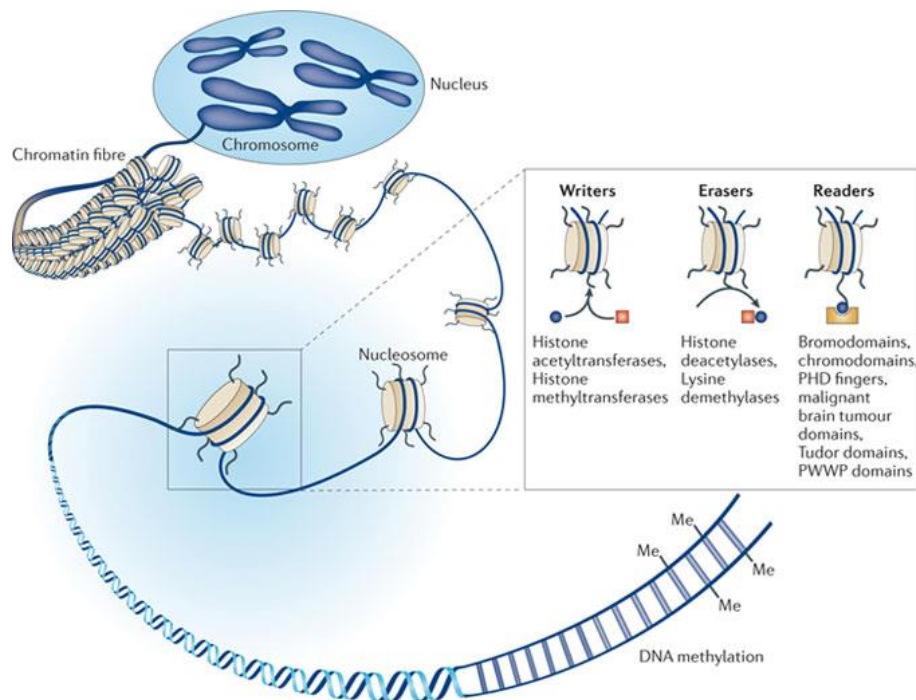
Figure 9. Epigenetic regulation of gene expression by histone modifications. Permission obtained from Copyright Nature Publishing Group (Arrowsmith et al., 2012).

1.7.3   Non-coding RNA

A non-coding RNA is a functional RNA molecule that is transcribed from DNA but is not translated into a protein (Collins et al., 2011). Epigenetically related classes of ncRNAs include 1) long ncRNAs, and 2) short ncRNAs including miRNA and siRNA (Collins et al., 2011; Kurokawa et al., 2009). All these ncRNAs are also called RNAi. The short and long ncRNAs are involved in chromatin-mediated gene silencing and DNA rearrangements (Collins and Penny, 2009; Gangaraju and Lin, 2009). A study by Wang et al. demonstrated that maize organ-specific distributions of canonical miRNAs and endogenous siRNAs have been linked to epigenetic modifications on the level of DNA methylation and the histone code (Wang et al., 2009). It was demonstrated that long ncRNAs and miRNAs are involved in diabetic complications (Beltrami et al., 2015; Prattichizzo et al., 2015a; Reddy et al., 2015).

1.8 Epigenetic control of macrophage activation

All three epigenetic mechanisms contribute to the development of differentiated macrophage phenotypes. Whole-genome bisulfite sequencing revealed that human primary monocytes undergo significant DNA demethylation during their differentiation into macrophages (Wallner et al., 2016). 114 DMRs (differentially methylated regions) that have a methylation difference equal or greater than 0.3 were identified, where 110 out of 114 were demethylated during monocyte-macrophage differentiation. This study demonstrated that DNA demethylation is a critical process for the differentiation of monocytes to macrophages, providing conditions for the next level of regulation of chromatin structure and function by the modification of the histone code (Wallner et al., 2016). In macrophages, M1 and M2 activation and differentiation states were demonstrated to depend significantly on post-translational modifications of histones (Kapellos and Iqbal, 2016b; Saeed et al., 2014). Histone modifications such as methylation, acetylation, demethylation and deacetylation influence the development of macrophage phenotypes (Medzhitov and Horng, 2009; Saeed et al., 2014; Takeuch and Akira, 2011). It was demonstrated that IL4 stimulated bone marrow derived macrophages induce the STAT6-dependent jmjd3 protein, which leads to a decrease in H3K27me2/3 levels and increases the H3K4me3 levels on M2 macrophage markers' promoter regions (Ishii et al., 2009; Takeuch and Akira, 2011). ChIP-qPCR demonstrated changes of histone marks on M2 markers (chi3I3, retnla and Arg 1) (Ishii et al., 2009). This study also demonstrated that a decrease in H3K27 methylation is associated with increased recruitment of Jmjd3 in M2 macrophages (Ishii et al., 2009). Epigenetic changes during differentiation of human monocytes into naïve macrophages (without stimulation), trained immunity macrophages (stimulated with beta-glucan) and innate immune macrophages (stimulated with LPS) were found at promoter sites (Saeed et al., 2014). A dynamic change for the association of activating histone marks H3K4me1, H3K4me3 and H3K27ac with chromatin was demonstrated during monocyte to macrophage differentiation in all three settings (Saeed et al., 2014). It was also shown that H3K27 acetylation was decreased on 1240 promoters and increased on 1307 other promoters, further supporting the importance of histone modifications in the regulation of gene expression during macrophage differentiation (Saeed et al., 2014). It was also demonstrated that a miRNA signature was responsible for the polarisation of human monocyte-derived macrophages under stimulation with IFNg + TNF-alpha (M1), IL4 (M2a) and IL10 (M2c) (Cobos Jimenez et al., 2014).

1.9 Epigenetics in diabetes

Epigenetic mechanisms are involved in the development of diabetic complications and can be a major mechanism underlying the phenomenon of metabolic memory (Prattichizzo et al., 2015a; Reddy et al., 2015). Lifestyle factors such as nutrition, the level of physical activity and cigarette smoking can induce T2D by influencing epigenetic factors (Reddy et al., 2015). Diabetes and metabolic factors can induce several growth factors and lipids that can stimulate multiple signalling pathways, transcription factors (TFs) and crosstalk with epigenetic networks (Prattichizzo et al., 2015a; Reddy et al., 2015). These events can change the structure of chromatin and regulate the pathological genes relevant to diabetic complications. Persistence of such epigenetic aberrations, including a change in DNA methylation, histone code and ncRNA profiles, may lead to metabolic memory, which results in an increased risk of developing diabetic complications even after normalisation of hyperglycaemia (Reddy et al., 2015).

Diabetes and diabetogenic agents such as high glucose, AGEs, Ang II, TGF-beta and oxidized lipids have detrimental effects on several cell types involved in vascular dysfunction, such as endothelial cells, vascular smooth muscle cells and renal cells (Figure 10) (Beckman et al., 2002; Natarajan and Nadler, 2004; Reddy et al., 2015). These diabetogenic agents activate several signalling pathways that lead to the activation of transcriptional factors, such as NF-kB and SMADs. Activation of NF-kB and SMADs results in the production of growth factors, inflammatory chemokines and cytokines involved in dysfunction of endothelial cells and inflammation, that lead to the progression of vascular complications (Figure 10) (Reddy et al., 2015). Although hyperglycaemia can be controlled by medications, exercise and dietary modifications, many patients still continue to experience numerous vascular complications (Reddy et al., 2015). This could be due to so-called "metabolic memory" of prior exposure of target cells to hyperglycaemia, where epigenetic mechanisms play an essential role (Figure 10) (Reddy et al., 2015).

Diabetes–associated epigenetic mechanisms were mostly studied in endothelial cells. It was demonstrated that in primary human aortic endothelial cells that transient hyperglycaemia causes persistent atherogenic effects by inducing long-lasting changes in chromatin remodelling through the recruitment of the histone methyltransferase SET7. Recruitment of SET7 is associated with increased H3K4 monomethylation at the NF-κB promoter leading to increased expression of p65, MCP-1 and VCAM-1 (El-Osta et al., 2008). Brasacchio et al.

have demonstrated that transient hyperglycaemia induced the enrichment of SET7 (responsible for monomethylation of H3K4) on the NFkappaB-p65 promoter in bovine aortic endothelial cells; knockdown of SET7 attenuated glucose-induced activation of the p65 gene (Brasacchio et al., 2009). Transient hyperglycaemia stimulated lysine-specific demethylase 1 (LSD1) resulting in reduced H3K9 methylation and increased NFκB-p65 expression (Brasacchio et al., 2009). However, whether the same epigenetic mechanisms that were identified in the endothelial cells are induced by hyperglycaemia in other cell types, including macrophages, remain to be identified.
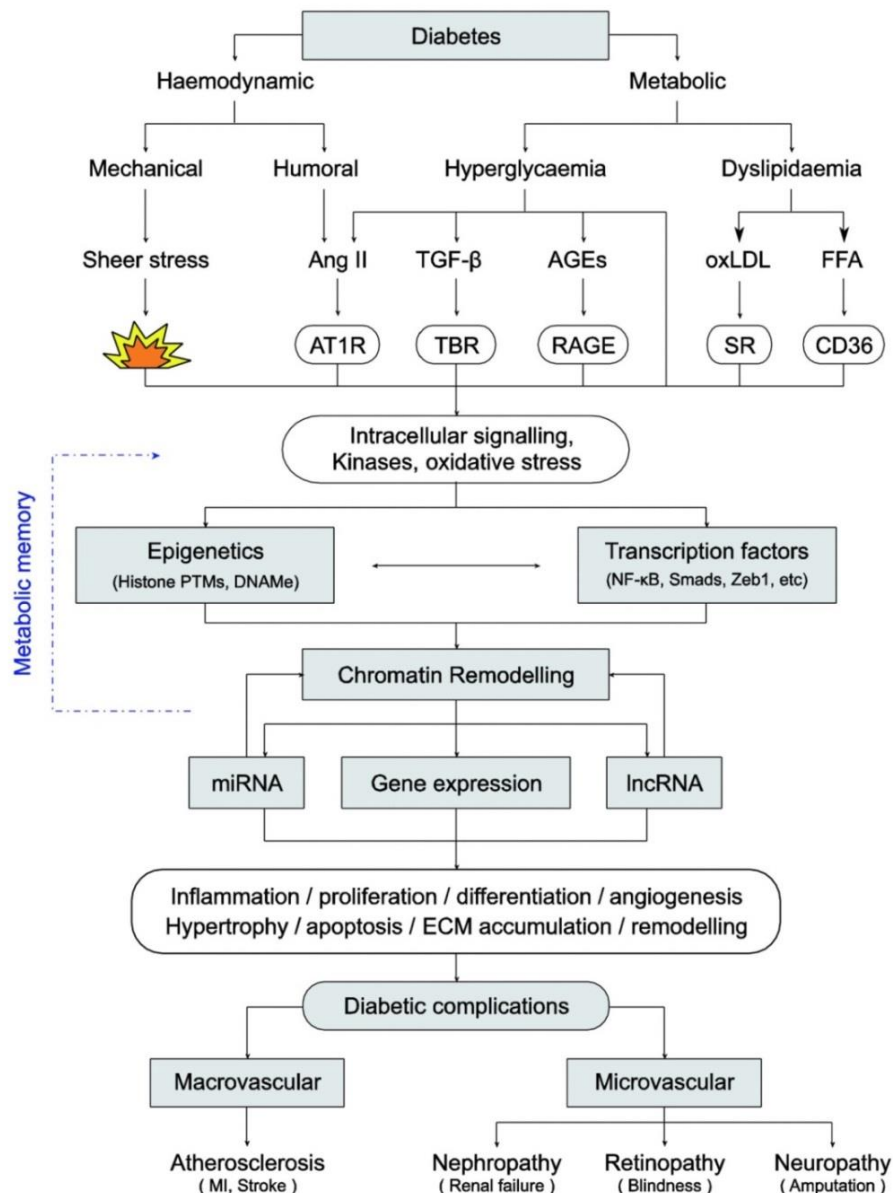
Figure 10. Signalling and epigenetic networks mediating the pathogenesis of diabetic complications and metabolic memory. AT1R (Ang II type 1 receptor); MI (myocardial infarction); oxLDL (Oxidized-LDL); RAGE (Receptor for AGEs); SR (scavenger receptors); TBR (TGF-β receptor). Permission obtained from copyright Springer (Reddy et al., 2015).

1.10 The aims and objectives of the project

The major goal of the current study was to analyse the effect of hyperglycaemia on the programming of primary human macrophages related to the development of diabetes and its vascular complications. Despite recognising the critical role of inflammation in the progression of diabetes and the development of diabetic vascular complications, the direct effect of hyperglycaemia on the epigenetic programming of pro-inflammatory activities of human primary macrophages remained to be identified. In this study, for the first time, we established a model system for the analysis of primary human monocyte to macrophage differentiation in normal and hyperglycaemic conditions that enabled the analysis of the effect of hyperglycaemia on the activation and epigenetic programming of macrophages.

The specific aims of the present study were:

1. Identification of hyperglycaemia-induced changes in gene expression and secretion of major M1 and M2 marker cytokines in human primary M0, M1 and M2 macrophages.

2. Identification of the complete program of transcriptional changes induced by hyperglycaemia in human primary M0, M1 and M2 by Affymetrix DNA microarray.

3. To analyse functional changes in macrophages under hyperglycaemic conditions

4. To analyse the role of the histone code in the regulation of hyperglycaemia-induced gene expression by chromatin immunoprecipitation assays (ChIP).

2. <u>Materials and Methods</u>

This work was written with the operating system *Windows 10* and the text processing program *Microsoft Office Word 2010*. The presented pictures, as well as numerous reports, have been processed using the programs *Microsoft Office Excel 2010*, *GraphPad Prism 5*, *ImageJ*, *Corel Draw Graphics Suite 12* and *Photoshop CS6*

2.1   Chemicals, reagents and kits

Product	Company
0.05% Trypsin/EDTA solution	Biochrom AG
10x Incomplete PCR buffer	BIORON
30% Acrylamide/Bis Solution, 37.5:1	Bio-rad
4′.6-diamidino-2-phenylindole (DAPI)	Roche
50x Tris-Acetate EDTA (TAE) buffer	Eppendorf
Agarose	Roth
Amersham Hyperfilm ECL	GE Healthcare
Ammonium persulfate (APS)	Merck Millipore
Bovine Serum Albumin (BSA)	Sigma
Calf Intestinal Alkaline Phosphatase	Thermo Scientific
Complete, EDTA-free Protease Inhibitor Cocktail Tablets	Roche
Dako Fluorescent Mounting Medium	Dako Cytomation
Deoxyribonucleotides (dNTPs) 10M	Fermentas
DEPC Water	Thermo Scientific
Dimethylsulfoxide (DMSO)	Sigma
DMEM medium	Life Technologies
DNase Buffer(10x)	Thermo Scientific
DNase I lyophilized	Roche
DNase I RNase free 1U/μl solution	Fermentas
DRAQ5	Biostatus Ltd.
Ethanol	Roth
Expand High Fidelity PCR Kit	Roche
Foetal calf serum (FCS)	Biochrom AG
Gel Code Blue stain reagent	Pierce
GeneRuler DNA ladder	Fermentas
Glycerol	Sigma
Hepes buffer	Sigma
Isopropanol	Merck Millipore
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Sigma
Laemmli sample buffer	Bio-Rad
LB broth / agar (Lennox)	Sigma
Loading dye 6x	Fermentas
Methanol	Merck Millipore
$MgCl_2$	Sigma
$MnCl_2$ 1 M	Sigma

Product	Company
Gel Red DNA Gel Stain	Biotium
Oligo(dt) primer	Thermo Scientific
Omnipure-OLS kit	Omnilife Science
Page Ruler Plus Prestained Protein Ladder (10-250 kD)	Fermentas
Paraformaldehyde (PFA)	Fluke
PBS Dulbecco, w/o Ca $^{2+}$, Mg $^{2+}$	Biochrom AG
PCR primers	Eurofins MWG Operon
PCR probes	Eurofins MWG Operon
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Phosphate buffered saline (D-PBS), sterile 1x	Invitrogen
Polybrene	Sigma
Poly-D-Lysine	Sigma
Protein Assay Reagent	Bio-Rad
Protein G Sepharose	Amersham Biosciences
Sodium dodecyl sulphate (SDS) 10%	Bio-Rad
Sodium pyruvate (100mM)	Sigma
ß-Mercaptoethanol	Sigma
Subcloning Efficiency DH5α Competent Cells	Life Technologies
Taq polymerase 5 u/µl	BIORON
TEMED	Genaxxon
Triton X-100	Sigma
Tween 20	Sigma

2.2 Consumables

Product	Company
22x22mm coverslips	Marienfeld
22µm filters	Fisherbrand
Cell culture plates	Greiner
Cryovials	Nunc
Glass slides	Servoprax
Parafilm	American National Can
PCR tubes	Star Labs
Petri dishes	Star Labs
Pipette tips	Eppendorf
Pipettes	Gilson, Eppendorf
Plastic wrap	Topics
Safe-Lock Eppendorf Tubes, 1.5ml	Eppendorf
Scalpel	Feather
Sterile Pipette tips	Avantguard, Star Labs, Nerbeplus
Tubes	Falcon

2.3 Equipment

Equipment used in the study

Product	Company
Agarose electrophoresis unit i-Mupid	Erogentec
Autoclave VX-95	Systec
BD FACSCanto II	BD
Cell counter	Scharfe system
Centrifuge 5415 D	Eppendorf
Centrifuge 6K15	Sigma
Centrifuge RC 5C Plus	Sorvall
Cryo freezing container	Nalgene
Deep freezer (-80°C)	Sanyo
Electrophoresis comb	Peqlab
Electrophoresis power supply	Peqlab
Freezer (-20°C)	Liebherr
Glass plates	Peqlab
Glass syringe	Hamilton
Homogenizer Ultra Turrax T8	Ika-werke
Ice machine	Scotsman AF100
Incubator 37°C	Edmund Bühler GmbH
Inverted microscope	Leica
Laminar flow hood	Thermo
Confocal software	Leica
TCS SP2 laser scanning spectral confocal microscope	Leica
Magnetic stirrer.MR3000	Heidolph
Microwave oven	Sharp
Neubauer haemocytometer	Assistent
Pipette Controller	Accu Jet Pro, Brand
Roller	Ortho Diagnostic Systems
Rotator	Neolab
SDS-PAGE chamber	Peqlab
SDS-PAGE gel comb	Peqlab
SDS-PAGE power unit Power-Pac 200	Bio-Rad
SDS-PAGE unit	Biometra
Shaker KS 260 basic	IKA
Sorvall RC5C Plus ultracentrifuge	Thermo Scientific
Staining Dish	Neolab
Staining rack	Neolab
Tecan Infinite 200	Tecan
Thermocycler DNA Engine PTC220 Dyad	MJ Research
Thermomixer 5436	Eppendorf
Thermomixer comfort	Eppendorf
Tweezers	Neolab
Ultracentrifuge tubes 50ml	Thermo Scientific

Product	Company
UV fluorescent light	Peqlab
UV/Stratalinker 1800	Stratagene
UV/Visible spectrophotometer Ultraspec 3000	Amersham
Vortex Genie 2	Scientific Industries
Water bath	Memmert
Water bath SW-21	Julabo
Sonicator (Bioruptor)	Diagenode

2.4 Kits

Following kits were used in the study

E.Z.N.A. Total RNA Kit I	Omega bio-tek
QIAquick PCR Purification Kit	Qiagen
RevertAid H Minus First Strand Synthesis Kit	Fermentas
RNAeasy mini kit	Qiagen

2.5 Buffers and solutions

Buffers used for chromatin immunoprecipitation are listed in the order of application

ChIP buffer	50 mM HEPES-KOH pH7.5 140 mM NaCl 0.1% SDS 1% Triton X-100 0.1% sodium deoxycholate 1 mM EDTA pH8 Protease inhibitors (Cat: 04693159001 Roche), freshly added to the buffer
RIPA Buffer	50 mM Tris-HCl pH8 150 mM NaCl 0.1% SDS 1% NP-40 0.5% sodium deoxycholate 2 mM EDTA pH8 Protease inhibitors (Cat: 04693159001 Roche), freshly added to the buffer
Low Salt Wash Buffer	20 mM Tris-HCl pH 8.0 150 mM NaCl 0.1% SDS 1% Triton X-100 2 mM EDTA
High Salt Wash Buffer	20 mM Tris-HCl pH 8.0 500 mM NaCl 0.1% SDS 1% Triton X-100 2 mM EDTA
LiCl Wash Buffer	10 mM Tris-HCl pH 8.0 0.25 M LiCl

	1% NP-40
	1% sodium deoxycholate
	1 mM EDTA
TE Buffer	10 mM Tris pH 8
	0 1 mM EDTA
Elution Buffer	100 mM NaHCO3
	1% SDS

2.6 Isolation of human CD14+ monocytes from buffy coats

Buffy coats were provided by the German Red Cross Blood Service Baden-Württemberg – Hessen. Buffy coats were obtained from healthy blood donors after informed consent. For each buffy coat isolation (each donor) a unique number was given. The volume of buffy was usually 30 ml. Buffy coats were provided in protective bags. CD14+ monocytes were isolated out of buffy coats according to the following procedure, performed under sterile conditions. During the isolation procedure, on several steps, sterile PBS without Ca^{2+} and Mg^{2+} was used (indicated as PBS in the protocol).

1) First gradient: Biocoll gradient was prepared by addition of 15 ml of biocoll separating solution in a 50 ml falcon tube.

2) Buffy coat package was cut, the contents were placed in a sterile T-75 flask and diluted 1:1 with PBS.

3) The diluted contents of the buffy coat was added to the 1st gradient falcon tube and centrifuged at 420 rcf for 30 min without breaks.

4) First washing step: the upper layer (serum) was discarded and the second layer (enriched PBMC fraction) was collected in a fresh 50 ml falcon tube and filled up with PBS to 50 ml. Centrifugation was performed at 420 rcf for 10 min with breaks (first wash).

5) Second washing step: the supernatant was discarded, and the cell pellet was resuspended in 3 ml of PBS and filled up to 50 ml with PBS. Centrifugation was performed at 420 rcf for 10 min.

6) Third washing step and cell counting: supernatant was discarded, and the cell pellet was resuspended in 3 ml PBS and filled up to the 50 ml with PBS. Centrifugation was performed at 420 rcf for 10 min.

7) An aliquot of 10 µl was taken for cell counting from the 50 ml suspension and mixed with 10 ml of casytone solution.

8) Second gradient: The Percoll gradient solution was prepared by mixing 13.5 ml Percoll, 15 ml MEM medium and 1.5 ml 10x Earle´s salt solution per 30 ml sample and mixed well.

9) After the final wash, the cell pellet was resuspended in 4 ml PBS and the cell suspension was applied on the top of the percoll gradient very slow and carefully. The cells were centrifuged at 420 rcf for 30 min without breaks.

10) The PBMC fraction (upper layer) was carefully collected and transferred to a 50 ml falcon tube and filled up to 50 ml with PBS and centrifuged at 420 rcf for 10 min with breaks.

11) The supernatant was discarded, the cell pellet was thoroughly resuspended in 2 ml of PBS and transferred into a 15 ml falcon tube. PBS was added to obtain a volume of 10 ml; centrifugation was performed at 420 rcf for 10 min.

12) The cell pellet was resuspended in 95 µl of MACS buffer (0.5% BSA, 2 mM EDTA dissolved in PBS) and 5 µl CD14 microbeads per 10^7 cells (for the selection of CD14 positive monocytes).

13) The cells were incubated for 15 min on a rotator at 4°C. The final volume was made up to 10 ml with MACS buffer and centrifuged at 420 rcf for 10 min. The cell pellet was resuspended in 1000 µl of MACS buffer.

14) An LS separation column was placed in the magnetic separation unit and washed with 3 ml MACS buffer. A fresh collecting tube was placed, and the cell suspension (1000 µl) was applied onto the LS column.

15) The column was washed 3 times with 3 ml MACS buffer. The LS column was removed from the magnetic separation unit and placed on a fresh 15 ml falcon tube.

16) The CD14+ monocytes were eluted from the column with 5 ml MACS buffer in a 15 ml falcon tube. The cells were sedimented by centrifugation at 420 rcf for 10 min.

17) The cell pellet was resuspended in SFM (serum-free medium) medium at a concentration of 1×10^6 cells/ml. Cells were seeded in cell culture dishes and stimulated with different factors: IFNg at 100 ng/ml, IL4 at 10 ng/ml and M-CSF at 5 ng/ml were used as indicated in each specific experimental setup in the section results.

2.6.1 Culturing primary human macrophages

Glucose-free SFM medium was custom-made and purchased from Life Technologies. Glucose stock was added to the medium until a final concentration of 5mM and 25mM. Glucose concentration was controlled by an Accu-Chek glucose monitor.

1) Isolated cells were cultured in SFM normal glucose medium (5mM) or high glucose medium (25mM) with 5 ng/ml of M-CSF.

2) Cells were suspended at 1×10^6 cells per ml of medium.

3) Cells were plated in 10cm dishes (10-15ml).

4) Cells were plated and stimulated with specific stimulations to differentiate into specific subtypes of macrophages [non-stimulation (M0), IFNg (M1) and IL4 (M2)] in normal and high glucose for 6 days.

2.6.2   Collecting supernatants

1) Supernatants were harvested on day 1, day 3 and day 6.

2) These supernatants were frozen in a -80°C freezer until analysis by ELISA.

2.7   RNA-related methods

2.7.1   Isolation of total RNA

For RNA Isolation, the E.Z.N.A. Total RNA Kit I was used.

The lysis buffer was prepared by addition of 10 µl of β-mercaptoethanol to 1 ml of the TRK buffer (guanidine isothiocyanate containing buffer which suppresses RNase activity) and mixed well.

1) Cells ($3-5X10^6$ cells) were harvested and lysed in 350 µl of lysis buffer. Cells were completely disrupted by passing the lysate 10-15 times through a needle fitted with a syringe. An equal volume of 70% ethanol was added to the lysate. Each sample was vortexed and applied to a HiBind RNA spin column placed into a 2 ml collection tube.

2) The column was centrifuged at 10,000 g for 1 min. After centrifugation, the flow-through was discarded, and the column was washed once with 500 µl of RNA wash buffer I and two times with 500 µl of RNA wash buffer II.

3) After the last centrifugation, the column was placed in a fresh RNase-free microcentrifuge tube. The RNA was eluted 2 times with 50 µl of RNase-free water preheated at 70°C.

4) The concentration of isolated RNA was determined with a Tecan Infinite 200. The quality of the obtained RNA samples was analysed on a 1.5% agarose gel. Isolated samples were stored at -20°C until use.

2.7.2   RNA preparation for Affymetrix GeneChip analysis

Total RNA was prepared using the E.Z.N.A. Total RNA Kit I as described above.

1) The volume of RNA sample was adjusted to 100 µl.

2) 12 µl of 10X DNAase I buffer with $MgCl_2$ (Fermentas) and 10 µl of RNAse free DNAse I (Fermentas) were added to the sample and mixed well by pipetting.

3) DNA digestion was done at 37°C for 40 min in a thermal block followed by a clean-up procedure with the Rneasy kit from Qiagen.

4) 350 µl of RLT buffer was added to the sample and mixed well. 250 µl of 100% ethanol was added to the sample and mixed by pipetting.

5) The sample was applied to an RNeasy mini column and centrifuged at 10,000 g for 1 min. After centrifugation, the flow-through was discarded, and the column was washed two times with 500 µl of buffer RPE.

6) After the last centrifugation, the column was placed in a fresh RNase-free microcentrifuge tube. The RNA was eluted 2 times with 50 µl of RNase-free water preheated at 70°C.

7) The concentration of the RNA was measured by using a Tecan Infinite 200

8) The quality of RNA was examined by gel electrophoresis.

9) An aliquot of RNA was used for cDNA synthesis for RT-PCR or store separately from the total amount of RNA to avoid repetitive freeze/thaw cycles.

10) The rest of the isolated RNA was stored at $-80^{\circ}C$.

2.7.3 Hybridization and statistical analysis of gene chip microarray

Total RNA samples were provided to the core facility of the Medical Research Centre of the Medical Faculty Mannheim, University of Heidelberg. A final confirmation of RNA quality was performed using an Agilent 2100 Bioanalyzer by Ms. Carolina de la Torre in the core facility of the Medical Research Centre of the Medical Faculty Mannheim, University of Heidelberg. Next, synthesis of cDNA and hybridization was performed. For the hybridization, Human gene 1.0 from Affymetrix (cat N 901086) was used. Scanning of chips were performed by the technical assistant Ms. Carolina de la Torre in the core facility of the Medical Research Centre of the Medical Faculty Mannheim, University of Heidelberg. Statistical analysis was done by Dr. Carsten Sticht in the core facility of the Medical Research Centre of the Medical Faculty Mannheim.

2.7.4 First strand cDNA synthesis for RT-PCR

All RNA samples were digested with DNase I to remove genomic DNA contamination.

The following reaction set-up was used:

Total RNA	5 µl (1 µg)
10X DNAseI buffer with $MgCl_2$ (Fermentas)	1 µl
RNAse free DNAse I (Fermentas)	1 µl
Distilled water (RNAse free)	3 µl

Digestion was done at 37°C for 40 min and followed by inactivation of the enzyme at 70°C for 10 min.

For the cDNA synthesis, the RevertAid H Minus First Strand Synthesis Kit (Fermentas) was used. After DNAse I digestion, cDNA was synthesised according to the following protocol:

1) 1 µl of Oligo dT primer was added to the above RNA sample.

2) The volume was adjusted with ddH$_2$O up to 12 µl and annealing of the primer was done at 70°C for 5 min.

3) The samples were placed on ice for 1 min.

4) The following reagents were then added:

5X buffer for polymerase	4 µl
Ribolock RNAse inhibitor	1 µl
10 mM dNTP mix	2 µl
RevertAid H minus reverse transcriptase	1 µl

5) The reaction was incubated at 42°C for 1 h and activity of the enzyme was stopped by an additional incubation at 70°C for 10 min.

6) The cDNA sample was diluted 10 times with ddH$_2$O.

7) The PCR reaction and agarose gel electrophoresis with 2% agarose gel were done as described above.

2.7.5   Real-time PCR with Taqman probe

All primers and dual-labelled probes were obtained from Eurofins (Germany). Dual-labelled probes contained FAM on the 5' end and a BHQ1 quencher at the 3' end of the sequence. A list of used primers is shown in Table 10. All primer sequences are shown from the 5' end to 3' end direction.

2.7.6   Optimisation of primers for qRT-PCR

1) A primer optimisation assay was done for each gene to obtain the optimal probe and primer concentration to generate the lowest Ct value.

2) A combination of three concentrations (50nM, 300nM and 900 nM) of both forward and reverse primers with a constant probe concentration (250nM) was used.

3) The combination showing the lowest Ct value for forward and reverse primers was fixed, and TaqMan probe optimisation was optimised with five different TaqMan probe concentrations (50nM, 100nM, 150nM, 200nM and 250nM).

4) The combination of primer and TaqMan probe concentration that yielded optimal assay performance was chosen for further experiments.

Table 1. List of primers used for Real-Time PCR. Primer concentrations were optimised according to section 2.7.6. Primers for coding sequence (cs) or promoter region (promoter) were used in the study

Name of gene	Primer names	Primer sequence	Primer concentration
Human GAPDH Cs	FP848	TCCATGACAACTTTGGTATCGT	18 μM
	RP848	CAGTCTTCTGGGTGGCAGTGA	6 μM
	Pr849	AAGGACTCATGACCACAGTCCATGCC	4 μM
CCL18 cs	FP2020	ATACCTCCTGGCAGATTCCAC	18 μM
	RP2020	GCTGATGTATTTCTGGACCCAC	6 μM
	Pr2020	CAAGCCAGGTGTCATCCTCCTAACCAAGAGAG	4 μM
TNF-alpha Cs	FP896	TCTTCTCGAACCCCGAGTGA	18 μM
	RP896	AGCTGCCCCTCAGCTTGA	18 μM
	Pr896	AAGCCTGTAGCCCATGTTGTAGCAAACC	5 μM
IL-1beta Cs	FP2150	ACAGATGAAGTGCTCCTTCCA	18 μM
	RP2150	GTCGGAGATTCGTAGCTGGAT	18 μM
	Pr2150	CTCTGCCCTCTGGATGGCGG	5 μM
IL-1Ra Cs	FP2145	GAAGATGTGCCTGTCCTGTGT	18 μM
	RP2145	CGCTCAGGTCAGTGATGTTAA	18 μM
	Pr2145	TGGTGATGAGACCAGACTCCAGCTG	5 μM
CCR2 Cs	FP2241	GACCAGGAAAGAATGTGAAAGTGA	18 μM
	RP2241	GCTCTGCCAATTGACTTTCCT	18 μM
	Pr2241	CACAAGGACTCCTCGATGGTCGTGG	4 μM
18srRNA	FP2242	CCATTCGAACGTCTGCCCTAT	6 μM
	RP2242	TCACCCGTGGTCACCATG	18 μM
	Pr2242	ACTTTCGATGGTAGTCGCCGTGCCT	5 μM
CCR2 promotor	FP2244	GCACAACGATTGTCAGGAAA	18 μM
	RP2244	TTGGTGGAAGCTTCTTAGGG	18 μM
	Pr2244	CGTCACCCTACTTGGAGCAGAGCA	5 μM
IL-1beta promotor	FP2245	AAAGACTCCAAGCCCACAAT	18 μM
	RP2245	CCTAACCTCTAGCCCAGCAG	18 μM
	Pr2245	TCCCTCTCCCTCCAAGCCACA	5 μM

A TaqMan primer mix for each gene was prepared according to the concentration in Table 1. For analysis of gene expression, a real-time PCR reaction was set up with the following components (for single reaction):

TaqMan Gene Expression Master Mix	5 μl
TaqMan primer mix (target)	0.5 μl
TaqMan primer mix (reference)	0.5 μl
cDNA	1 μl
Distilled water	3 μl

Usually, master mix without cDNA was prepared for the planned amount of reactions (20-90). Each cDNA sample was analysed in triplicates. For normalisation, the expression of 18srRNA mRNA was used for each reaction. Amplification was performed using a LightCycler 480 instrument. The following program was used:

1 cycle	95°C	3 min
50 cycles	95°C	10 sec
	60°C	30 sec

2.8 Protein techniques

2.8.1 ELISA

ELISA was used to identify concentrations of secreted factors in macrophage conditioned supernatants (described as "samples" in the protocols). Sample preparation is described in Table 4. All procedures were performed at RT.

1) A 96 well Plate was coated with capture antibody provided in the kit for overnight incubation.

2) The next day these plates were washed with wash buffer (PBS with 0.01% Tween 20) and incubated with 1% BSA/PBS for 1 hour for blocking.

3) After blocking, the 96 well plate was washed 3 times; 50µl of 1% BSA/PBS and 50µl of the sample was added to each well.

4) The standard was added according to the manufacturer's instructions and incubated for 2 hours.

5) After incubation, the plate was washed 3 times with wash buffer.

6) 100µl of detection antibody was added according to the manufacturer's instructions and incubated for 2 hours

7) 100µl of human-specific cytokine conjugated was added to each well and incubated for 20 min.

8) The substrate solution was added to each well and incubated for 20min.

9) Stop solution was added to stop the reaction.

10) The change of colour was measured in a Tecan Infinite 200 with the wavelength set according to the manufacturer's instructions.

Table 2. Sample preparation of ELISA. Macrophage supernatants were used in the non-diluted or diluted form to fit in a standard curve of ELISA. The optimal dilution was identified for each cytokine and each individual donor-derived macrophage culture.

Secreted cytokine analysed by ELISA	Macrophage stimulation	Dilution of supernatant
TNF-alpha	NS, IFNg, IL4	Non-diluted, all days analysed
IL-1beta	NS, IFNg, IL4	Non-diluted, all days analysed
IL-1Ra	IL4	Dilutions: 1:10 or 1:50, day 6
CCL18	IL4	Dilutions: 1:10 or 1:50, day 3 Dilutions 1:500, 1:1000 or 1:5000, day 6

2.9 Cell migration assay

1) For performing of the migration assay, the transwell inserts with 5μm pore membrane filters (BD Biosciences, San Jose, CA) were used.

2) Primary human monocyte-derived macrophages were placed on ice for 30 min to detach the cells and these cells were harvested for migration assays.

3) These cells were counted with the CASYton cell counter and $1*10^5$cells/100μl/well were loaded onto the upper chamber. CCL2 was used as a chemoattractant at a concentration of 100ng/ml (600 μl/well into the lower well compartment).

4) After overnight incubation at 37°C, the upper sides of the filters were carefully washed with cold PBS, and the remaining non-migrated cells were removed with a cotton swab.

5) The cells were fixed with ice-cold methanol (800μl /well) and incubated at -20°c for 5 min.

6) Methanol was removed and washed with ddH$_2$O 3 times for 10 min each at RT on a shaker.

7) The cells were stained with DAPI. The insert was removed, and the filter was cut with the help of a needle, and the filter was transferred onto a glass slide.

8) Each experiment was performed in triplicate with 6 individual donors.

9) The slides were allowed to dry in the dark and cells were counted under the fluorescence microscope with a 10x objective.

2.10 Chromatin immunoprecipitation assay (ChIP)

2.10.1 Cross-linking and cell harvesting

1) 10-15 million monocytes were plated in a 10cm cell culture Petri dish and cultured for 6 days in normal and high glucose conditions.

2) On day 6, conditioned medium was partially discarded. 5ml of the medium was left in the Petri dishes.

3) A total volume 312 μl of 16% Formaldehyde was added dropwise directly to the remaining medium to reach a final concentration of 1% and gently rotated at room temperature for 10 min.

4) 1M of glycine was prepared with ddH$_2$O water and added to the medium to make a final concentration of 325mM and incubated with shaking for 5min at RT.

5) The cells were washed 2X with 10 ml cold PBS.

6) The cells were scraped in 5 ml of cold PBS and transferred into a 10 ml tube.

7) The collected cells were centrifuged for 10 min at 420 g.

8) The supernatant was carefully aspirated, and the pellet was resuspended in ChiP lysis buffer (750µl per 10-15 million cells).

2.10.2 Sonication

1) The cell lysates were sonicated to shear DNA to an average size between 200 to 1000 bp. This step has to be optimised for different subtypes of macrophages.

2) In a biorupture sonicator, 15 cycles were used to sonicate the M1 macrophage samples.

3) After sonication, the samples were centrifuged for 10 min, 4°C, 8000 g.

4) Supernatants were transferred to a new 2 ml Eppendorf tube and used for immunoprecipitation (IP). The samples were stored at -80°C.

5) An aliquot of 50µl for each sonicated sample, containing fragmented chromatin ("Chromatin sample") was used as an input for the qRT-PCR control and was used to measure the DNA concentration and identify fragment size by gel electrophoresis.

2.10.3 Determination of amount of DNA and size of DNA fragments

1) The sonicated chromatin samples were used to calculate the concentration of DNA for subsequent IPs and measure DNA fragment size.

2) 70µl of elution buffer was added to the 50µl of "Chromatin sample".

3) 2µl of RNase A (0.5mg/ml) was added.

4) In order to remove the cross-links, the sample was incubated at 65°C with shaking overnight.

5) 2µl of RNase A (10mg/ml) and 2µl proteinase K (20mg/ml) were added and incubated at 45°C for 1hour.

6) Fragmented DNA was purified using QIAquick PCR Purification Kit according to the manufacturer's instructions. The final DNA was eluted in EB buffer. The concentration was determined with a Tecan Infinite 200.

7) The samples were stored at -20°C.

2.10.4 Immunoprecipitation

1) Sonicated chromatin samples were allocated equally to all the tubes (one tube per antibody).

2) Each sample was diluted 1:10 with RIPA buffer.

3) 4µg of primary antibody was added to each sample and incubated at 4°C for 1 hour with rotation.

4) For each specific antibody, an immunoprecipitation reaction using isotype matching IgG control was done in parallel.

5) Preparation of protein A/protein B beads: Salmon sperm DNA coated Protein A (Merck Millipore, Germany) and Salmon sperm DNA coated Protein G (Merck Millipore, Germany) were mixed equally and washed three times with RIPA buffer.

6) RIPA buffer was added at twice the volume of beads.

7) 100µl of protein A/G beads were added to all the samples and incubated overnight with rotation at 4°C.

8) Immunoprecipitated samples were centrifuged for 3 min at 1000 g and the supernatant was discarded.

9) The following washings were performed: once in low salt wash buffer, once with high salt wash buffer, once in LiCl wash buffer.

10) After each wash, samples were centrifuged for 3 min at 1000 g and supernatants were discarded.

2.10.5 Elution and removal of cross-linking

1. The DNA was eluted by adding 150µl of elution buffer to the protein A/G beads and vortexed slowly for 20 min at 30°C.

2. The tubes were centrifuged for 3 min at 1000 g and the supernatants were collected into fresh tubes.

3. 10µl of 5M NaCl was added to the supernatants and incubated at 65°C overnight with shaking.

4. 2 µl RNase A (10 mg/ml) and 2 µl proteinase K (20 mg/ml) were added and incubated while shaking at 45°C for 1 hour.

5. The DNA was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions.

6. The samples were stored at -20°C.

2.10.6 ChIP-qPCR

1. The amount of precipitated genomic DNA concentration was measured with a Tecan Infinite 200.

2. All the samples were diluted to make 1ng concentration of DNA, including IgG and input sample DNA. Relative expression of qChIP was normalised to IgG.

3. Primers and probes were designed using online tools. Diluted genomic DNA were quantitatively measured by real-time PCR.

2.10.7 ChiP antibodies

Table 3. Antibodies used for ChiP assay.

Name of the antibody	Name of the Company
H3K4me1	Abcam (Cat N ab8895)
H3K4me3	Merck Millipore (Cat N 07-473)
H3K9me3	Merck Millipore(Cat N 17-625)
H3K27me3	Merck Millipore(Cat N 17-622)
acetylated H3	Merck Millipore(Cat N 06-599)

2.11 Statistical analysis

2.11.1 Statistical analysis for qRT-PCR-and migration assays

To define the significance of the difference between groups of experimental data, paired two-tailed t-tests were performed using Microsoft Excel 2010. A p-value less than 0.05 was considered statistically significant. Standard error mean (SEM) was calculated for error bars.

2.11.2 Statistical analysis for Affymetrix Chip assay

Statistical analysis of microarray data was done by Dr. Carsten Sticht from the Affymetrix Core Facility of the Medical Research Centre, Medical Faculty Mannheim. For statistical analysis of differential expression during the microarray assay, the SAS software package microarray solution, version 1.3 was used. To map the probes to genes, a custom chip definition file was applied. Gene expression was analysed using a log-linear mixed model of perfect matches. To set the level of significance, a false discovery rate of a=0.05 with Bonferroni-correction for multiple testing was applied.

3. Results

3.1 Design of an in vitro model system to examine the effect of hyperglycaemia on the activation of primary human macrophages

To analyse the detrimental effect of high glucose on the differentiation and activation of monocytes and macrophages, a unique model system based on primary human monocyte-derived macrophages was established. CD14+ primary human monocytes isolated out of buffy coats were cultivated in serum–free medium (SFM). The SFM medium was produced on custom request as glucose free and supplemented with 5mM or 25mM glucose prior to use. M-CSF at a concentration of 5 ng/ml was added to optimise viability of macrophages in culture conditions. Three prototypes of macrophage subpopulations were generated subsequently: M0 (no cytokine stimulation), M1 (stimulated with IFNgamma) and M2 (stimulated with IL4). The general experimental design is schematically illustrated in Figure 11.
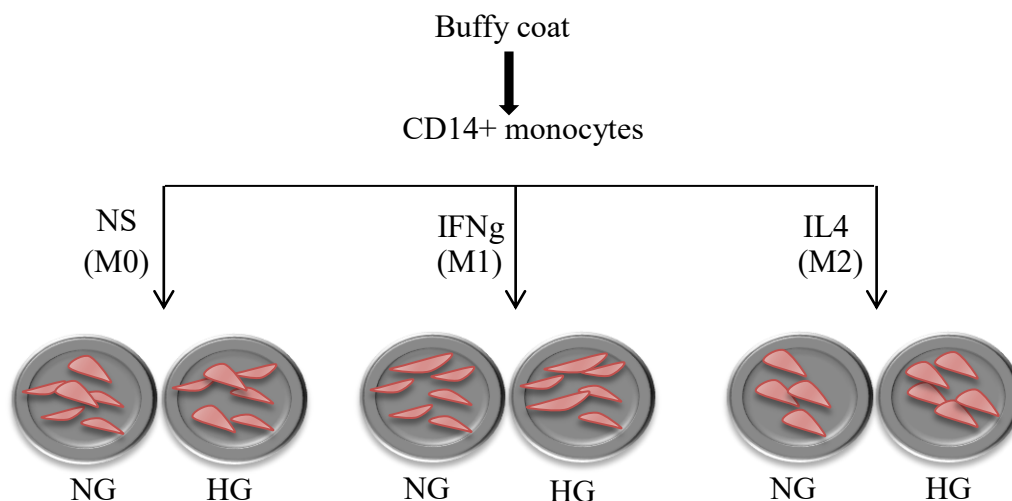
Techniques used: RT-PCR, ELISA, DNA microarray, ChIP assay, trans-well assay

Figure 11. Schematic diagram of model system developed to analyse macrophage responses to hyperglycaemia. Monocytes were isolated from buffy coats using density gradient centrifugation followed by CD14+ magnetic separation. Monocytes were cultivated in serum-free SFM medium supplemented with M-CSF (5 ng/ml) in the presence of normal glucose (NG, 5mM) or high glucose (HG, 25mM). M0: no additional cytokines added; M1: stimulated with IFNg; M2: stimulated with IL4. Gene expression was analysed on day 6 of cultivation by RT-PCR and DNA microarray. Secretion of cytokines was analysed in the supernatants harvested after 6h, 24h, 3 days and 6 days. The presence of histone marks on the promoter of selected genes were analysed by chromatin immunoprecipitation (ChIP) on day 6. Transmigration toward inflammatory stimuli was analysed in trans-well assays on day 6.

3.2 Effect of hyperglycaemia on TNF-alpha production in M0, M1 and M2 macrophages

TNF-alpha is a key inflammatory cytokine produced by macrophages, and its production is stimulated by IFNgamma (Martinez and Gordon, 2014a). Elevated TNF-alpha levels were found in diabetic conditions in humans (Mirza et al., 2012) and rats (Quan et al., 2011). However, it remains unclear whether hyperglycaemia can be an inducing factor for macrophages to continuously produce TNF-alpha in patients with metabolic syndrome and diabetes. The effect of hyperglycaemia on TNF-alpha release was examined in M0, M1 and M2 macrophages derived from eight individual donors. The inducing effect of hyperglycaemia on TNF-alpha secretion was found in 5 out of 8 analysed donors in M0, in 4 out of 8 donors in M1, and in 1 out of 8 donors in M2 (Figure 12). The effect was stronger after 24 hours of macrophage cultivation (up to 0.5 to 4 times elevation in M0 and 4 times in M1, Figure 12). The stimulatory effect of hyperglycaemia declined after 3 days and 6 days of macrophage cultivation (Figure 12). However, hyperglycaemia didn't show a stimulatory effect on TNF-alpha secretion from day 1 until day 6 in 3 donors in M0, 4 donors in M1 and 7 donors in M2 (Figure 12). RT-PCR analysis demonstrated that there is only a very slight increase (up to 40% in 3 donors) of TNF-alpha gene expression on day 6 (Figure 13). TNF-alpha is an acute inflammatory response factor induced during the first hours after stimulation with exogenous pathogens or adhesion events, therefore, it was further examined whether hyperglycaemia has an immediate effect on the gene expression of TNF-alpha (Figure 14). The mRNA levels of TNF-alpha in M0, M1 and M2 macrophages obtained from an additional 6 healthy donors have been analysed after 6 and 24 hours of exposure to hyperglycaemia. The stimulatory effect of high glucose on TNF-alpha mRNA levels in M1 after 6 hours was found in 5 out of 6 donors (Figure 14). The effect was more pronounced in M1 macrophages with the lowest levels of TNF-alpha mRNA in normal glucose conditions (Figure 14, donor 12 (2 times) and donor 13 (3.5 times)). The stimulatory effect of high glucose in M1 was not detectable after 24 hours, suggesting the activation of a classical negative feedback induced by acute inflammatory responses (Hu and Ivashkiv, 2009). These data indicate that hyperglycaemia has a clear stimulatory effect on TNF-alpha gene expression and release in primary human monocyte-derived macrophages, and this effect is more pronounced in INFg stimulated M1 macrophages.
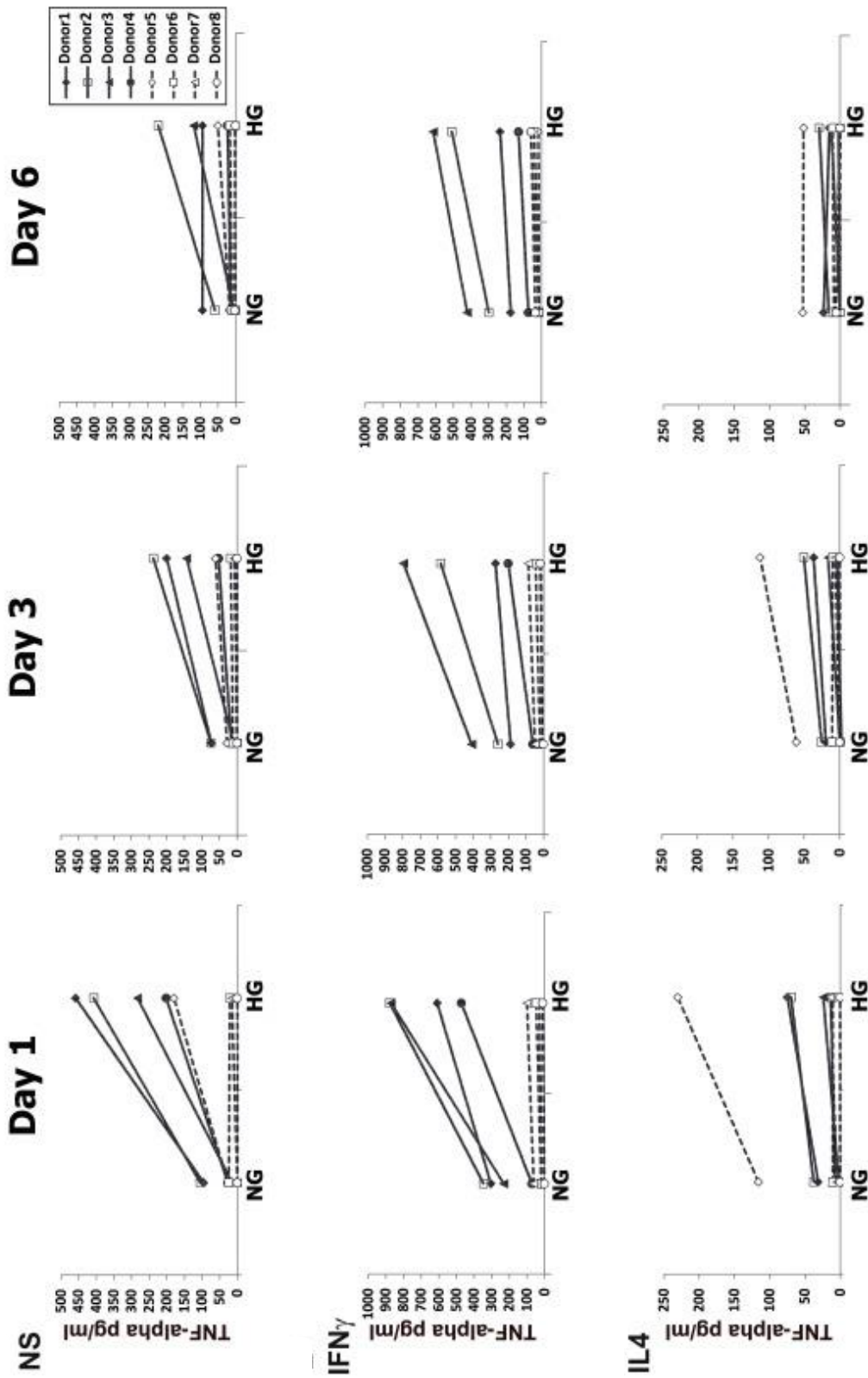
Figure 12. Effect of hyperglycaemia on TNF-alpha secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions Supernatants were collected on day 1, 3 and 6. ELISA analysis of TNF-alpha production by differently stimulated macrophages was measured in normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions. All experiments were performed in duplicates. The results are presented for individual donors (n=8) in normal and high glucose conditions. NS (M0), IFNg (M1) and IL4 (M2).
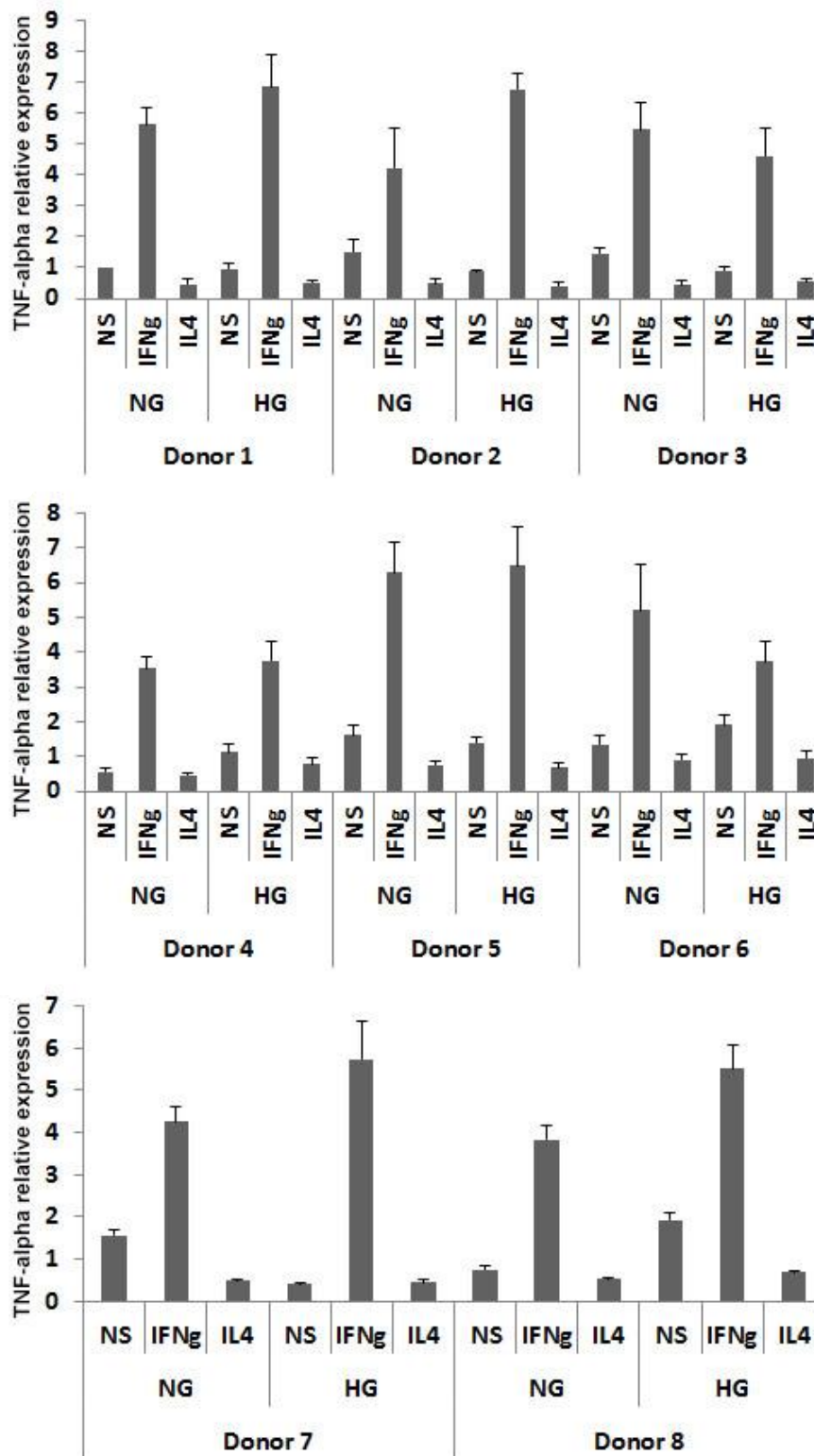
Figure 13. Effect of hyperglycaemia on TNF-alpha mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of TNF-alpha mRNA expression in differently stimulated macrophages cultured in normal (NG, 5mM) and high glucose (HG, 25mM) condition for 6 days. The experiments were performed in duplicates. Individual donors are indicated as Donor 1 to Donor 8. NS (M0), IFNg (M1) and IL4 (M2).
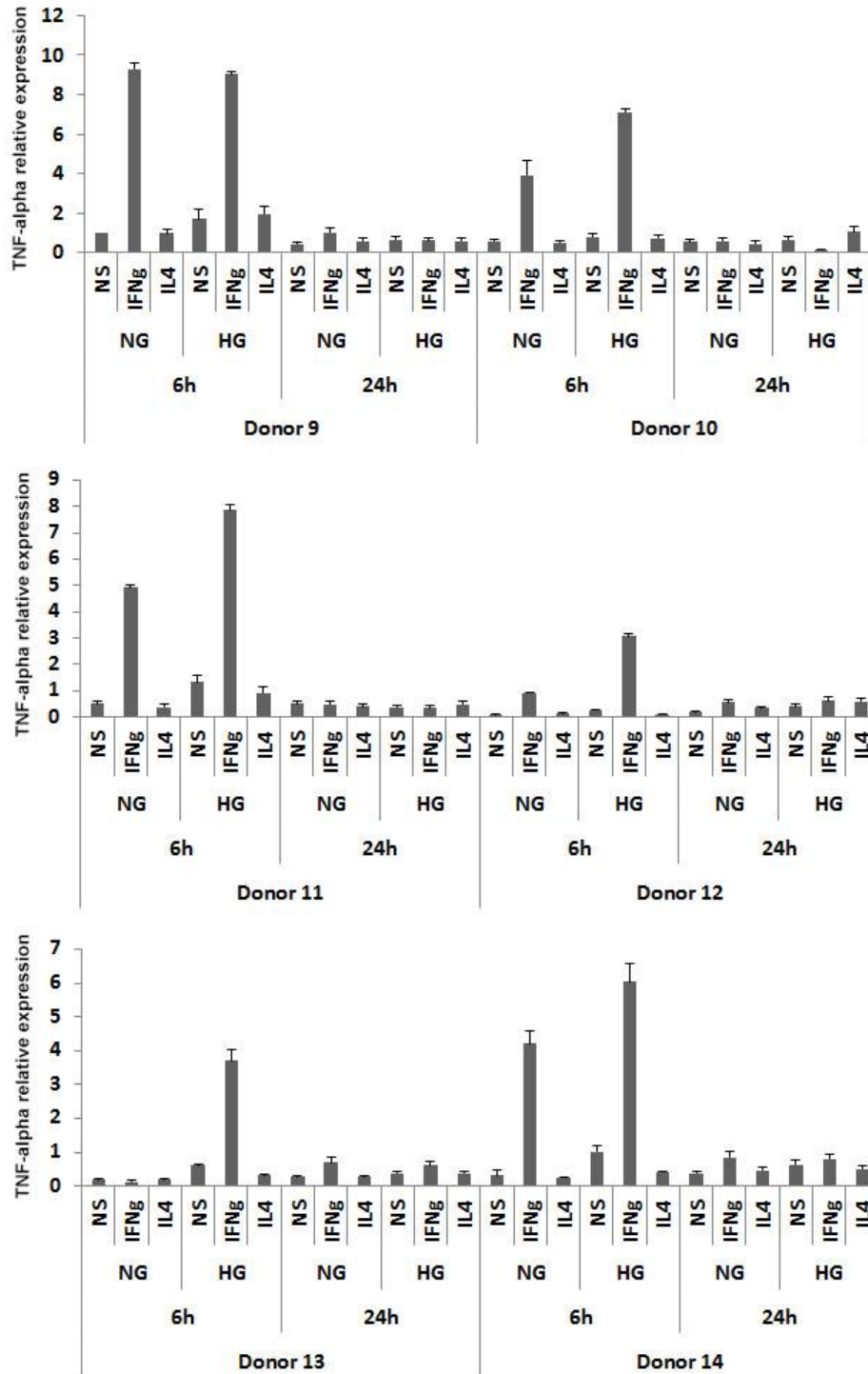
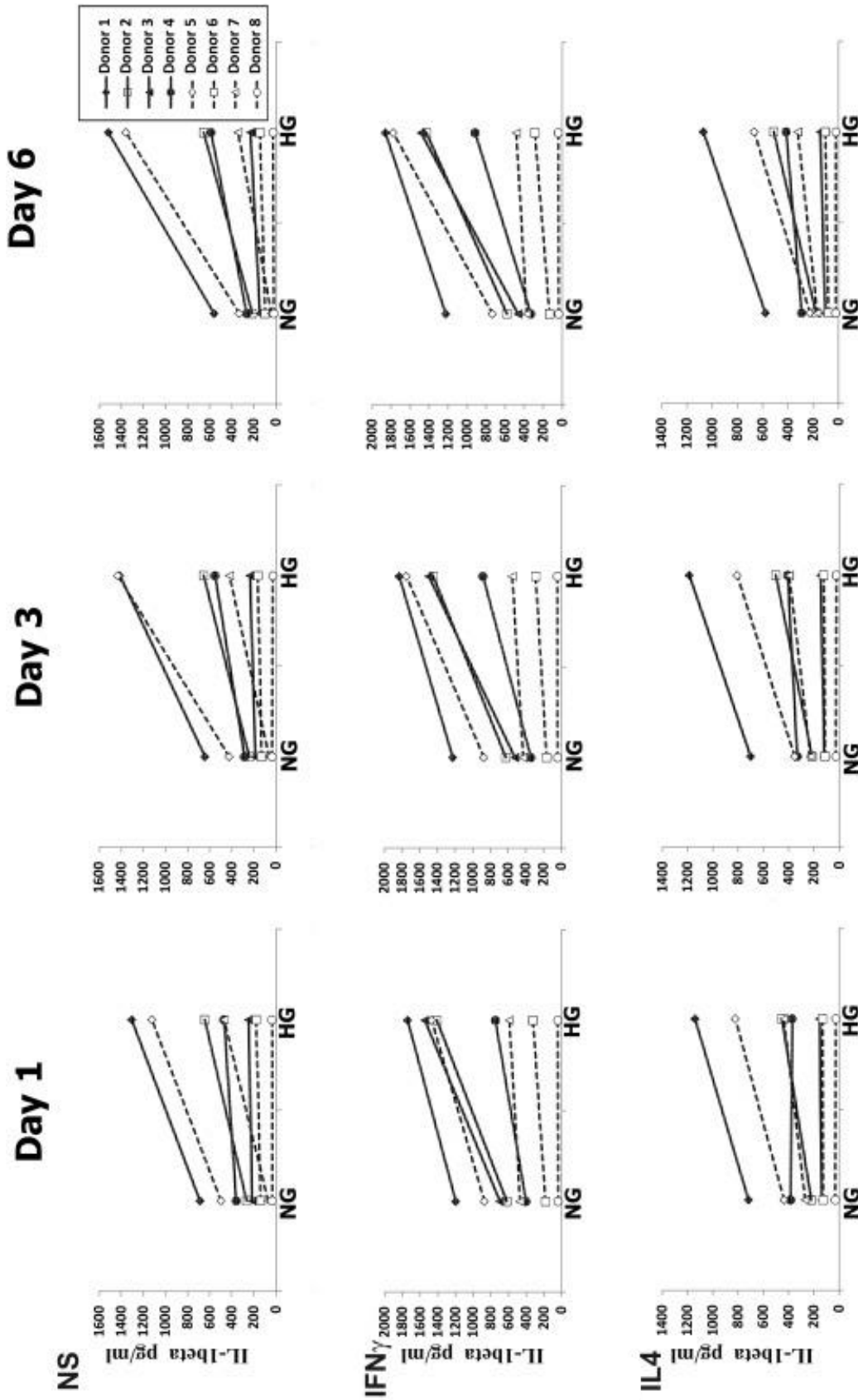**Figure 14. Effect of hyperglycaemia on TNF-alpha mRNA expression in macrophages after 6h and 24h.** RT-PCR analysis of TNF-alpha mRNA expression in monocytes without cytokine stimulation (NS) and stimulated with IFNg or IL4. Cultivation was done for 6h and 24h in normal (NG, 5mM) and high glucose (HG, 25mM) conditions. The experiments were performed in duplicates. Individual donors are indicated as Donor 9 to Donor 14.

3.3 Hyperglycaemia leads to increased gene expression and secretion of IL-1beta in human M0, M1 and M2

IL-1beta is an inflammatory cytokine known to be produced by IFNgamma stimulated macrophages, and is considered as a marker for M1 macrophages (Kzhyshkowska et al., 2016; Martinez and Gordon, 2014b). Therefore, it was next examined how hyperglycaemia affects differentiation of macrophages towards a pro-inflammatory phenotype by ELISA analysis of IL-1beta release on day 6. As expected, the highest IL-1beta levels are produced by M1 macrophages stimulated with IFNgamma (Figure 15). Hyperglycaemia increased IL-1beta release on all days analysed (day 1, 3 and 6) in 7 out of 8 donors, ranging from 0.5 to 3.2 times in M1. Hyperglycaemia also had an inducing effect on IL-1beta release in M0 and M2, with the strongest effect on donors 1, 2, 5 and 7 (Figure 15). RT-PCR analysis demonstrated that hyperglycaemia had an inducing effect on the gene expression level of IL-1beta in M1 (on day 6) in 4 out of 8 donors (donors 1, 2, 3, and 5) (Figure 16). Next, it was examined whether high glucose has an immediate effect on the stimulation of IL-1beta gene expression (Figure 17) and secretion (Figure 18). Monocytes derived from 6 donors have been analysed for IL-1beta mRNA levels and cytokine release after 6 and 24 hours of exposure to hyperglycemic conditions. In all analysed donors, we found the stimulatory effect of high glucose on IL-1beta mRNA levels in M1 after 6 hours, and the strongest response was detected in donor 10 (89 times increase) (Figure 17). After 6 h, hyperglycaemia also stimulated IL-1beta expression in M0 of all analysed donors as well as in M2 in 5 out of 6 donors (Figure 17). Taken together, the stimulatory effects of hyperglycaemia were strongly pronounced in M0 and M1 macrophages. The total levels of mRNA differ between donors with highest (17A), intermediate (17B), and lowest (17C) expression levels in hyperglycaemic conditions. An induction of IL-1beta release was detected in M0, M1 and M2 in 4 out of 6 donors after 6 h and 24 h, mostly corresponding to the HG-induced IL-1beta RNA levels (Figure 18).

Figure 15. Effect of hyperglycaemia on IL-1beta secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions. Supernatants were collected on day 1, 3 and 6 as indicated in methods. ELISA analysis of IL-1beta production by differently stimulated macrophages was measured in normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions. All experiments were performed in duplicates. The results are presented for individual donors (n=8) in normal and high glucose conditions. NS (M0), IFNg (M1) and IL4 (M2).

Figure 16. Effect of hyperglycaemia on IL-1beta mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of IL-1beta mRNA expression in differently stimulated macrophages cultured in normal (NG, 5mM) and high glucose (HG, 25mM) conditions for 6 days. The experiments were performed in duplicates. Individual donors are indicated as Donor 1 to Donor 8.
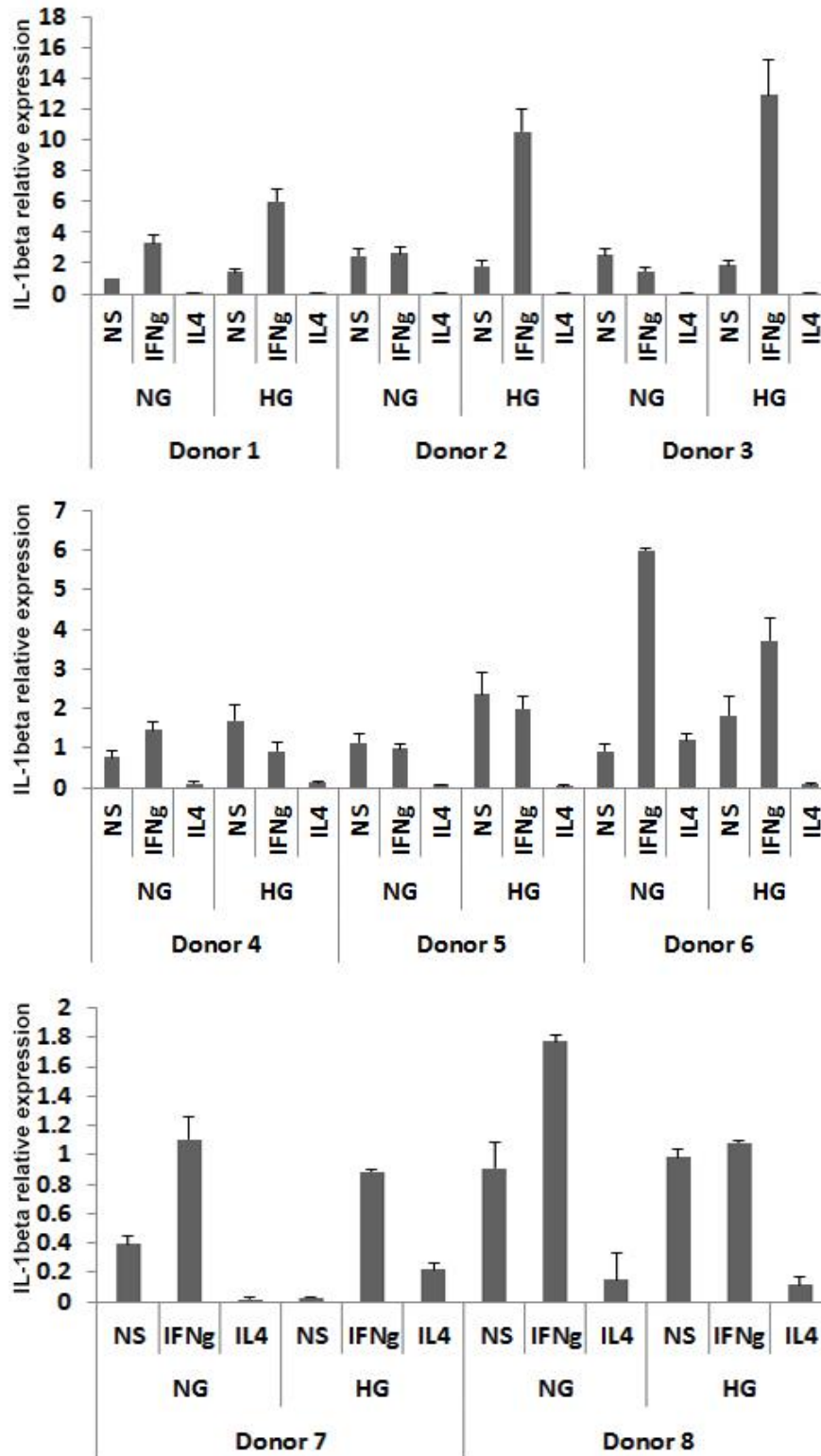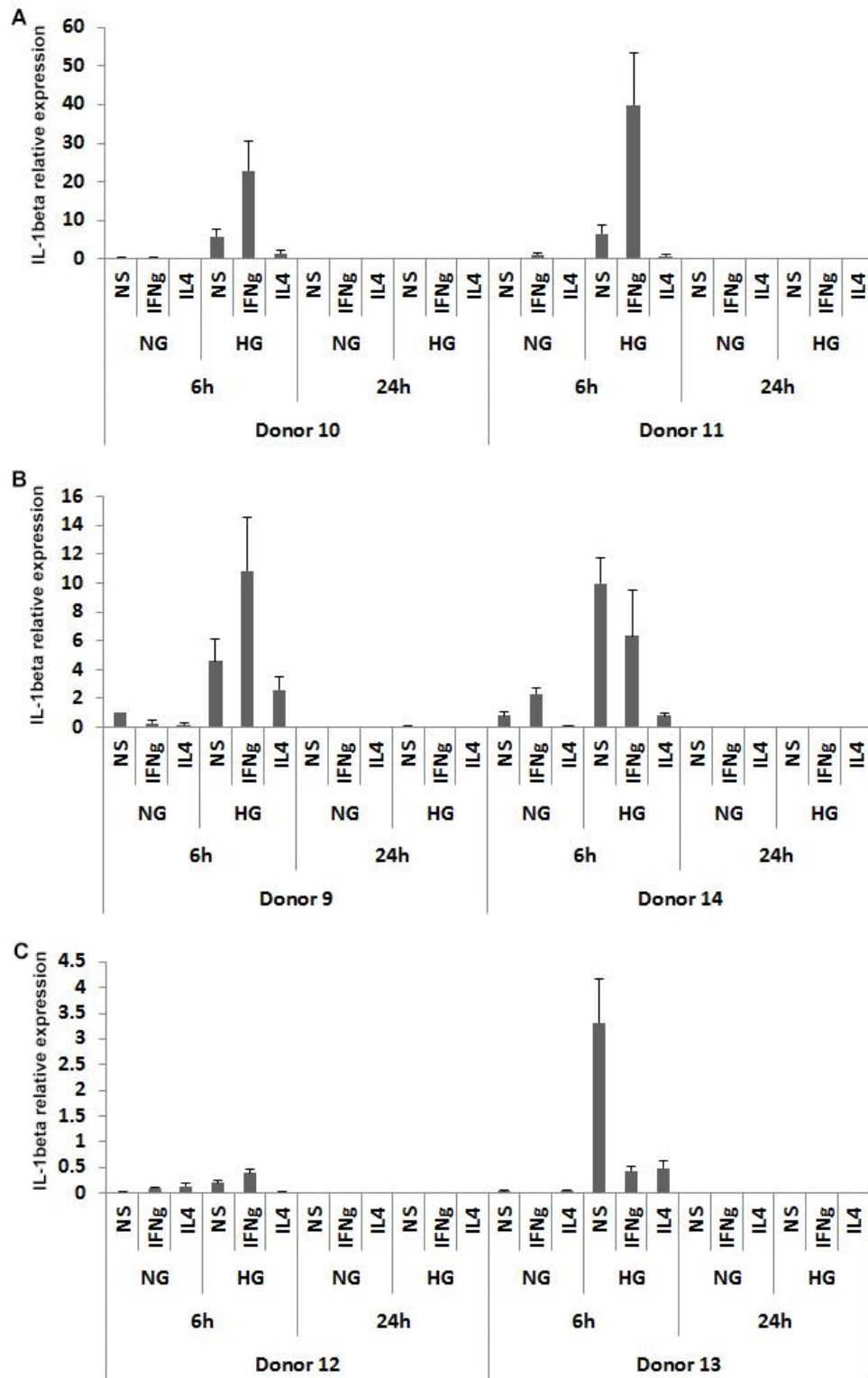
Figure 17. Effect of hyperglycaemia on IL-1beta mRNA expression in macrophages after 6h and 24h. RT-PCR analysis of IL-1beta mRNA expression in monocytes without cytokine stimulation (NS), stimulated with IFNgamma or IL4. Cultivation was done for 6h and 24h in normal (NG, 5mM) and high glucose (HG, 25mM) conditions. The experiments were performed in duplicates. Individual donors are indicated as Donor 9 to Donor 14. (A) Donors 10 and 11 with highest mRNA levels of IL-1beta in HG; (B) Donors 9 and 14 with intermediate mRNA levels of IL-1beta in HG, and (C) Donors 12 and 13 the lowest mRNA levels of IL-1beta in HG.
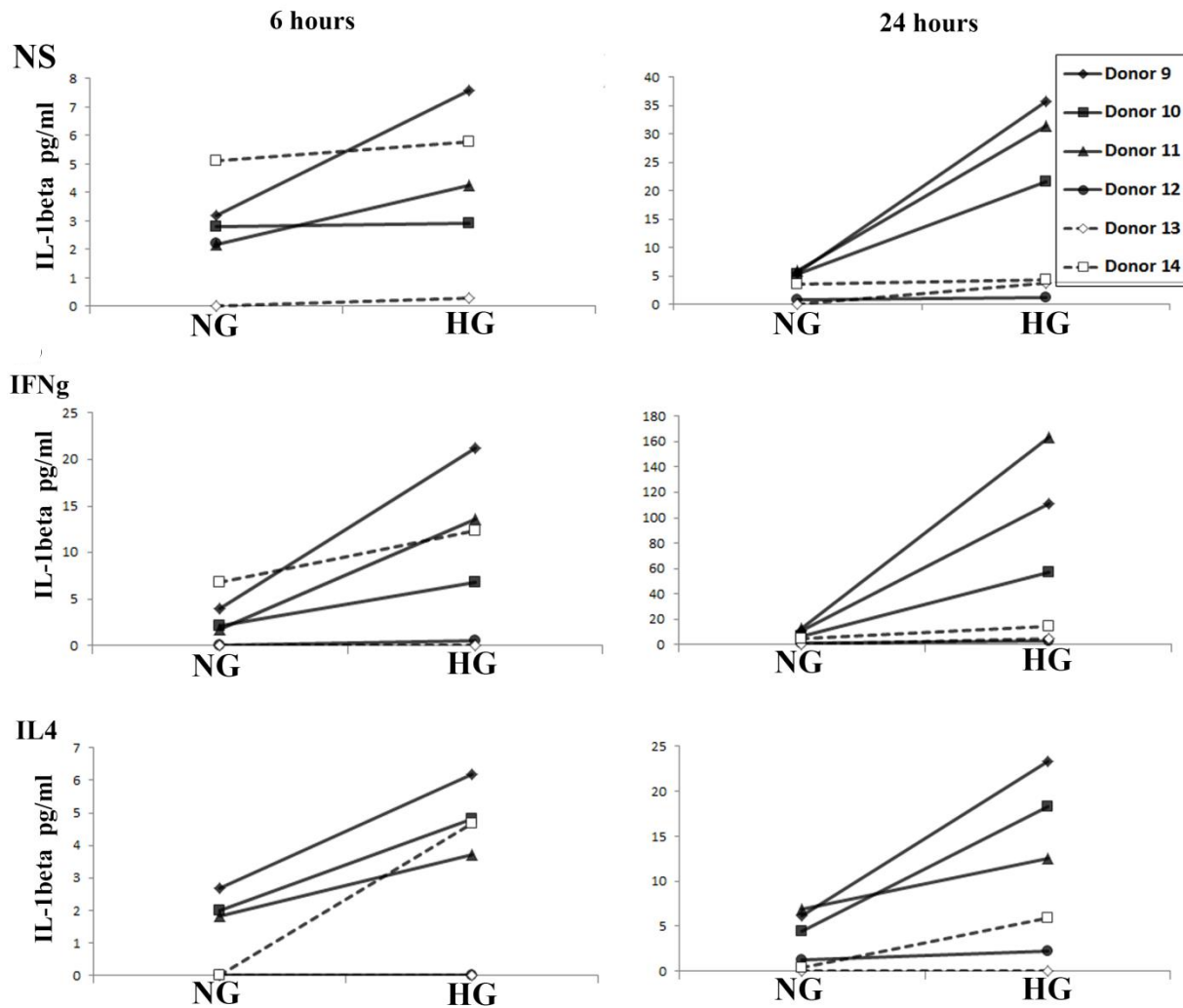
Figure 18. Effect of hyperglycaemia on IL-1beta secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions. Supernatants of macrophages cultured in NG and HG conditions were harvested after 6h and 24h. Concentrations of secreted IL-1beta were measured by ELISA. All measurements were performed in duplicates. The results are presented for individual donors (n=6) in NG and HG conditions. Non-stimulated (NS), IFNgamma-stimulated and IL4 stimulated macrophages were analysed.

3.4 High glucose can induce IL-1Ra release in human M0, M1 and M2 independent of gene expression activation

IL-1Ra is a cytokine produced by macrophages in response to IL4, known to block the pro-inflammatory effects of IL-1beta (Palomo et al., 2015 38). However, elevated levels of IL-1Ra were found to be associated with increased risk of developing type 2 diabetes (Carstensen et al., 2010). IL-1Ra is suggested to be involved in the inflammatory responses in prediabetes (Herder et al., 2009), and the role of IL-1Ra in diabetic complications remains to be controversial. However, IL-1Ra is considered as a marker cytokine for M2 macrophages.

Therefore, it was next examined how hyperglycaemia affects the differentiation of macrophages towards M2 by the analysis of IL-1Ra release on day 6. The highest IL-1Ra levels were produced by M2, stimulated with IL4 on day 6 (Figure 19). Hyperglycaemia increased the release of IL-1Ra in M2 on all days analysed (day 1, 3 and 6). The stimulatory effect of HG increased up to day 6 in 5 out of 8 donors (donors 1, 2, 5, 6 and 8) ranging from 0.5 to 3 times (Figure 19). Hyperglycaemia also strongly induced IL-1Ra release in M0 and M1 macrophages, with the strongest effect on day 6 observed in M0 for donors 1, 3, 4, 5 and 7 (Figure 19). In M1 macrophages, the inducing effect of HG on the IL-1Ra release was found in donor 5 (Figure 19). RT-PCR analysis demonstrated that on day 6, hyperglycaemia did not show any significant effect on IL-1Ra gene expression (Figure 20). Therefore, it was considered that elevated levels of secreted IL-1Ra on day 6 are a result of the accumulation of the cytokine in the medium. It was hypothesised that expression of IL-1Ra mRNA is induced at an early stage of macrophage differentiation as an immediate response of exposure to HG conditions, and is already downregulated on day 6. Therefore, it was examined whether high glucose has an immediate effect on the stimulation of IL-1Ra gene expression (Figure 21) and secretion (Figure 22). The mRNA levels, as well as the cytokine release of IL-1Ra of monocytes derived from 6 healthy donors, were analysed after 6 and 24 hours of exposure to HG in M0, M1 and M2 conditions. In 4 out of 6 donors (donors 9, 10, 11 and 13) the stimulatory effect of high glucose on IL-1Ra mRNA levels was found in M0, M1 and M2 after 6 hours. Donor 12 didn't show an elevated mRNA expression level in M1, but in M0 and M2 (Figure 21). The induction mediated by HG was more pronounced in M1 macrophages compared to M0 and M2 with the highest upregulation up to 88 times in donor 11 (Figure 21). After 6 hours, ELISA demonstrated the stimulatory effect of hyperglycaemia on IL-1Ra release in M1 and M2 in all 6 donors, and in M0 in 5 out of 6 donors (Figure 22). After 24 hours the stimulatory effect of hyperglycaemia was observed in M0, M1 and M2 in all donors (Figure 22). In summary, these data demonstrate that hyperglycaemia has a stimulatory effect on the IL-1Ra gene expression and release, independent of IL4, and the inducing effect of IL4 strongly depends on high glucose levels.
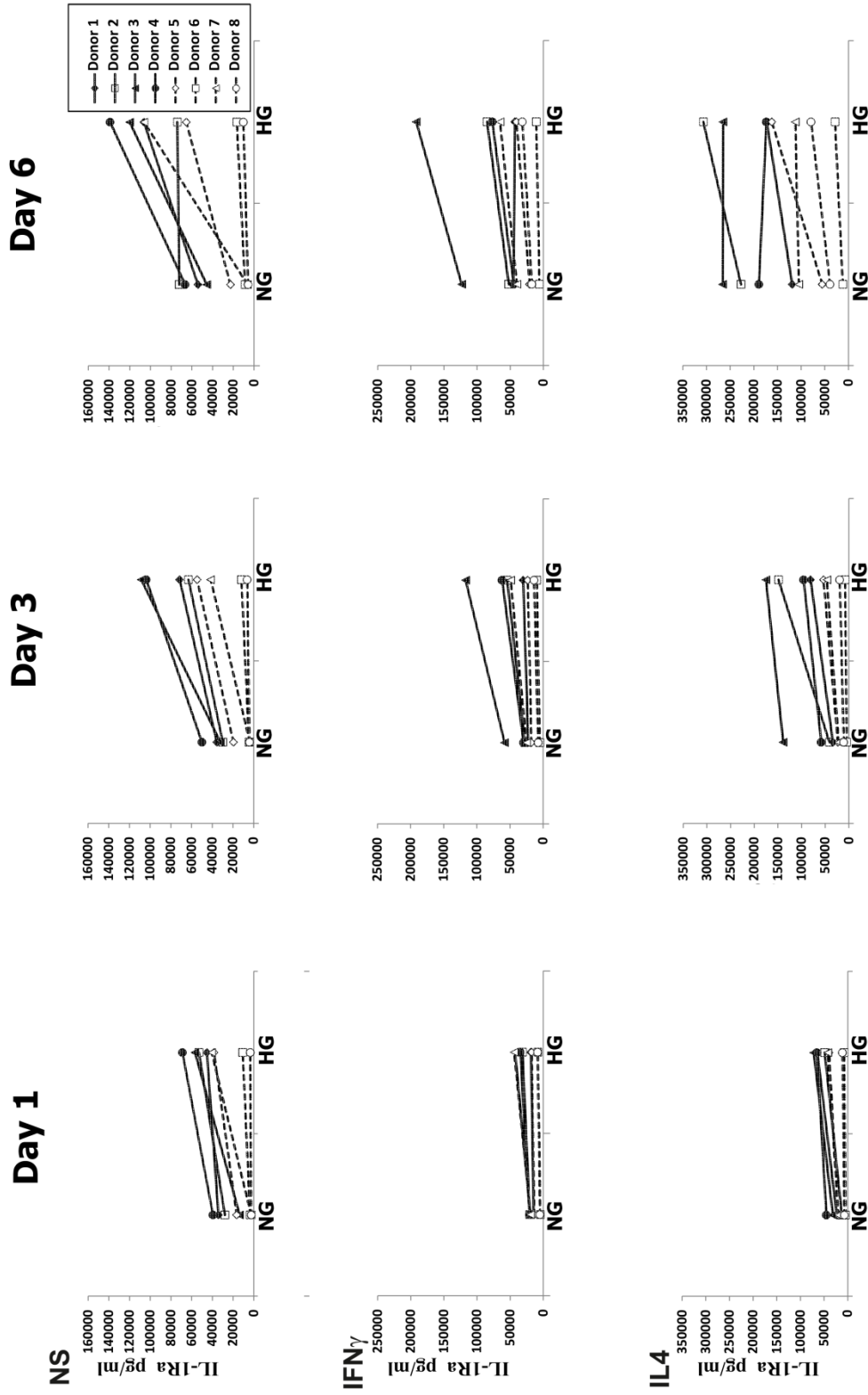
Figure 19. Effect of hyperglycaemia on IL-1Ra secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions. Supernatants were collected on day 1, 3 and 6 as indicated in methods. Enzyme-linked immunosorbent assay (ELISA) analysis of IL-1Ra production by differently stimulated macrophages was measured in normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions. All experiments were performed in duplicates. The results are presented for individual donors (n=8) in normal and high glucose conditions. NS (M0), IFNg (M1) and IL4 (M2).
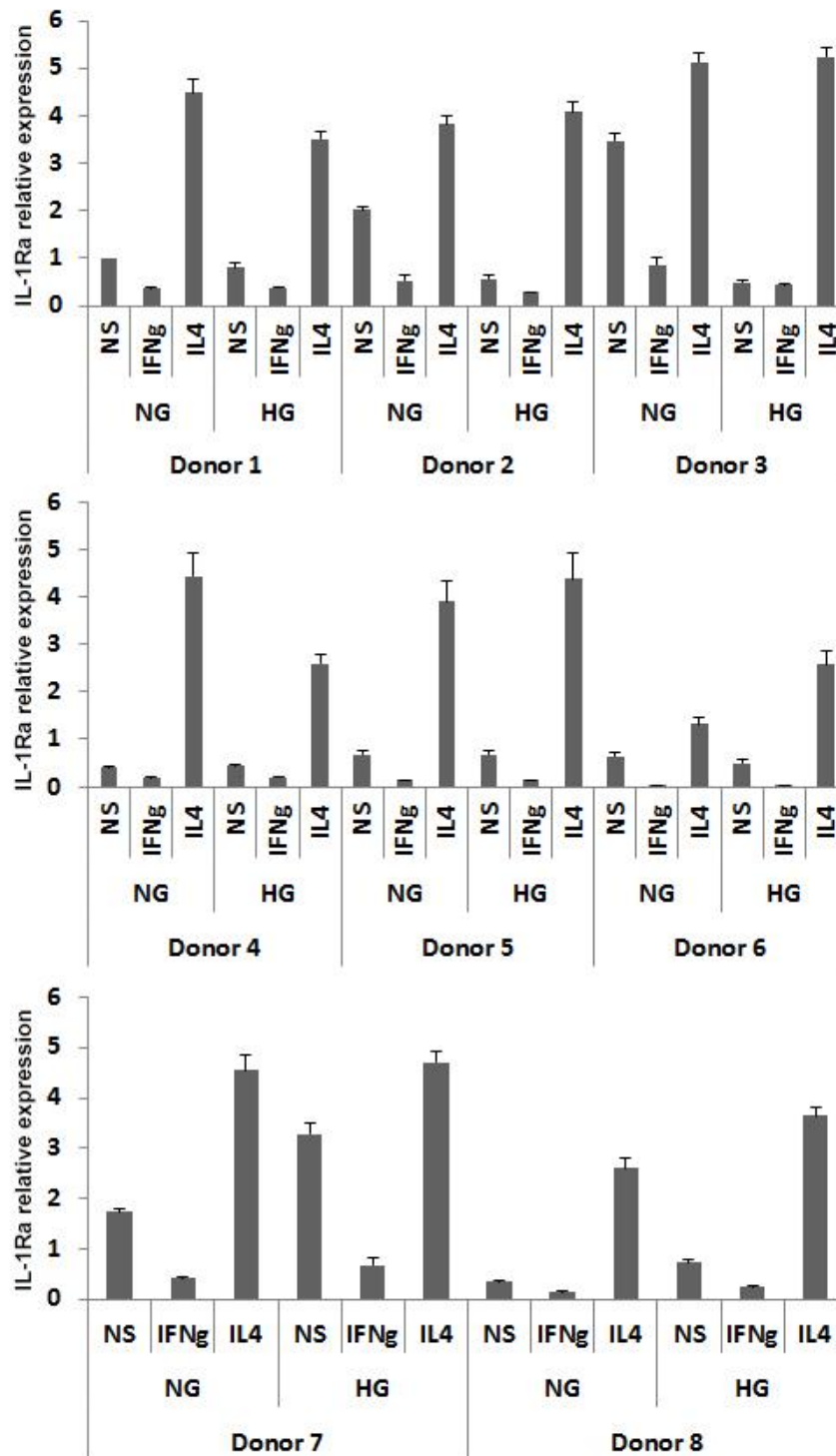
Figure 20. Effect of hyperglycaemia on IL-1Ra mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of IL-1Ra mRNA expression in differently stimulated macrophages cultured in normal (NG, 5mM) and high glucose (HG, 25mM) conditions for 6 days. The experiments were performed in duplicates. Individual donors are indicated as Donor 1 to Donor 8.

Figure 21. Effect of hyperglycaemia on IL-1Ra mRNA expression in macrophages after 6h and 24h. RT-PCR analysis of IL-1Ra mRNA expression in macrophages without cytokine stimulation (NS), stimulated with IFNg or IL4. Cultivation was done for 6h and 24h in normal (NG, 5mM) and high glucose (HG, 25mM) conditions. The experiments were performed in duplicates. Individual donors are indicated as Donor 9 to Donor 14.
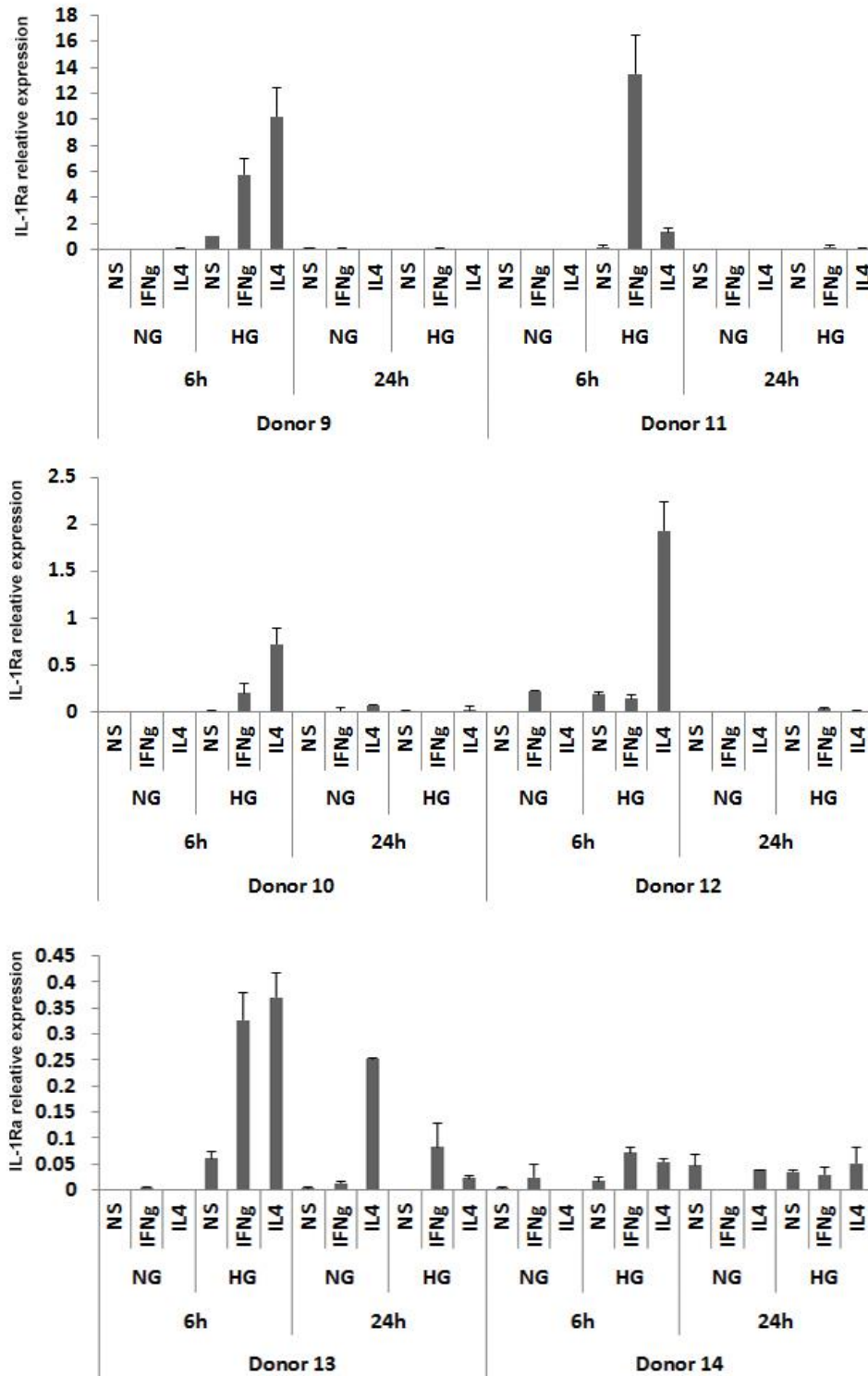
Figure 22. Effect of hyperglycaemia on IL-1Ra secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions. Supernatants of macrophages cultured in NG and HG conditions were harvested after 6h and 24h. Concentrations of secreted IL-1Ra were measured by ELISA. All measurements were performed in duplicates. The results are presented for individual donors (n=6) in normal and high glucose conditions. Non-stimulated (NS), IFNgamma-stimulated and IL4 stimulated macrophages were analysed.

3.5 High glucose suppresses CCL18 production in human monocytes/macrophages

CCL18 is one of the cytokines expressed by M2 macrophages upon stimulation with IL4 in late stages of macrophage differentiation (Kodelja et al., 1998). Therefore, it was next examined how hyperglycaemia affects differentiation of macrophages towards M2 by analysis of CCL18 expression. M0, M1 and M2 of 8 individual donors were analysed for CCL18 gene expression on day 6. RT-PCR analysis demonstrated that hyperglycaemia has a suppressive effect on CCL18 expression in M2 of all 8 donors (range between 0.24 to 16.7 times) (Figure 23). In M0 and M1, the levels of CCL18 mRNA were very low in both NG and HG conditions. Next, it was investigated whether HG has an effect on the release of CCL18. CCL18 secretion levels were measured on days 1, 3 and 6 by ELISA (Figure 24). The IL4

stimulated macrophages secreted a high amount of CCL18 on day 6 of cultivation under normal glucose conditions. Hyperglycaemia suppressed CCL18 release on day 6 in most of the donors in M2 macrophages (Figure 24). Hyperglycaemia had a slightly inducing effect of CCL18 release on day 6 in M0, however the absolute levels of secreted CCL18 in M0 were very low compared to M2, and biologically not significant. The suppression mediated by HG on CCL18 mRNA levels and cytokine release in M2 correlated for most of the donors. The suppressive effect of HG on CCL18 was much more pronounced on the level of gene expression compared to the levels of secreted cytokine. The suppression was most prominent in M2 of donors with the lowest base values of CCL18 mRNA levels under normal glycaemic conditions on day 6.
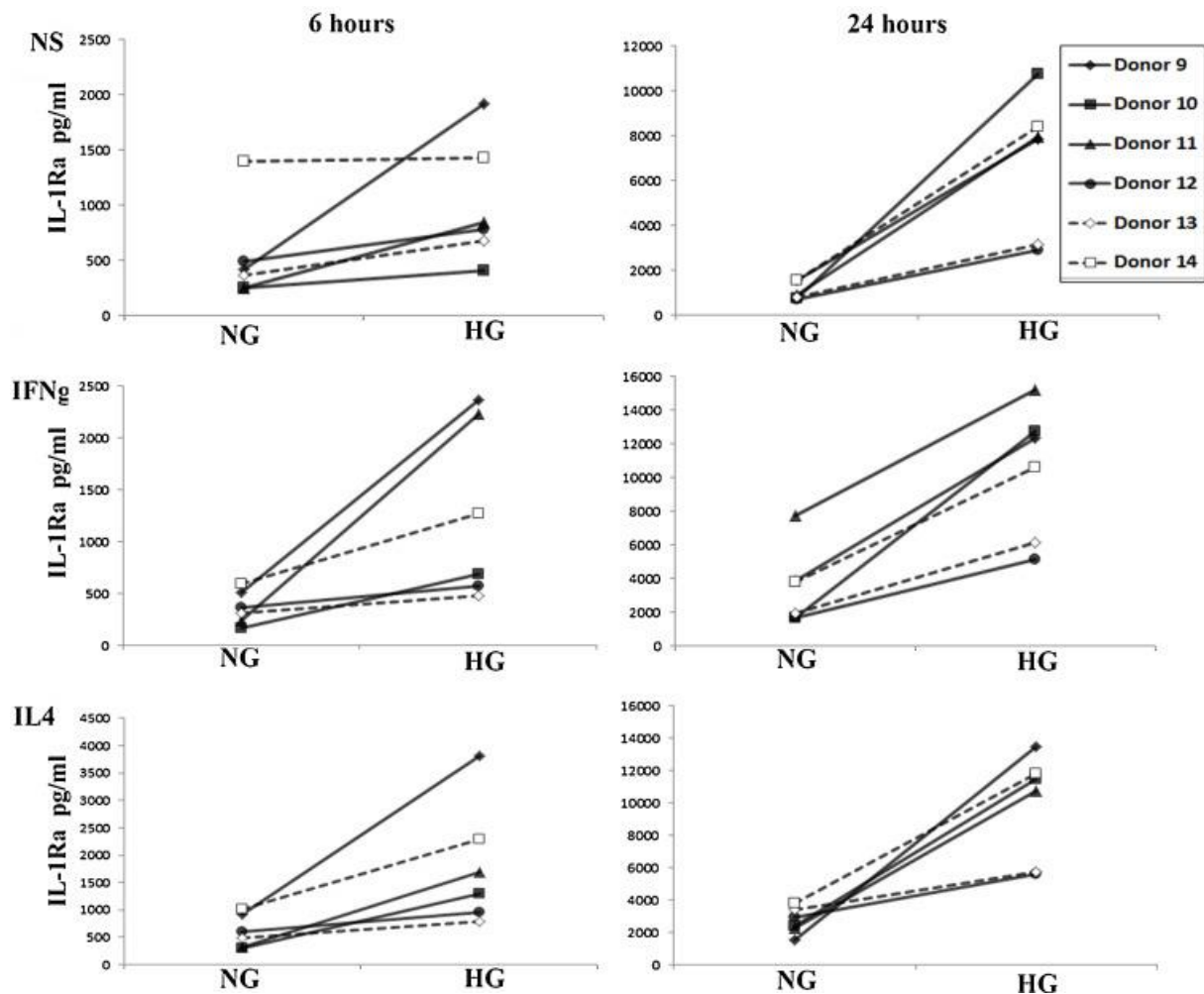
Figure 23. Effect of hyperglycaemia on CCL18 mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of CCL18 mRNA expression in differently stimulated macrophages cultured in normal (NG, 5mM) and high glucose (HG, 25mM) conditions for 6 days. The experiments were performed in duplicates. Individual donors are indicated as Donor 1 to Donor 8.
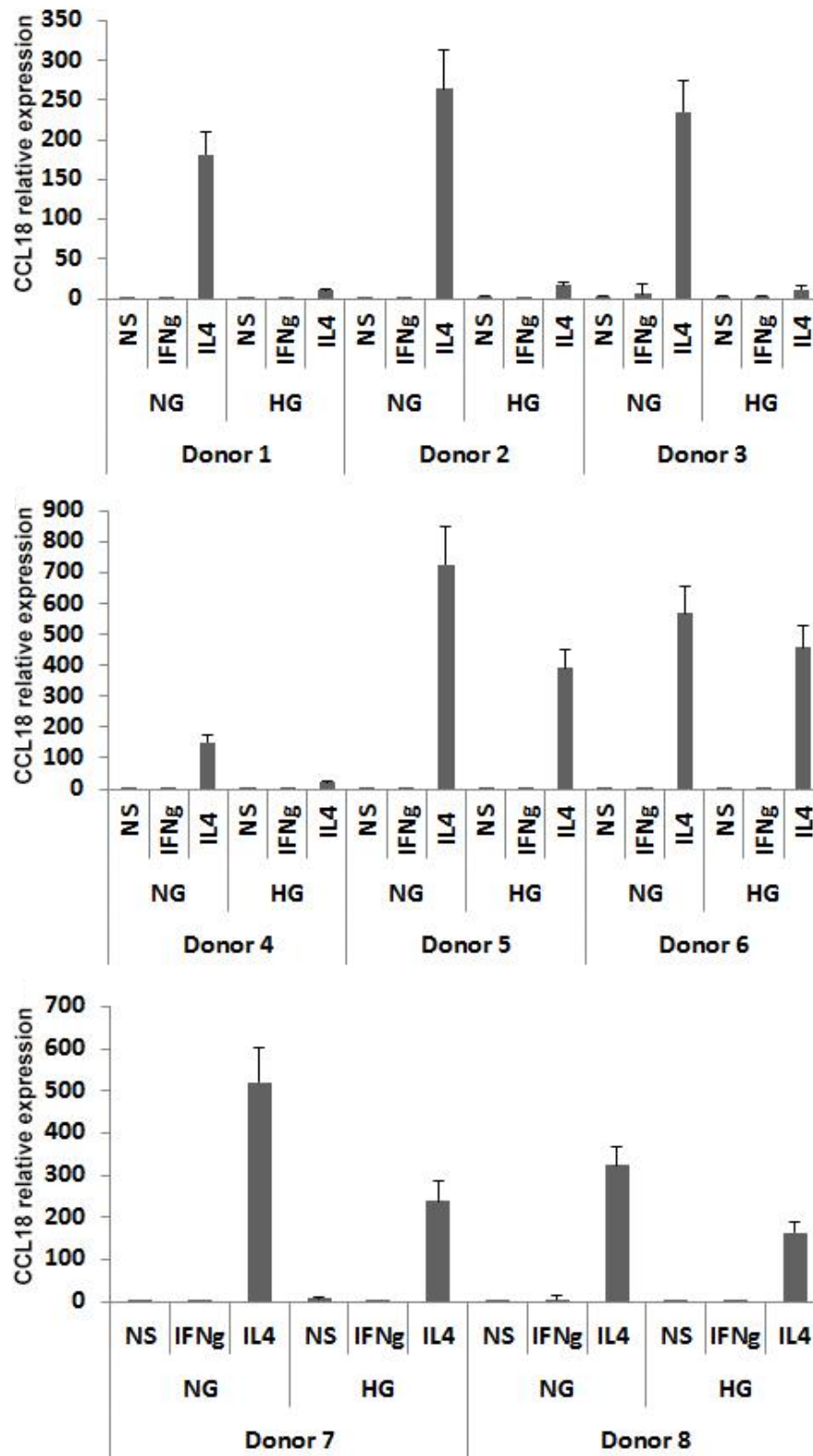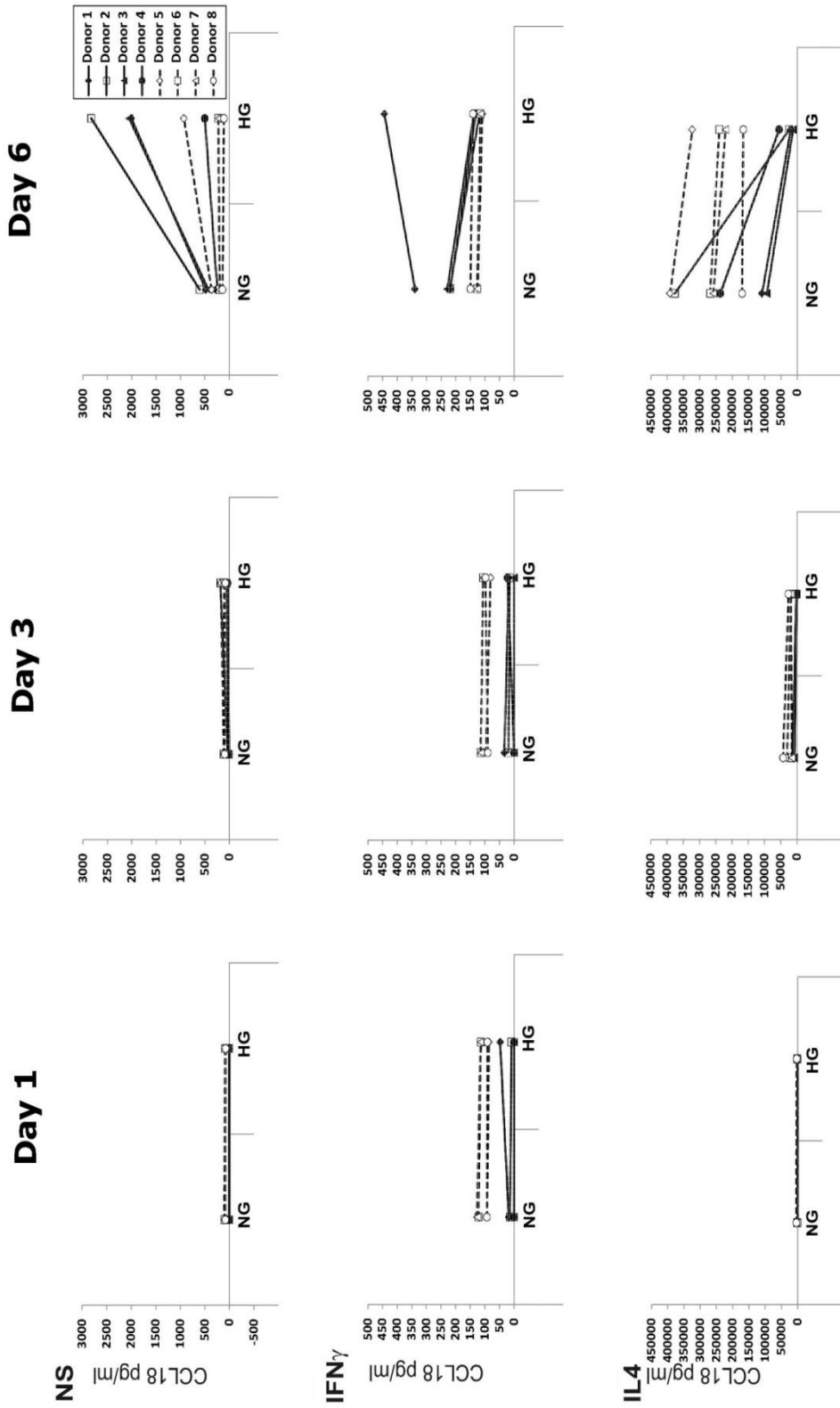
Figure 24. Effect of hyperglycaemia on CCL18 secretion in primary human M0, M1 and M2 macrophages in normal and high glucose conditions. Supernatants were collected on day1, 3 and 6 as indicated in methods. Enzyme-linked immunosorbent assay (ELISA) analysis of CCL18 production by differently stimulated macrophages was measured in normal glucose (NG, 5mM) and high glucose (HG, 25mM) conditions. All experiments were performed in duplicates. The results are presented for individual donors (n=8) in normal and high glucose conditions. NS (M0), IFNg (M1) and IL4 (M2).

3.6 Identification of genes induced by hyperglycaemia using Affymetrix DNA microarray

Analysis of the cytokine profile demonstrated that hyperglycaemia has not only simple pro-inflammatory effects on macrophage activation, but rather a complex mode of programming towards a chronic inflammatory state. Affymetrix GeneChips microarray was applied in order to identify the transcriptional program induced by hyperglycaemia in human primary M0, M1 and M2. Macrophages were cultivated in normal and hyperglycaemic conditions for 6 days. Macrophages from 4 donors (donors 1-4) with the strongest response to hyperglycaemia according to the suppression of CCL18 were used in the analysis. The total amount of genes differentially expressed in normal and hyperglycaemic conditions are demonstrated in Figure 25. A total of 2546 genes were differentially regulated by hyperglycaemia. Thereof 1173 genes were found statistically significant in M0 (NS), 1574 genes in M1 (IFNgamma) and 16 genes in M2 (IL4). Microarray analysis also determined that hyperglycaemia-induced differential regulation of 207 common genes in M0 (NS) and M1 (IFNgamma), 2 common genes in M1 (IFNgamma) and M2 (IL4), 1 common gene in M0 (NS) and M2 (IL4), and 2 common genes for all 3 activation states of macrophages (Figure 25).

The Gene Set enrichment analysis (GSEA) database was used to define the gene families. The Microsoft office excel VBA scripts were used to track the genes with a statistically significant difference ($P \leq 0.05$) in expression comparing normal and hyperglycaemic conditions. The major families of genes upregulated in hyperglycemic conditions are listed in Table 4 and included families of chemokines and cytokines that also included cytokine receptors (20 members), the ribonuclease RNase A family (9 members), the solute carrier family (28 members), the S100 calcium binding protein family (8 members), the transmembrane protein family (20 members) and the zinc finger family (37 members). The complete microarray data was deposited in the Gene Expression Omnibus database (GEO) with accession number GSE86298.
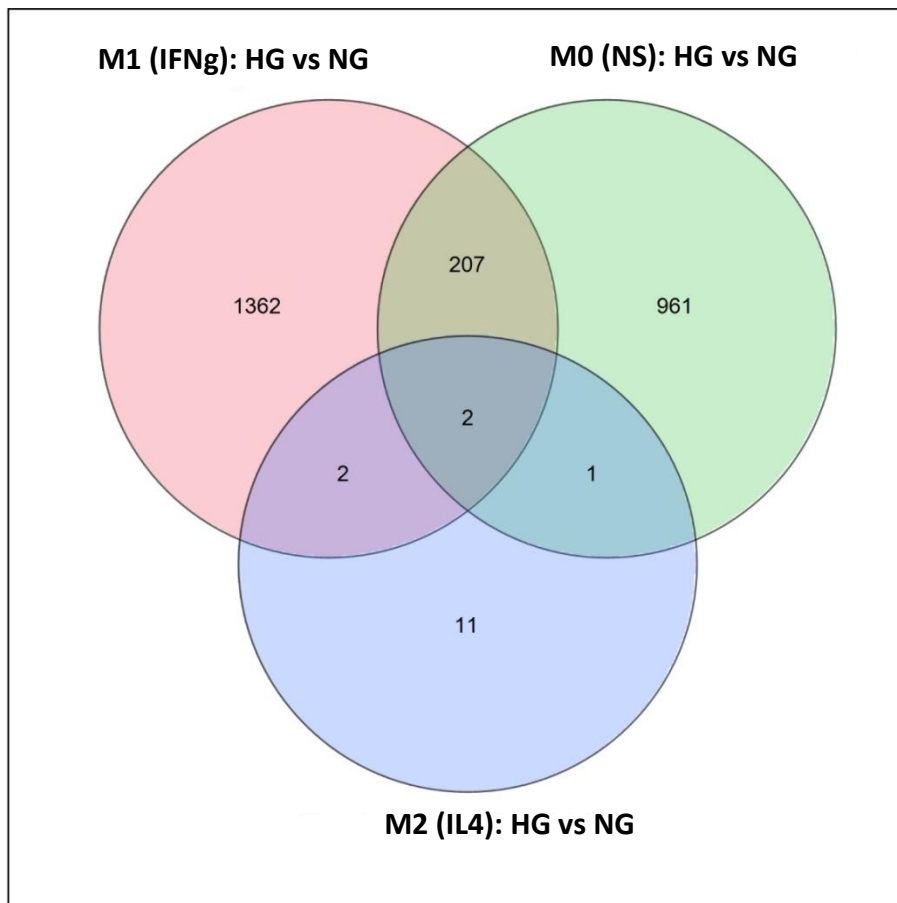
Figure 25. Affymetrix DNA microarray analysis of gene expression in macrophage cultured in NG and HG conditions for 6 days. Venn diagram of differentially expressed genes in macrophages cultured with NG and HG conditions. Each number represents the number of genes differentially expressed in response to hyperglycaemia. Each circle represents a population of macrophages with a different stimulation (Green – M0 (NS), Red – M1 (IFNgamma), Blue – M2 (IL4)). Intersecting points represent the number of genes differentially regulated by hyperglycaemia in 2 or more stimulations.

Table 4. Families of genes upregulated by high glucose in human macrophages.

Gene Family	No. of genes affected by HG			Most strongly affected family member
	M0	M1	M2	
Chemokines and Cytokines	2	18	0	CCR2 > 7.1 fold change (M1)
Ribonuclease, RNase A family	6	3	0	RNASE2 > 5 fold change (M0)
Solute carrier family	11	17	1	SLC4A7 > 1.8 fold change (M1)
S100 calcium binding protein family	1	7	0	S100A12 > 4.4 fold change (M1)
Transmembrane protein family	8	12	0	TMEM130 > 3.6 fold change (M1)
Zinc finger family	17	19	1	ZMYM3 > 1.8 fold change (M1)

Affymetrix microarray analysis revealed that hyperglycaemia induces expression of IL-1beta in M1 (3 fold change, P=0.0002) and M0 (1 fold change, P=0.9), which confirmed the results of the previous analysis by RT-PCR and ELISA. The strongest upregulating effect of hyperglycaemia within the family of cytokines and cytokine receptors was found for CCR2 [M0 (4.2 fold change, p=0.01) and M1 (7.1 fold change, p= 0.0008)].

Chemokine receptor CCR2 is the major receptor for monocyte chemoattractant protein 1 MCP1 (CCL2), and interaction of CCL2 with CCR2 expressed on monocytes is responsible for monocytes recruitment to the sites of inflammation, including fat tissues in obesity and vascular complications (Ali et al., 2008; Chu et al., 2014; Weisberg et al., 2006). In one study, it was demonstrated that silencing of CCR2 expression reduces the infiltration of macrophages into inflamed adipose tissue in a mouse model (Kim et al., 2016). The CCL2/CCR2 system was shown to be important for vascular complications such as atherosclerosis (Feria and Díaz-González, 2006).Therefore, CCR2 was selected to examine the effects of hyperglycaemia on its expression regulation and functional role in macrophage recruitment.

3.7   RT-PCR analysis of CCR2 expression in hyperglycaemic conditions

To confirm the results obtained by microarray analysis, expression of CCR2 was further examined by RT-PCR (Figure 26). CCR2 gene expression was analysed in M0, M1 and M2 of 8 individual donors. M0, M1 and M2 were cultivated in normal and hyperglycaemia conditions for 6 days (as illustrated by Figure 11). RT-PCR analysis of macrophages isolated from 8 individual donors (donor 1-8) demonstrated that hyperglycaemia induced an upregulation of CCR2 gene expression in M0 4.6 times (P=0.055) and in M1 3.8 times (P=0.012) (Figure 26). CCR2 gene expression was increased in hyperglycaemic conditions in M0 and M1 of all donors analysed, ranging from 1.7 to 35.7 times for M0, and 1.4 to 12.3 times in M1 (Figure 28). In M2, the levels of CCR2 mRNA were very low in both NG and HG conditions (Figure 26). CCR2 was shown to be expressed in monocytes and is responsible for monocyte response to MCP1/CCL2. RT-PCR analysis of 5 individual donors (donor 4-8) demonstrated that monocytes express much lower levels of CCR2 mRNA compared to M0 and M1 in HG conditions, indicating that hyperglycaemia induces CCR2 expression during monocyte to macrophage differentiation (Figure 27).
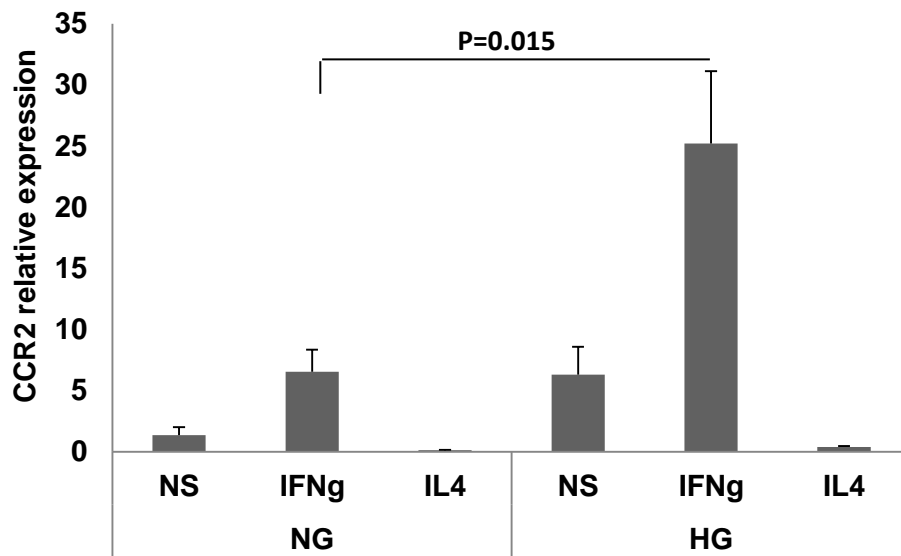
Figure 26. Effect of hyperglycaemia on CCR2 mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of CCR2 mRNA expression in differently stimulated macrophages cultured in normal (NG, 5mM) and high (HG, 25mM) glucose conditions. Each column represents the mean ± SEM normalised to 18srRNA levels. The experiments for each donor were performed in duplicates (total number of donors n=8).
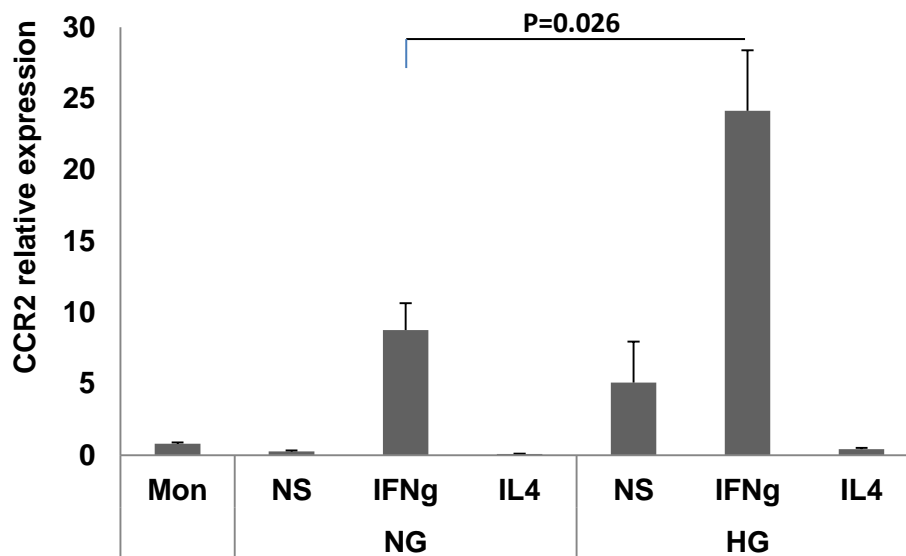
Figure 27. Comparison of CCR2 mRNA expression between monocytes and mature macrophages cultured in normal and hyperglycaemic conditions. CCR2 mRNA expression in freshly isolated monocytes (Mon) and differently stimulated macrophages cultured in NG and HG conditions for 6 days was analysed by RT-PCR. Each column represents the mean ± SEM normalised to 18srRNA levels. Monocytes and macrophages from 5 individual donors were analysed. The RT-PCR experiments for each donor were performed in duplicates (total number of donors n=5).

Figure 28. Effect of hyperglycaemia on CCR2 mRNA expression on day 6 of macrophage cultivation. RT-PCR analysis of CCR2 mRNA expression in differently stimulated macrophages cultured in NG and HG condition for 6 days. The experiments were performed in duplicates. Individual donors are indicated as Donor 1 to Donor 8.

3.8 Hyperglycaemia facilitates macrophage migration towards CCL2

To address the functional relevance of elevated expression of CCR2 in hyperglycaemic conditions, migration of M0 and M1 generated in normal and high glucose conditions toward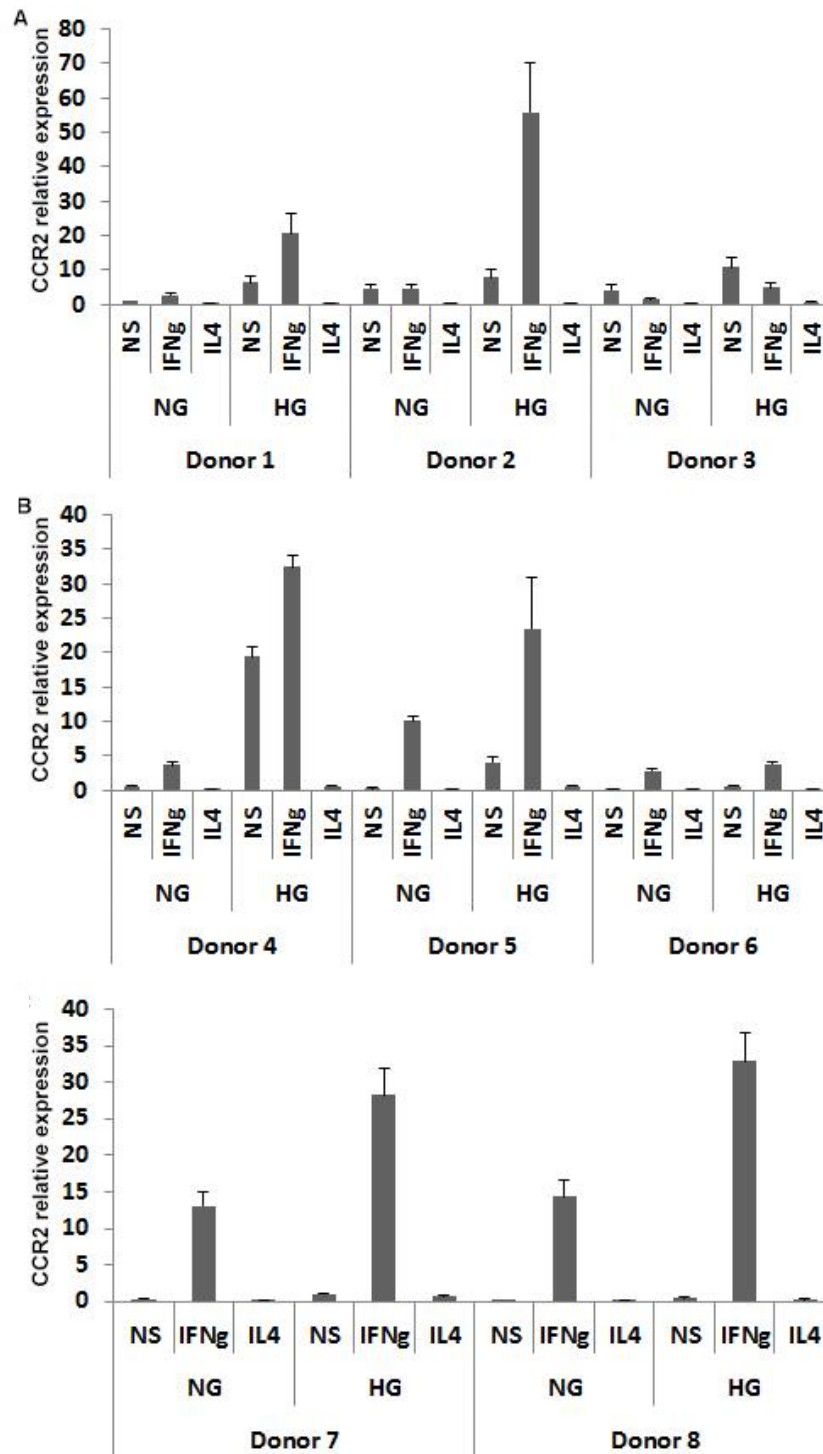s a CCL2 gradient was analysed. The migration assay was performed with a transwell system with a 5-µm pore size membrane. Macrophages were cultivated in normal (NG) or hyperglycaemia (HG) conditions in the absence of cytokines (M0) or under stimulation of IFNgamma (M1) as illustrated in Figure 11. On day 6, macrophages were collected and 1 x 10^5 were seeded in serum-free medium in the upper chamber of a transwell, while CCL2 was added to the lower chamber at a concentration of 100 ng/ml. After 16h incubation, migrated cells on the lower surface of the membrane were fixed, stained with DAPI and quantified with image J. Analysis of M0 and M1 from 5 independent donors demonstrated that CCL2 induced migration was strongly stimulated by hyperglycaemia (Figure 29). For M0 macrophages, the stimulatory effect of hyperglycaemia was 7.6 times (P=0.007), and for M1 it was increased 11.2 times (P=0.001) (Figure 29). Next, it was hypothesised that elevated expression of CCR2 induced by hyperglycaemia can make macrophages more sensitive to lower concentrations of CCL2. Therefore, a range of different concentrations of CCL2 (100 ng/ml, 25 ng/ml, 6.25 ng/ml and 1.56 ng/ml) was examined for the ability to induce migration of M1 macrophages in a transwell system for 3 additional donors. For 100 ng/ml CCL2, the stimulatory effect of hyperglycaemia on M1 macrophages was 11.3 times (P=0.15), for 25 ng/ml CCL2 it was 14.4 times (P=0.057), for 6.25 ng/ml CCL2 it was 11.6 times (P=0.036) and for 1.56 ng/ml CCL2 it was 8.4 times (P=0.0059) (Figure 30).These data indicate that even very low concentrations of CCL2 can induce migration of inflammatory macrophages in hyperglycemic conditions.
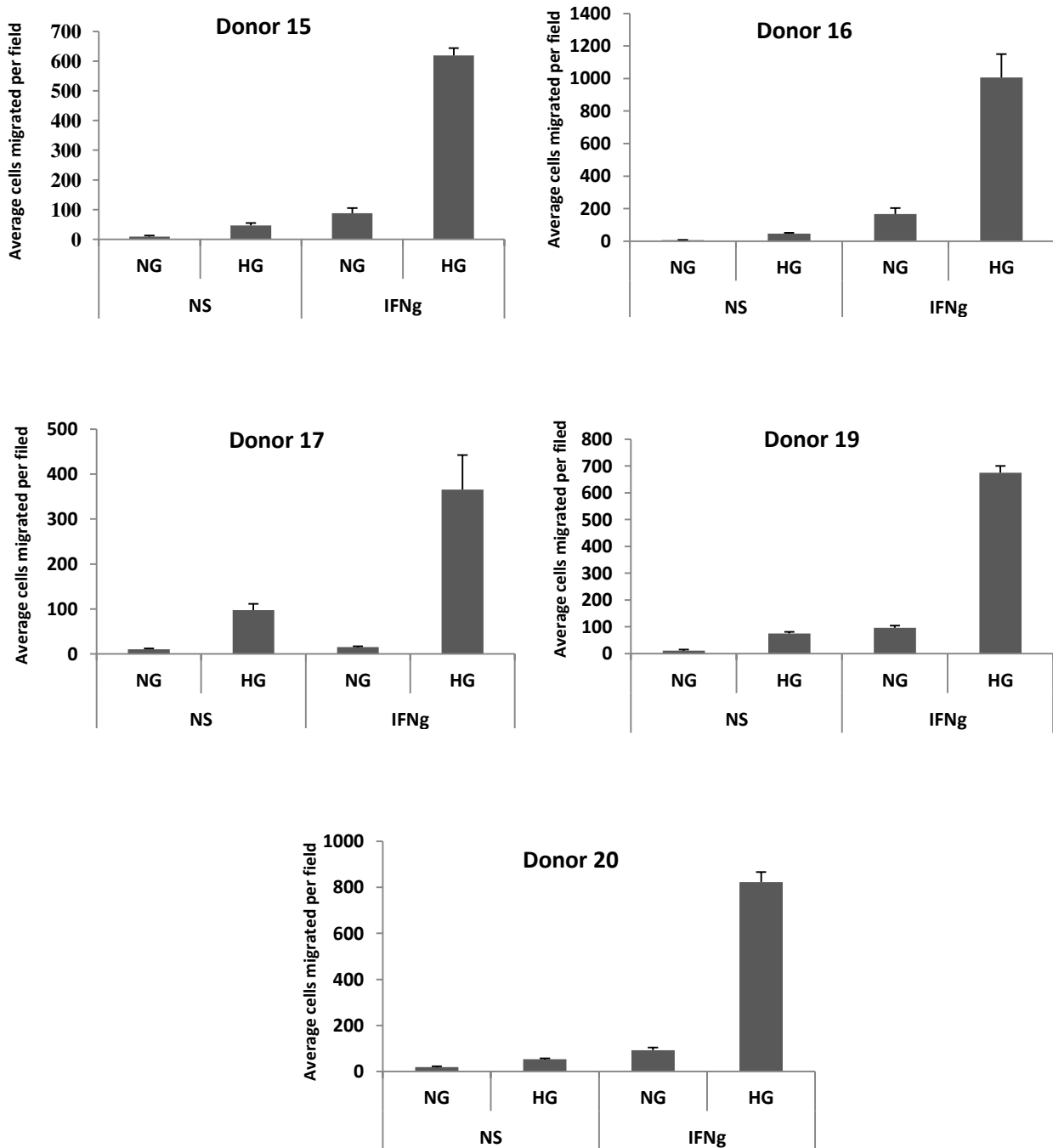
Figure 29. Effect of hyperglycaemia on M0 and M1 trans-migration induced by CCL2. Macrophages were cultured in NG and HG conditions in the absence of stimulation (NS) or the presence of IFNgamma for 6 days. The migration assay was performed in a transwell system with CCL2 (added to the lower chamber at the concentration of 100 ng/ml). Data are presented for macrophages of individual donors.
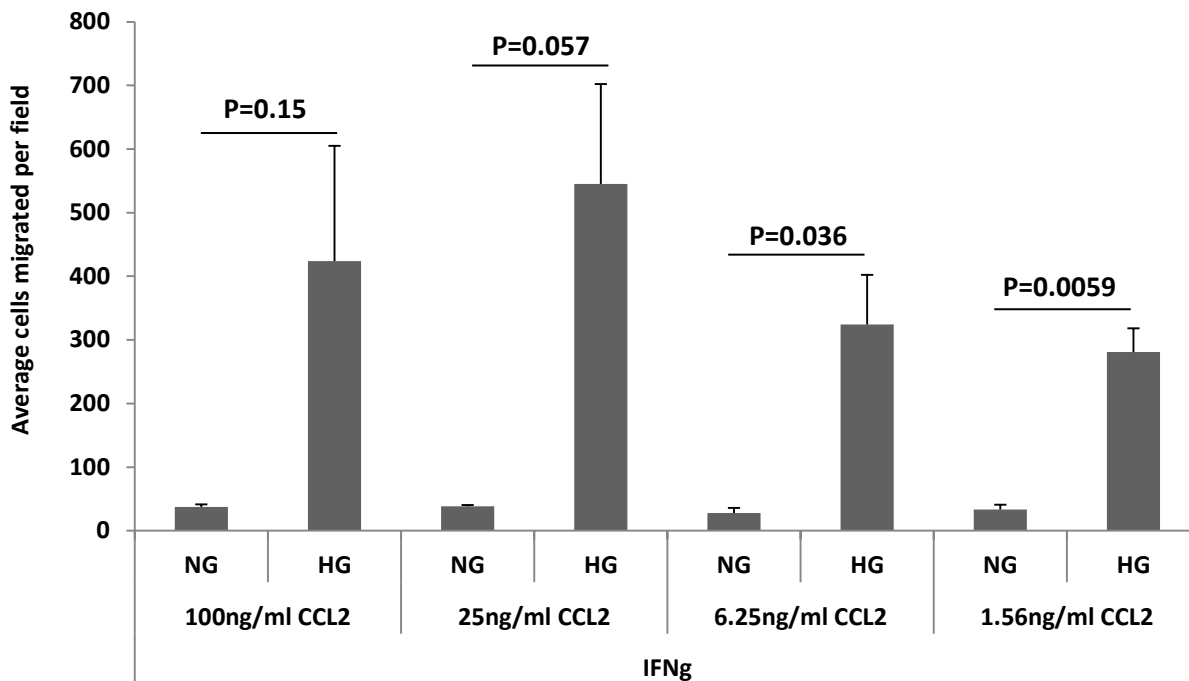
Figure 30. Effect of hyperglycaemia on M1 trans-migration towards different concentrations of CCL2. Macrophages were cultured in NG and HG conditions under stimulation with IFNgamma for 6 days. The transwell migration assay was performed in a transwell system using a range of concentrations of CCL2 (100 ng/ml, 25 ng/ml, 6.25 ng/ml and 1.56 ng/ml) in the lower chamber. The total amount of donors analysed (n=3).

3.9 Epigenetic regulation of CCR2 and IL-1beta expression

An increasing number of studies demonstrate that epigenetic mechanisms contribute to the development of diabetic vascular complications (Prattichizzo et al., 2015b; Reddy et al., 2015). The histone code that controls chromatin structure was shown to be affected by diabetic conditions in endothelial cells (Prattichizzo et al., 2015b; Reddy et al., 2015). Increased acetylation of histone H3 and mono and tri-methylation of H3 at K4 (lysine 4) are associated with gene activation (Heintzman et al., 2007; Kim et al., 2005; Koch et al., 2007), while increased tri-methylation of H3 at K9 or K27 (lysine 9 or 27) were linked with gene repression (Heintzman et al., 2007).

Since hyperglycaemia has strong effects on the expression of CCR2 and IL-1beta (section 3.3 and 3.7), the promoters of these genes were selected for the analysis of the histone code under hyperglycemic conditions. Bioinformatic analysis of CCR2 and IL-1beta promoters using Roadmap Epigenomics Visualization Hub (VizHub)) was used to select the histone marks. Three activating histone marks (H3K4me1, H3K4me3 and acetylated H3) and 2 repressing histone marks (H3K9me3 and H3K27me3) were chosen for chromatin immunoprecipitation experiments.

3.10 Effect of hyperglycaemia on the presence of histone marks on the CCR2 promoter

The impact of hyperglycemic conditions on histone modifications at the CCR2 promoter was studied with the Chromatin Immuno Precipitation assay (ChIP). Macrophages were cultivated in normal (NG) or hyperglycaemia (HG) conditions in the presence of IFNgamma (M1) as illustrated in Figure 11. On day 6, macrophages were treated with formaldehyde in order to cross-link the DNA with protein (chromatin). The cross-linked chromatin was sonicated in order to obtain fragments with a size between 200-1000bp. Fragmented chromatin was incubated overnight at 4 degrees with specific antibodies against: 1) acetylated H3 (Acetyl H3), 2) monomethylation of lysine 4 on histone H3 (H3K4me1), 3) trimethylation of lysine 4 on histone H3 (H3K4me3), 4) trimethylation of lysine 9 on histone H3 (H3K9me3) and 5) trimethylation of lysine 27 on histone H3 (H3K27me3). The immune-complex was collected by Protein A/G beads and precipitated DNA was purified. The purified DNA was quantified by RT-PCR (qChIP). qChIP analysis of M1 macrophages demonstrated that levels of H3k4me1 ($p=0.006$, $n=10$) (Figure 31a) and H3K4me3 ($p=0.008$, $n=10$) (Figure 31b) significantly increased under HG compared to NG conditions at the CCR2 promoter. An increase in Acetyl H3 (Figure 31c) was observed, however it didn't reach significance ($p=0.13$, $n=4$). Hyperglycaemic conditions didn't affect H3K9me3 ($p=0.42$, $n=6$) (Figure 31d) and H3K27me3 ($p=0.78$, $n=6$) (Figure 31e). The present data provides the evidence that hyperglycaemic conditions induce a dramatic increase in the expression of the CCR2 gene along with epigenetic modifications in the nucleus.
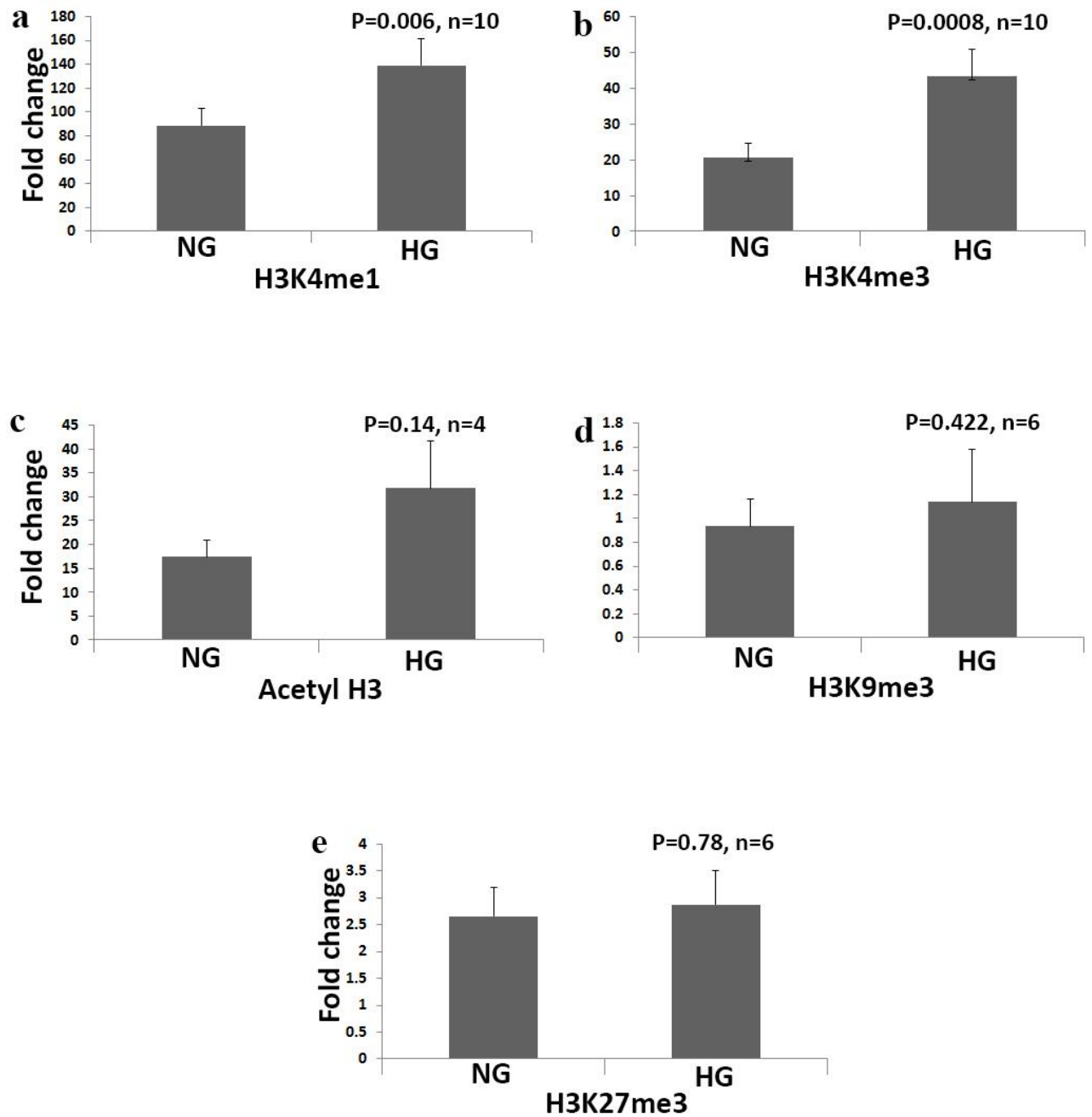
Figure 31. Analysis of histone marks associated with the CCR2 promoter using qChIP. ChIP was done using antibodies specific for a) histone H3 monomethylated at lysine 4 (H3K4me1), b) histone H3 trimethylated at lysine 4 (H3K4me3), c) acetylated histone H3 (AcH3), d) histone H3 trimethylated at lysine 9 (H3K9me3) and e) histone H3 trimethylated at lysine 27 (H3K27me3) at the CCR2 promoter in primary human M1 macrophages cultured in NG and HG. RT-PCR was used to amplify the CCR2 promoter fragment. Each column represents the mean ± SEM. (n=4-10 (which is mentioned in Figure) donors per group; three independent experiments), fold change compared to IgG.

3.11 Histone modification levels correlate to CCR2 gene expression in M1 macrophages

It is known that histone activation marks are indicative of the formation of transcriptionally active chromatin. It was next verified whether the activating histone marks identified on the promoter of CCR2 gene lead to an increase in CCR2 expression.

The ChIP assay for histone mark presence on the CCR2 promoter and RT-PCR analysis of CCR2 gene expression were performed in parallel for M1 macrophages exposed to hyperglycaemic conditions derived from four independent healthy donors. In 3 out of 4 donors (donor 21, 23 and 24), levels of all three histone marks (H3K4me1, H3K4me3 and Acetyl H3) on the CCR2 promoter were increased and correlated with a strong upregulation of CCR2 gene expression (Figure 32). In donor 22, levels of only two histone marks, H3K4me1 and H3K4me3 were increased, and the level of Acetyl H3 was decreased, which correlated with only a slight upregulation of CCR2 gene expression (Figure 32). These data indicate that cooperative action of all three activating histone marks, H3K4me1, H3K4me3 and Acetyl H3, is required for efficient stimulation of CCR2 gene expression in hyperglycaemic conditions.

3.12 Effect of hyperglycaemia on the presence of histone marks on the IL-1beta promoter

The impact of hyperglycaemic conditions on histone modifications at the IL-1beta promoter was studied by ChIP using the same macrophage samples as for analysis of the CCR2 promoter (Section 3.10). Levels of H3k4me1 ($p=0.049$, n=10) (Figure 33a) and H3K4me3 ($p =0.041$, n=10) (Figure 33b) on the IL-1beta promoter were significantly increased in M1 under HG conditions as compared to NG conditions. An increase in Acetyl H3 (Figure 33c) was observed, however it wasn't significant ($P=0.063$, n=4). Hyperglycaemic conditions didn't affect H3K9me3 ($p=0.79$, n=6) (Figure 33d) and H3K27me3 ($p=0.16$, n=6) (Figure 33e). The present data provides that hyperglycaemic conditions induce the expression of the IL-1beta gene along with epigenetic modifications in the nucleus.
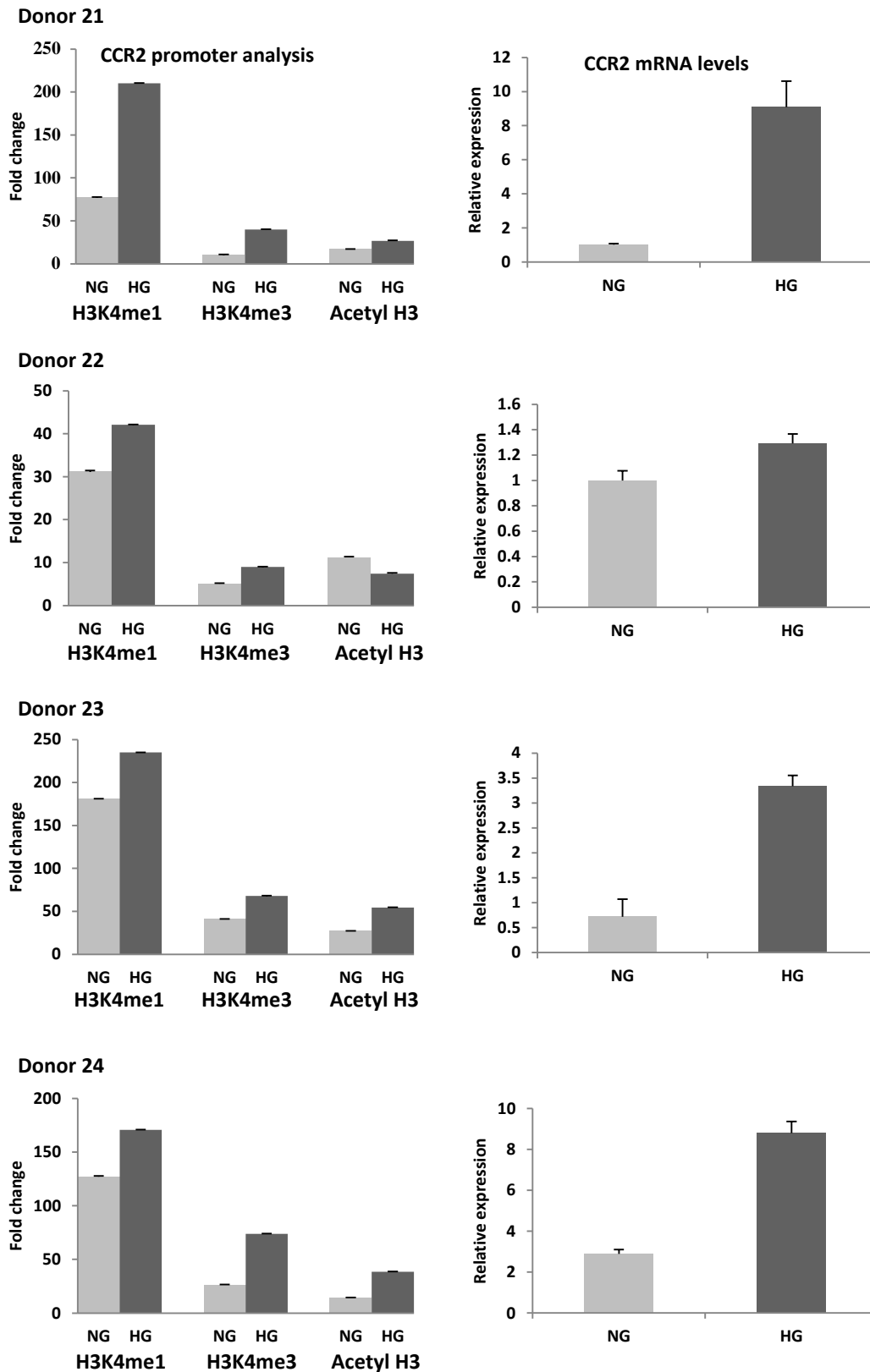
Figure 32. Comparison of the presence of histone marks on the CCR2 promoter with CCR2 gene expression. Macrophages stimulated for 6 days with IFNgamma were used. The left panel shows the results of chromatin immunoprecipitation (ChIP) analysis using antibodies for specific histone marks (H3K4me1, H3K4me3, AcetlyH3) and the right panel shows the results of RT-PCR. qChIP (fold change compared to IgG) and RT-PCR are presented for 4 individual donors (donor 21, 22, 23 and 24). Each row represents an individual donor**.** The experiments were performed in triplicates. NG: 5mM glucose, HG: 25mM glucose.
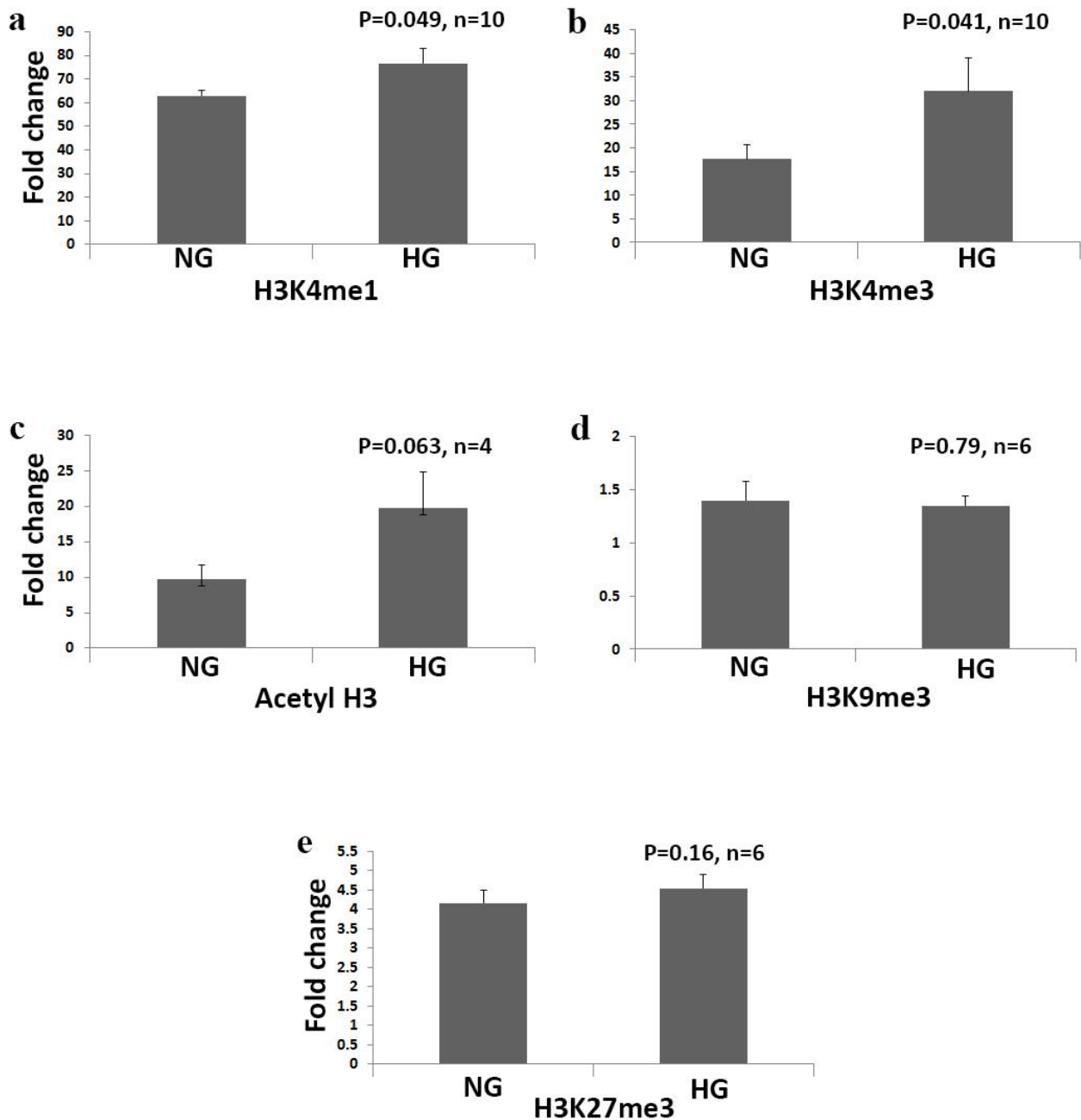
Figure 33. Analysis of histone marks associated with the IL-1beta promoter using qChIP. ChIP was done using antibodies specific for a) histone H3 monomethylated at lysine 4 (H3K4me1), b) histone H3 trimethylated at lysine 4 (H3K4me3), c) acetylated histone H3 (Acetyl H3), d) histone H3 trimethylated at lysine 9 (H3K9me3), and e) histone H3 trimethylated at lysine 27 (H3K27me3) at the IL-1beta promoter in the primary human M1 macrophages that had been cultured in normal (5mM) and High (25mM) glucose. Each column represents the mean $\pm$ SEM (n = 4-10 (mentioned in each Figure) donors per group; three independent experiments) fold change compared to IgG.

## 3.13 Histone modification levels correlate IL-1beta gene expression in M1 macrophages

It was next verified if the activating histone marks identified on the promoter of IL-1beta gene correlate with the increase in IL-1beta gene expression.

The ChIP assay for histone marks present on the IL-1beta promoter and RT-PCR analysis of IL-1beta gene expression were performed in parallel for M1 macrophages exposed to hyperglycaemic condition derived from four independent healthy donors. In 3 out of 4 donors (donor 21, 23 and 24), the levels of all three histone marks, H3K4me1, H3K4me3 and Acetyl H3, on the IL-1beta promoter were increased and correlated with the upregulation of IL-1beta expression (Figure 34). In donor 22, the level of Acetyl H3 was increased, the level of H3K4me1 was not changed (very slight tendency towards increased) and the level of H3K4me3 was decreased, which correlated with downregulation of IL-1beta gene expression (Figure 34). These data suggest that H3K4me1 and H3K4me3 are required for stimulation of IL-1beta gene expression in hyperglycaemic conditions.

Donor 21

Donor 22

Donor 23

Donor 24
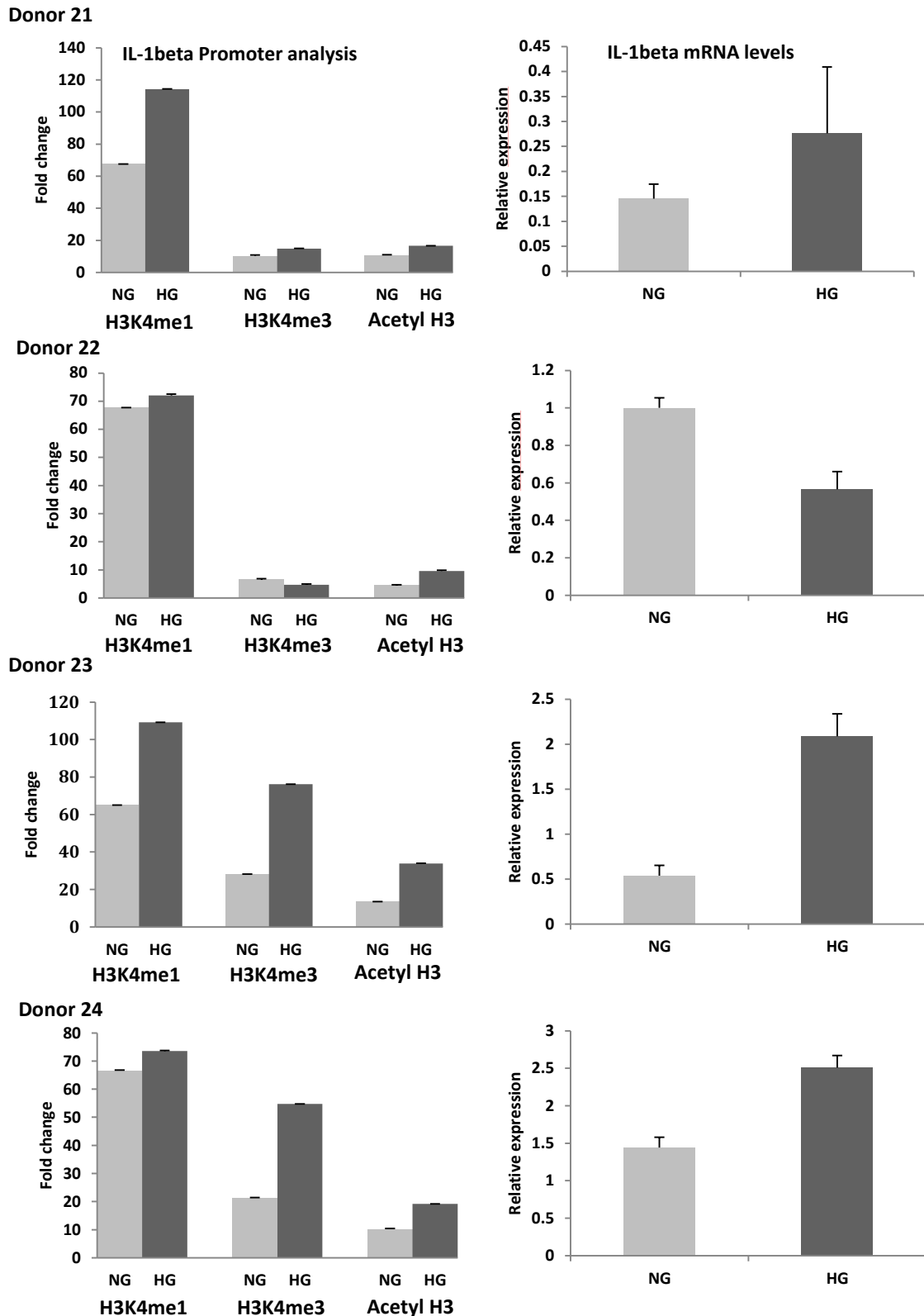
Figure 34. Comparison of the presence of histone marks on the IL-1beta promoter with IL-1beta gene expression in hyperglycaemic conditions. Macrophages stimulated for 6 days with IFNgamma were used. The left panel shows the results of chromatin immunoprecipitation (ChIP) analysis using antibodies for specific histone marks (H3K4me1, H3K4me3, AcetylH3) and the right panel shows the results of RT-PCR. qChIP (fold change compared to IgG) and RT-PCR are presented for 4 individual donors (donor 21, 22, 23 and 24). Each row represents an individual donor. The experiments were performed in triplicates. NG: 5mM glucose, HG: 25mM glucose.

4. Discussion

4.1 Effect of hyperglycaemia on cytokine production during monocyte to macrophages differentiation

Macrophages play a key role in inflammatory reactions in metabolic syndromes, diabetes and vascular complications. However, very limited attention was given until now to the investigation of the role of hyperglycaemia as a separate factor in the programming of primary human monocyte-derived macrophages. The limitation was caused by the absence of a model system to compare the molecular profile and functions of primary human macrophages in serum-free conditions in the presence of normal and high glucose. In the current study, we have established such a system and first analysed how hyperglycaemia affected the production of key cytokines characteristic of the M1 macrophage phenotype (TNF-alpha, IL-1beta) and M2 macrophage phenotype (IL-1Ra, CCL18) (Kzhyshkowska et al., 2016).

Among the analysed cytokines and chemokines, TNF-alpha production is known to induce microvascular complications associated with diabetes (Demircan et al., 2006). In addition to triggering acute and chronic inflammation, TNF-alpha controls glucose and lipid metabolism and prevents insulin production in pancreatic beta cells (Pickup, 2004). It was previously shown that newly diagnosed diabetic patients have increased levels of TNF-alpha in serum compared to non-diabetic controls (Lee et al., 2005; Mirza et al., 2012; Stentz et al., 2004). However, the cellular sources that contribute to increased circulating TNF-alpha levels are still questionable. In this study, it was demonstrated that hyperglycaemia has an immediate effect on the stimulation of TNF-alpha expression and secretion in monocytes (6 hours and 24 hours respectively). Moreover, TNF-alpha secretion during monocyte/macrophage differentiation in hyperglycaemic conditions decreased in a time-dependent manner, probably reflecting the degradation of already secreted cytokine while no additional secretion was induced at later time points when gene expression of TNF-alpha was already down-regulated. Thus, monocytes exposed to high glucose in the blood, but not mature macrophages, can be the source of increased TNF-alpha levels in the circulation of diabetic patients.

Macrophage-derived IL-1beta induces insulin resistance in obesity, mediates beta-cell destruction and impairs insulin secretion (Maedler et al., 2009). In this study, hyperglycaemia was found to stimulate both gene expression and release of IL-1beta in M0 and M1 at all analysed time points (beginning from 6 hours until 6 days). However, while the inducing

effect of hyperglycaemia at early time points was identified for all donors analysed, long-term up-regulation of IL-1beta gene expression was found only in part of the donors, suggesting additional mechanisms that control IL-1beta release in hyperglycaemic conditions. Furthermore, also in M2, hyperglycaemia-induced short-term gene expression of IL-1beta (after 6h), while the elevated release of the cytokine was detected up to 6 days. The possible explanation for the prolonged stimulatory effect of hyperglycaemia on the release of IL-1beta is the involvement of the NLRP3 inflammasome that acts as a platform for the maturation and secretion of IL-1beta (Grishman et al., 2012; Stutz et al., 2009). The NLRP3 inflammasome is an important sensor of metabolic dysregulation and is involved in the regulation of glucose and insulin homoeostasis (Grant and Dixit, 2013). Genetic deletion of components of the NLRP3 inflammasome results in improved glucose tolerance of high-fat diet fed mice (Vandanmagsar et al., 2011a). It was shown that the NLRP3 inflammasome was involved in the progression of diabetes and microvascular complications (Grant and Dixit, 2013; Lee et al., 2013; Shi et al., 2015). Our data suggest that hyperglycaemia has an effect on the maturation of IL-1beta in the NLRP3 inflammasome, not only in M0 and M1, but also in M2 macrophages. Of note, IL-1beta secretion levels were not significantly affected by the type of macrophage polarisation and were comparable for M0, M1 and M2 macrophages at all analysed time points. The data on the upregulation of IL-1beta secretion in hyperglycemic conditions are in line with other studies on different cell types such as bovine retinal endothelial cells (Kowluru and Odenbach, 2004), LPS-activated human monocytes (Orlinska and Newton, 1993), human pancreatic islets (Maedler et al., 2002) and human aortic endothelial cells (Asakawa et al., 1997). In contrast to TNF-alpha, IL-1beta production did not undergo downregulation by the negative feedback mechanism. A study by Spranger et al. demonstrated that increased levels of secreted IL-1beta can predict the development of type 2 diabetes in obese patients (Spranger et al., 2003). Recently, it was demonstrated that islet amyloid induces the production of IL-1beta in cultured human pancreatic islets resulting in beta cell apoptosis (Park et al., 2017). In addition, T1DM patients with kidney failure have higher levels of pro-inflammatory monocytes and circulatory inflammatory mediators such as IL-1beta, IL-1Ra and VEGF compared to patients with T1DM alone (Kolseth et al., 2017). Furthermore, bariatric surgery-induced weight loss was shown to reduce IL-1beta expression in subcutaneous fat (Moschen et al., 2011). Our data suggest that mature macrophages, even being polarised in the M2 direction, can respond to hyperglycaemia by the production of IL-1beta that supports chronic inflammation in different tissues, including sub-endothelial regions and fat tissue.

IL-1Ra is one of the main cytokines released by M2 macrophages acting as a natural inhibitor of IL-1beta by a competitive binding to the receptor of IL-1beta (IL-1R1), and blocking its activation (Palomo et al., 2015). The evidence for the beneficial effect of IL-1Ra was provided by application of a recombinant human IL-1Ra (Anakinra) in type 2 diabetic patients. Treatment with Anakinra resulted in improved beta cell secretory function; normalisation of glycaemic conditions and reduced markers of systemic inflammation C-reactive protein and IL6 (Larsen et al., 2007). However, the role of IL-1Ra in the development of diabetes remains to be controversial since increased levels of IL-1Ra were found to be associated with an elevated risk of type 2 diabetes and insulin resistance in the obese patients (Carstensen et al., 2010; Feve and Bastard, 2009; Herder et al., 2009; Strandberg et al., 2006). Thus, two follow-up studies performed on Whitehall II cohort (UK) identified that systemic levels of IL-1Ra are associated with incident type 2 diabetes indicating the complexity of immune reactions that support the development of diabetes (Carstensen et al., 2010; Herder et al., 2009). In one of these studies it was demonstrated that elevated levels of IL-1Ra can be detected 6 years before type 2 diabetes diagnoses (Carstensen et al., 2010). However, the role of hyperglycaemia in the induction of IL-1Ra was not considered in these studies. The present study for the first time demonstrates that primary human macrophages can respond to hyperglycaemia by the production of elevated levels of IL-1Ra. Moreover, hyperglycaemia not only increased the levels of IL-1Ra in M2 macrophages, but was also able to increase its production in M0 and M1 macrophages, suggesting that hyperglycaemia can induce IL-1Ra in chronic inflammatory conditions that precede type 2 diabetes development.

Production of CCL18 in macrophages is highly specifically induced by IL4, and this cytokine is one of the most pronounced secreted M2 markers (Kodelja et al., 1998; Kzhyshkowska et al., 2016). However, despite being produced by M2 macrophages that primarily function in tissue remodelling and healing, overexpression of CCL18 can have detrimental pro-fibrotic activities. Increased amounts of CCL18 were identified in alveolar macrophages and bronchoalveolar lavage of patients with scleroderma lung disease (Luzina et al., 2002). In a mouse model, overexpression CCL18 using adenoviral delivery into the lungs resulted in a massive perivascular and peribronchial infiltration of T lymphocytes, destruction of alveolar architecture and collagen accumulation (Luzina et al., 2006). It was recently demonstrated that secreted levels of CCL18 from white adipose tissue positively correlate with insulin resistance (Eriksson Hogling et al., 2016). CCL18 was suggested as a biomarker for diabetic nephropathy to monitor treatment efficiency (Tam, 2008). Analysis of the role of CCL18 in

the progression of coronary artery disease in patients with cardiac angiography demonstrated that increased CCL18 levels were associated with coronary calcification (CAD) (Versteylen et al., 2013). In our primary monocytes-based experimental system we demonstrated for the first time that hyperglycaemia suppresses the release of CCL18 in M2 macrophages in most of the donors analysed. These data suggest that elevated levels of CCL18 in diabetic conditions are not a consequence of hyperglycemic activation of macrophages, and other metabolic parameters should be considered for the induction of CCL18 production in monocytes and macrophages.

Altogether, the analysis of the cytokine release indicated that hyperglycaemia itself, independent of other metabolic factors, associated with metabolic syndrome or diabetic conditions, can induce a mixed M1/M2 cytokine secretion profile that can support the progression of diabetes and vascular complications. Out of four analysed cytokines, IL-1beta was the only cytokine for which not only secretion levels, but also gene expression was constitutively induced during 6 days of macrophage maturation that suggested a possible epigenetic level of expression regulation in hyperglycaemic conditions.

4.2 Effect of hyperglycaemia on the transcriptional profile of mature M0, M1 and M2 macrophages

To assess the effect of hyperglycaemia on global transcriptional profiles of differentially polarised macrophages, the Affymetrix microarray assay was used. Human primary monocyte-derived macrophages differentiated for 6 days without stimulation (M0), stimulated with IFNg (M1) and stimulated with IL4 (M2) were used for analysis.

Affymetrix microarray expression profiling demonstrated that hyperglycaemia primarily influences gene expression in pro-inflammatory M1 macrophages, followed by M0, and has only a minor effect on M2 macrophages. In M1 macrophages, 771 genes were upregulated and 803 genes were downregulated, in M0, 518 genes were upregulated and 655 genes were downregulated, and in M2, 9 genes were upregulated and 7 genes were downregulated. These findings were consistent with the major differences in metabolism of M1 and M2 macrophages, where M1 utilise glucose as an energy source, while M2 utilise fatty acids as an energy source (Mills and O'Neill, 2016).

The major groups of genes that were upregulated in M0 and M1 macrophages included 1) cytokines and chemokine receptors; 2) the RNase A family; 3) the solute carrier family; 4) the S100 calcium binding protein family; 5) the transmembrane protein family, and 6) the zinc finger family.

Cytokines and their receptors are major regulators of inflammatory processes both on a systemic level and during cell-cell communication during the development of diabetes and diabetic vascular complications. The strongest effect of hyperglycaemia was found in the expression of CCR2, IL-1beta, CSF1R, IL6ST and IL10RA genes. In recent studies in a mouse model, it was shown that blockade of CCL2/CCR2 signalling protected from diabetic nephropathy (Seok et al., 2013). It was demonstrated that increased levels of secreted IL-1beta can predict the development of type 2 diabetes in obese patients (Spranger et al., 2003). During pancreatitis, CSF1 dependent macrophages were shown to promote islet angiogenesis and maintain beta cell population in a mouse model (Tessem et al., 2008). However, the direct effect of hyperglycaemia on the expression of these genes in primary macrophages was demonstrated by us for the first time. The role of members of the S100 calcium binding protein family in diabetes and vascular complications was also shown previously (Donato et al., 2013a). S100 can be produced by neutrophils, macrophages, fibroblasts, osteoblasts and melanoma cells (Donato et al., 2013b). It was demonstrated that S100A12 was strongly upregulated in diabetic patients (Kosaki et al., 2004). Here for the first time, we found that primary macrophages can respond to hyperglycaemia by elevation of S100A9 and S100A12 expression levels. Hyperglycaemia also stimulated expression of members of the RNase A family members including RNASE4, RNASE1, RNASE6 and RNASET2 genes. It was recently suggested that the overexpression of RNASEK may mediate the effect of genotype on diabetes (Traurig et al., 2016), however, the role of these RNA degrading enzymes in the pathogenesis of diabetes remains to be investigated. Hyperglycaemia also stimulated expression of members of the solute carrier family group including various types of transporters for carnitine fatty acid, sodium bicarbonate, glutamate and the fatty acid sodium/myo-inositol. Hyperglycaemia-induced expression of transporters for SLC4A7 (transporter for sodium bicarbonate), SLC17A7 (transporter for glutamate transport), SLC5A3 (sodium/myo-inositol cotransporter), SLC25A20 (transporter for carnitine-fatty acid) and SLC27A3 (transporter for fatty acid). Hyperglycaemia also stimulated expression of members of the transmembrane protein family groups including TMEM130, TMEM45B, TMEM173, TMEM65 and TMEM256. These proteins mainly act as gateways to permit the

transfer of specific substances through the membrane. The overexpression of transmembrane protein 2 suggests that it can influence the accumulation of lipid droplets in cellular triglyceride in fat tissue (Miranda et al., 2014). Hyperglycaemia also stimulated expression of members of the zinc finger family groups including ZMYM3, ZNF467, ZNF436, ZNF652 and JAZF1 genes. These proteins play a role in stabilisation of the fold by coordinating zinc ions. It was demonstrated that ZNF236 was strongly upregulated in hyperglycaemia in human mesangial cells, suggesting it as a marker for diabetic nephropathy (Holmes et al., 1999).

In order to identify the role of hyperglycaemia in the inflammatory programming of macrophages, we focused on the most strongly affected genes from the family of cytokines and cytokine receptors: CCR2 and IL-1beta. Since chemokine receptor CCR2 plays an important role in trafficking of leukocytes during inflammation and the CCR2/CCL2 axis is involved in multiple immunological processes (O'Connor et al., 2015; Yamasaki et al., 2012), the role of CCR2 in hyperglycemic conditions was further analysed at the functional (migration assay) and epigenetic levels.

4.3 Hyperglycaemia induces CCR2 expression and migration of macrophages towards a CCL2 gradient

Chemokines and cytokines play a role in the pathogenesis of diabetes (Navarro-Gonzalez and Mora-Fernandez, 2008; Shanmugam et al., 2003b). CCR2 belongs to the superfamily of the G-protein-coupled seven-transmembrane receptors (Sallusto et al., 1998) and is known to recruit monocytes to the sites of inflammation through interaction with its ligand CCL2 (Chu et al., 2014; Deshmane et al., 2009; Janssen et al., 2002). The effect of diabetic conditions, including hyperglycaemia, on CCL2 production and action, was extensively studied. High levels of CCL2 were found in many inflammatory conditions, and it is a major chemotactic signal for monocytes/macrophages in inflammation (Brodmerkel et al., 2005). Several studies demonstrated that CCL2 is strongly upregulated by hyperglycemic conditions (Panee, 2012; Tesch, 2008; Wood et al., 2014). Several cell types including endothelial cells, vascular smooth muscle cells, mesangial cells, fibroblasts and pancreatic beta cells were shown to secrete CCL2 (Bertuzzi et al., 2004; Martin et al., 2008; Monickaraj et al., 2014; Piemonti et al., 2002). It was also shown that human pancreatic islets secrete CCL2 during diabetic conditions (Bertuzzi et al., 2004; Martin et al., 2008; Piemonti et al., 2002). Another study demonstrated that human retinal endothelial cells secrete CCL2 during diabetic retinopathy (Monickaraj et al., 2014). Recent studies in a mouse

model demonstrated that blockade of CCL2/CCR2 signalling has a protective effect in diabetic nephropathy (Seok et al., 2013). However, the role of hyperglycaemia in the regulation of CCR2 expression is controversial and can be cell-type specific. Thus, high glucose was previously shown to downregulate CCR2 expression in primary human mesangial cells (Janssen et al., 2002) and to induce CCR2 expression by podocytes in human diabetic nephropathy patients (Tarabra et al., 2009). The results of this study for the first time demonstrate that primary human macrophages react to hyperglycaemia by strong upregulation of CCR2 expression. Inconsistent with the strong induction of CCR2 expression, hyperglycaemia also induced migration of M1 macrophages towards a CCL2 gradient even when CCL2 was used in very low concentrations. Therefore, even slight increases of CCL2 production in hyperglycaemic conditions without any additional strong inflammatory stimuli can be sufficient to stimulate migration of M1 macrophages due to their increased sensitivity to CCL2 mediated by elevated levels of CCR2. Since CCR2+ macrophages are recognised as inflammatory macrophages and positively correlate with the extent of inflammation (Weisberg et al., 2006), it was hypothesised that hyperglycaemia skews macrophages towards a pro-inflammatory state and may favour their enhanced migration in the areas of diabetes-related inflammation and in fat tissues. Since pancreatic islets secrete CCL2 (Martin et al., 2008) that may attract pro-inflammatory M1 macrophages leading to the destruction beta cells, it would be a promising approach to block the CCR2 receptor to attenuate M1 macrophage migration into pancreatic islets or fat tissues. Of note, it was previously shown that CCR2 antagonist treated macrophages significantly decreased migration towards CCL2 *in vitro* (Lee et al., 2010). The results of the current study suggested that, in perspective, CCR2 antagonists may be used to attenuate migration of CCR2 expressing macrophages in the areas of inflammation in diabetic patients.

4.4 Epigenetic regulation of CCR2 and IL-1beta expression

Several studies showed that gene expression in macrophages is regulated on the epigenetic level in the inflammatory conditions (Amit et al., 2016; Kapellos and Iqbal, 2016a; Lavin et al., 2014). Evidence accumulates demonstrating that epigenetic factors regulate gene expression in endothelial cells, vascular smooth muscle, retinal and cardiac cells in diabetic conditions (Reddy et al., 2013; Reddy et al., 2015; Villeneuve et al., 2011). In the current study, the hypothesis about the hyperglycaemia-mediated regulation of the expression of pro-inflammatory genes CCR2 and IL-1beta was tested experimentally. Bioinformatic analysis of CCR2 and IL-1beta promoters using Roadmap Epigenomics Visualization Hub (VizHub)) was used to select the histone marks. Three activating histone marks (H3K4me1, H3K4me3

and acetylated H3) and 2 repressing histone marks (H3K9me3 and H3K27me3) were selected for chromatin immunoprecipitation experiments.

Epigenetic level of regulation for CCR2 gene expression was previously demonstrated in limbic forebrain in mice treated with methamphetamine (Ikegami et al., 2010). In the present study, it was shown for the first time that hyperglycaemia induces the presence of activating histone marks (H3K4me1, H3K4me3 and AceH3) on the CCR2 promoter associated with increased expression of the CCR2 gene. Remarkably, two elevated level histone marks (H3K4me1 and H3K4me3) were found to be sufficient to increase the expression of the CCR2 gene. These results are comparable with the study in a mouse model used by Ikegami et al. where up-regulated CCR2 gene expression was shown to be accompanied by a significant increase in histone H3 lysine 4 (H3K4) trimethylation (Ikegami et al., 2010).

IL-1beta is a critical factor produced by M1 macrophage conditions and involved in the detrimental effects (Gao et al., 2014). Epigenetic regulation of IL-1beta DNA methylation was demonstrated in human articular chondrocytes (Hashimoto et al., 2009). It was also demonstrated that association of H3 serine10 phosphorylation on the IL-1beta promoter correlates with gene expression in LPS stimulated THP-1 cells during sepsis (Yoza and McCall, 2011). In this study, it was shown that the control of IL-1beta gene expression in M1 macrophages by hyperglycaemia is regulated through activating histone modifications H3K4me1, H3K4me3 and AceH3. Overall, the current study for the first time demonstrates that hyperglycaemia induces the association of activating histone marks with promoters of CCR2 and IL-1beta genes in primary human macrophages.

Each specific histone modification is mediated by an enzyme called transferases (for example, methyltransferases, acetyltransferases) (Arrowsmith et al., 2012). Several groups have demonstrated that SET7, the methyltransferase responsible for mono-methylation of H3K4, can mediate vascular damage in hyperglycaemic conditions (El-Osta et al., 2008; Li et al., 2008; Okabe et al., 2012; Paneni et al., 2015; Sun et al., 2010). It was shown that hyperglycaemia induces SET7 translocation into the nucleus of human endothelial cells (Okabe et al., 2012). Furthermore, it was demonstrated that hyperglycaemia-induced overexpression of pro-inflammatory cytokine IL8 requires functional SET7, since this effect was abrogated in SET7 knockdown endothelial cells. The active role of SET7 in hyperglycaemia-induced upregulation of IL8 expression was confirmed by the fact that hyperglycaemia increased monomethylation of H3K4 on the IL8 promoter in endothelial cells (Okabe et al., 2012). The study of Paneni et al. demonstrated that increased levels of SET7

transcripts were found in peripheral blood mononuclear cells isolated out of patients with type 2 diabetes compared with healthy controls that correlated with the monomethylation of H3K4 on the NF-kB promoter (Paneni et al., 2015). SET7/9 was also shown to be involved in the upregulation of inflammatory genes (TNF-alpha and CCL2) in macrophages from diabetic mice (Li et al., 2008). However, the direct effect of hyperglycaemia on the activation of SET7 in primary human monocytes was not demonstrated to date. The identified by us effect of hyperglycaemia on the increased association of H3K4me1 with promoters of inflammatory genes is consistent with the data obtained on endothelial and mononuclear cells. Moreover, for the first time, we demonstrated that hyperglycaemia induces increased association of H3K4me1 with promoters of CCR2 and IL-1beta in mature human macrophages. The role of SET7 in the monomethylation of H3K4 on CCR2 and IL-1beta in primary monocyte-derived macrophages in hyperglycaemic conditions remains to be identified.

Tri-methylation of H3K4 is mediated by another group of enzymes, MLL (Mixed lineage leukaemia) (Ruthenburg et al., 2007). MLL regulates mainly HOX gene (genes involved in embryogenesis) (Milne et al., 2002). The function of MLL was mainly studied in the context of leukaemia (Milne et al., 2002; Zhu et al., 2016). However, the effect of hyperglycaemia on the activation of MLL was not demonstrated to date. Identified in the current study, the effect of hyperglycaemia on the increased association of H3K4me3 with promoters of CCR2 and IL-1beta suggests the involvement of MLL enzymes in hyperglycaemia-mediated pro-inflammatory activation of different cell types. Evidence accumulates indicating that epigenetic editing (writers or erasers) can be used to target endogenous gene expression (de Groote et al., 2012). Currently, new strategies are under development based on the targeting of histone modifying enzymes to modulate gene expression for therapeutic purposes, while such strategies were mostly suggested for treatment of cancer (Altucci and Rots, 2016). In future, epigenetic editing can be a powerful tool to target gene expression of CCR2 to prevent the accumulation of M1 macrophages in pre-diabetic or diabetic lesions.

In summary, we found that hyperglycaemia-induced expression of CCR2 and IL-1beta on primary human macrophages is linked to the epigenetic modifications of the CCR2 and IL-1beta promoters by activating the histone code. Elevated levels of CCR2 resulted in a high sensitivity of macrophages to the chemotactic ligand CCL2. Our data suggest that hyperglycaemia can be a primary factor that induces attraction of pro-inflammatory macrophages into the sites of low grade inflammation that can affect the progression of vascular complications at very early stages.

5. <u>Summary</u>

Hyperglycaemia is the hallmark of diabetes that is related to the development of diabetic vascular complications. Macrophages are key innate immune regulators of inflammation that undergo two major vectors of functional polarisation: classically (M1) and alternatively (M2) activated macrophages. Both M1 and M2 types of macrophages play a role in diabetes. M1 are involved in the establishment and progression of insulin resistance and inflammatory processes leading to vascular complications, whereas M2 can have protective effects in diabetes by reducing inflammation, obesity and insulin resistance. However, the effect of hyperglycaemia on differentiation and functional programming of macrophages is poorly understood. In order to analyse the detrimental effects of high glucose on the differentiation and activation of monocytes and macrophages, we established a new model system based on primary human monocyte-derived macrophages cultured in serum-free conditions in the presence of 5mM and 25mM glucose. The effects of high glucose were examined in control (M0), classically (M1) and alternatively (M2) activated macrophages. Using RT-PCR and ELISA, the expression and release of TNF-alpha and IL-1beta (M1 cytokines) and IL-1Ra and CCL18 (M2 cytokines) were quantified. Hyperglycaemia stimulated the production of TNF-alpha, IL-1beta and IL-1Ra during macrophage differentiation. The effect of hyperglycaemia on TNF-alpha was acute, while the stimulating effect on the production IL-1beta and IL-1Ra was continuous during monocyte to macrophage differentiation. Production of CCL18 was suppressed in M2 macrophages by hyperglycaemia. Altogether, analysis of the cytokine release indicated that hyperglycaemia itself, independent of other metabolic factors, can induce a mixed M1/M2 cytokine secretion profile that can support the progression of diabetes and vascular complications. In order to identify differentially expressed genes in M0, M1 and M2 macrophages differentiated in normal and high glucose conditions, an Affymetrix DNA microarray was used. We found that hyperglycaemia-induced differential expression of 1171 genes in M0, 1573 genes in M1 and 16 genes in M2. The major affected groups of differentially expressed genes were: chemokines, cytokines, chemokine receptors, the glycoproteins family, the RNase A family, the S100 calcium binding protein family, the solute carrier family, the transmembrane protein family and the zinc finger family. Hyperglycaemia had a very strong inducing effect on the expression of CCR2, a major receptor for macrophage chemotactic factor CCL2 that mediates recruitment of macrophages in chronic inflammation. The ability of hyperglycaemia to enhance the trans-migratory activity of macrophages was analysed in a trans-well system. Significantly higher amounts of M0 (7.6 times increase) and M1 (11.2 times increase) transmigrated towards CCL2 (100

ng/ml) in hyperglycaemic conditions. In consistency with the strong induction of CCR2 expression, hyperglycaemia also induced migration of M1 macrophages towards CCL2 even when it was used in very low concentrations (up to 1.56 ng/ml).

The histone code was demonstrated to be an essential mechanism that controls macrophage differentiation in inflammatory conditions. However, the role of the histone code in the hyperglycaemia-mediated programming of human macrophages remained unknown. The chromatin immunoprecipitation assay (ChIP) was applied to examine the presence of histone marks on the promoters of these genes. Three active histone modifications (acetylation of histone H3(aceH3), H3K4me3 and H3K4me1) and two repressive histone modifications (H3K9me3 and H3K27me3) at the promoters of CCR2 and IL-1beta genes were analysed in primary human macrophages cultured in normal and hyperglycaemic conditions. It was demonstrated that hyperglycaemia caused a statistically significant increase in the level of histone activating marks H3K4me1 and H3K4me3 at the CCR2 promoter and IL-1beta promoters. Hyperglycaemia did not affect repressing histone marks on the CCR2 and IL-1beta gene promoters. Analysis of macrophages isolated from individual donors demonstrated that levels of activating histone marks on CCR2 and IL-1beta promoters corresponded to the level of up-regulation of their gene expression. The cooperation of H3K4me1, H3K4me3 and AcetylH3 was required for efficient stimulation of CCR2 gene expression, while cooperation of H3K4me1 and H3K4me3 was critical for stimulation of IL-1beta gene expression in hyperglycemic conditions. Tri-methylation of H3K4 is mediated by the MLL group of enzymes, and our study, for the first time, suggests that MLL enzymes can be involved in the hyperglycaemia-mediated epigenetic programming of macrophages.

In summary, we found that hyperglycaemia-induced expression of CCR2 and IL-1beta on primary human macrophages is linked to epigenetic modifications of CCR2 and IL-1beta promoters by activating the histone code. Elevated levels of CCR2 resulted in a high sensitivity of macrophages to the chemotactic ligand CCL2. Our data suggest that hyperglycaemia can be a primary factor that induces attraction of pro-inflammatory macrophages into the sites of low-grade inflammation that can affect the progression of vascular complications at very early stages.

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7. <u>Curriculum Vitae</u>

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2013-2016: PhD in Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, University of Heidelberg.

2009-2012: Research Project in Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Dortmund, Germany.

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Papers Published:

Kondaiah Moganti, Feng Li, Sarah Riemann, Harald Klüter, Martin Harmsen, Alexei Gratchev, Julia Kzhyshkowska. Hyperglycemia induces mixed M1/M2 cytokine profile in primary human monocyte-derived macrophages. Immunobiology 2016

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Julia Kzhyshkowska, Alexandru Gudima, **Kondaiah Moganti**, Alexei Gratchev, Alexander Orekhov. Perspectives for Monocyte/Macrophage-Based Diagnostics of Chronic Inflammation. Transfusion Medicine and Hemotherapy 2016;43:66–77.

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