THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Regulation of inflammation by adipocytes through interaction with macrophages and in atypical antipsychotic drug treatments

By Anitta Kinga Sárvári

Supervisor: Prof. László Fésüs

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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ABBREVIATIONS

AAPs	Atypical antipsychotics
ADFP	Adipose differentiation-related protein
Ags	Antigens
ARI	Aripiprazole
ATGL	Adipose triglyceride lipase
Bid	BH3-interacting domain death agonist
BMI	Body mass index
BrefA	BrefeldinA
cFLIP	Cellular FLICE-like inhibitory protein
CHX	Cycloheximide
CNTL	Control
CMFDA	Cell tracker green
CMTMR	Cell tracker orange
CytD	CytochalasinD
DIL	Death-inducing ligands
DISC	death inducing signaling complex
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DR	Death receptors
FACS	Flow cytometry
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FTO	Fat mass and obesity associated protein
HAL	Haloperidol

HSL	Hormone-sensitive lipase
IAPs	Inhibitor of Apoptosis
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IMDM	Modified Dulbecco's Media
LPS	Lipopolysaccharide
LSC	Laser-scanning cytometry
LXR	Liver X receptor
MAGL	Monoacylglycerol lipase
MCP-1	Monocyte chemoattractant protein-1
MCSF	Macrophage colony-stimulating factor
MFGE-8	Milk fat globule-EGF-factor 8
mTOR	Mechanistic target of rapamycin
MΦ	Macrophages
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OLZ	Olanzapine
PA	Primary adipocytes
PAI-1	Plasminogen activator inhibitor-1
PAMPs	Pathogen-associated molecular patterns
PBS	phosphate buffer saline
PPAR-γ	Peroxisome proliferator activated receptor gamma
PBS	Phosphate buffered saline
QUE	Quetiapine
RIS	Risperidone

SA	SGBS adipocytes
SGAs	Second-generation antipsychotics
SGBS	Simpson–Golabi–Behmel syndrome preadipocyte
SNPs	Single nucleotide polymorphisms
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
WAT	White adipose tissue
ZIP	Ziprasidone

1. INTRODUCTION

1.1Adipose tissue

Two general types of adipose tissue can be distinguished by histological and functional means in mammals, white and brown. White adipose tissue is a loose connective tissue which in humans is mainly localized under the skin (subcutaneous) and around the internal organs (visceral).White adipocytes store triglycerides and cholesterol in a single large lipid droplet (unilocular appearance), while brown adipocytes contain several smaller lipid droplets (multilocular appearance), express higher amount of uncoupling protein-1 and have a high number of mitochondria and oxidize fatty acids for heat production (non-shivering thermogenesis) (Hyvönen & Spalding 2014) .

White adipose tissue is a highly dynamic tissue, i.e. capable of rapidly changing its mass according to the body's energy status. Lipid-laden mature adipocytes constitute the majority of white adipose tissue mass and volume, they account for less than 20% of the total cells in the tissue (Eto et al. 2009); the other cells are a heterogeneous population of precursors (preadipocytes, stem cells), endothelial cells, macrophages, fibroblasts, and lymphocytes (Cinti et al. 2005). Although previously considered as a passive energy storage, white adipose tissue is now recognized as an important endocrine organ, secreting adipokines, which regulate important physiological functions such as appetite, energy expenditure, insulin sensitivity, inflammation and coagulation (Hauner 2007). White adipose tissue is characterized by a continuous turnover of the adipocytes with approximately 10% of annual renewal (Spalding et al. 2008). Old cells usually die by apoptosis and are removed by professional phagocytes such as macrophages to keep cell number in a regulated equilibrium/balance (Duvall et al. 1985). It is generally accepted that apoptotic cells (Kerr J. F. R., Wyllie A. H. 1972) have a strong anti-inflammatory potential preventing inflammation

in healthy tissue (John Savill & Fadok 2000)(Kurosaka et al. 2003). The prevalence of macrophages in white adipose tissue of lean and obese mice and humans is selectively localized to dead adipocytes forming a so called crown like structure (Cinti et al. 2005). In lean adipose tissue the ratio of number of macrophages is 5%, while, during obesity this rises up to 50%. The characteristics of macrophages are shifted towards a pro-inflammatory state in obese adipose tissue compared to lean individuals (Weisberg et al. 2003)(Xu et al. 2003)(Ortega et al. 2009). Interestingly, the number of macrophages also increases during weight loss, when adipocytes do not die but shrink losing their lipid content (Kosteli et al. 2010).

1.1.1 Adipose tissue – a metabolic organ

Long before, the function of white adipose tissue, as an energy storage depot was well established. Most of the energy stored comes from ingested triglyceride appearing in the circulation. Some fatty acids are also synthesized in the liver and the adipocytes through lipogenesis (*de novo* fatty acid biosynthesis from non-lipid substrates mainly carbohydrates). Between meals, in a state of negative energy balance, adipocytes release fatty acids into the circulation to provide energy for peripheral tissues. Triacylglycerol stores are mobilized in adipose tissue through the lipolysis process which refers to the hydrolysis of triacylglycerol into fatty acids and glycerol (Large et al. 2004). Lipolysis in the white adipose tissue of humans and rodents is regulated by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) (Arner 2005). Adipose tissue lipid metabolism (triacylglycerol storage and fatty acid release from adipocytes) is highly regulated by hormones (insulin and catecholamines) and other factors such as nutritional status

(feeding, fasting) and exercises. The integrity of the processes regulating the adipocytes metabolism is essential to maintain the body weight homeostasis (Large et al. 2004).

1.1.2 Adipose tissue – an endocrine organ

The idea that white adipose tissue is only a storage organ changed dramatically when leptin was discovered in 1994 (Zhang Yiying et al. 1994). Leptin is a hormone which is exclusively secreted by white adipocytes and is an important regulator of body weight. It decreases food intake and increases energy expenditure via hypothalamic pathways. The list of white adipose tissue secretion products which influence several physiological interactions contains more than 100 factors (Fischer-Posovszky et al. 2007). Besides metabolites (fatty acids), lipid substances (prostaglandins, steroids), growth factors and adipokines (adiponectin, resistin), the adipose tissue also releases inflammatory cytokines, chemokines and hormones (Trayhurn & Wood 2007) (Sakurai et al. 2013) (Figure 1).

White adipose tissue influences pathways regulating energy homeostasis, glucose and lipid metabolism, thyroid and reproductive system as well as immune response, blood pressure and bone mass (Fischer-Posovszky et al. 2007) (Rosen & Spiegelman 2006). White adipose tissue is now accepted as a very important, active endocrine organ; therefore it is well conceivable that a dysregulated white adipose tissue itself could contribute to obesity-associated disorders.

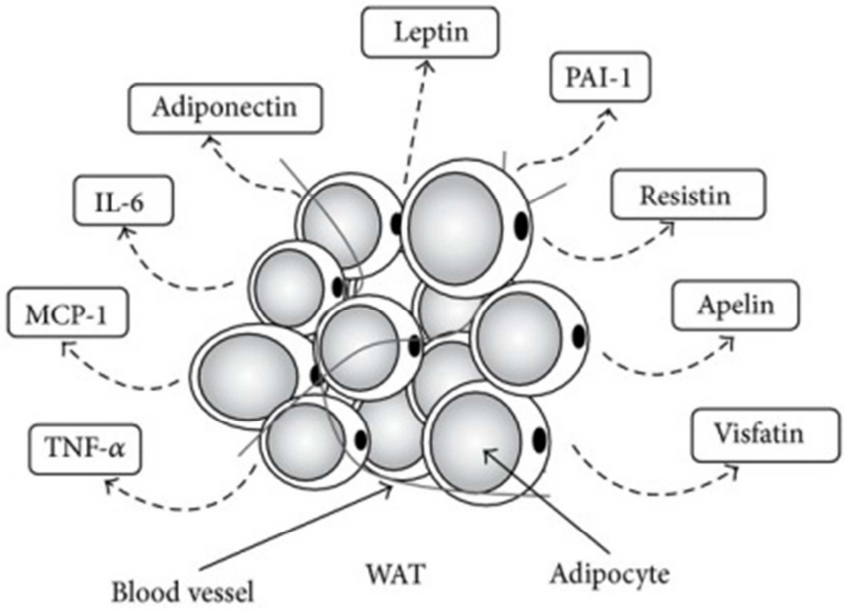

Figure 1. Adipokines secreted by white adipose tissue.

White adipose tissue (WAT) secretes various humoral factors called adipokines. Adipokines have important effects on lipid and glucose metabolism. Tumor necrosis factor-α (TNF-α); monocyte chemoattractant protein-1 (MCP-1); interleukin-6 (IL-6); plasminogen activator inhibitor-1 (PAI-1) (Sakurai et al. 2013).

1.2. Apoptosis

Apoptosis, the programmed cell death, is a well regulated mechanism which is characterized by distinct biochemical events. Apoptosis pathways can be triggered by stimuli originating from the outside of the cell (extrinsic pathway) or from intracellular signals. The extrinsic pathway is triggered by binding of death ligands to their specific receptors on the cell surface and mitochondria, while their associated proteins are in the center of the intrinsic pathway (Hengartner 2000). Triggering of the intrinsic pathway might occur through cellular stress.

Activation of apoptosis pathways eventually leads to activation of caspases that function as common death effector molecules (Degterev et al. 2003). Caspases are proteases belonging to cysteine-aspartic acid protease family that are synthesized as inactive proenzymes and become activated upon cleavage (Degterev et al. 2003). Upon binding of death ligands like tumor necrosis factor alpha (TNF-α), CD95L (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) to their corresponding death receptors, the adaptor molecule Fas-associated death domain (FADD) and the initiator caspase-8 are recruited (Fulda & Pervaiz 2010). Together they form the death inducing signaling complex (DISC) (Fulda & Pervaiz 2010). Cellular FLICE-like inhibitory protein (cFLIP) is a negative modulator of death receptor-induced apoptosis that blocks signal transduction from activated death receptors at the apex of the signaling cascade by preventing the recruitment and activation of caspase-8 at the DISC (Micheau 2003). During apoptosis induction, treatin adipocytes with TNF-α together with the protein biosynthesis inhibitor, cycloheximide (CHX), will block the synthesis of cFLIP. Without cFLIP at the DISC, caspase-8 is activated by cleavage (Lavrik et al. 2005). Activated caspase-8 in turn can either directly transmit apoptosis signals by activating effector caspase-3 or alternatively, may connect to the mitochondrial pathway via the cleavage of BH3-interacting domain death agonist (Bid) (Adams & Cory 2007). Bid is a protein of the Bcl-2 family, which upon cleavage translocates as tBid to mitochondria to stimulate mitochondrial outer membrane permeabilization. This leads to the release of apoptogenic factors like cytochrom C from the mitochondrial intermembrane space into the cytosol (Adams & Cory 2007)(Kroemer et al. 2007). The release of cytochrome C triggers caspase-3 activation through formation of the cytochrome C/ apoptotic protease activating factor-1/ caspase-9-containing apoptosome complex (Kroemer et al. 2007)(Riedl & Salvesen 2007). Both pathways wind up with activation of caspase-3 (Donald W. Nicholson 1997) ultimately

leading to fragmentation of cell macromolecules including DNA which finally results in cell death (Fulda & Pervaiz 2010) (Figure 2).

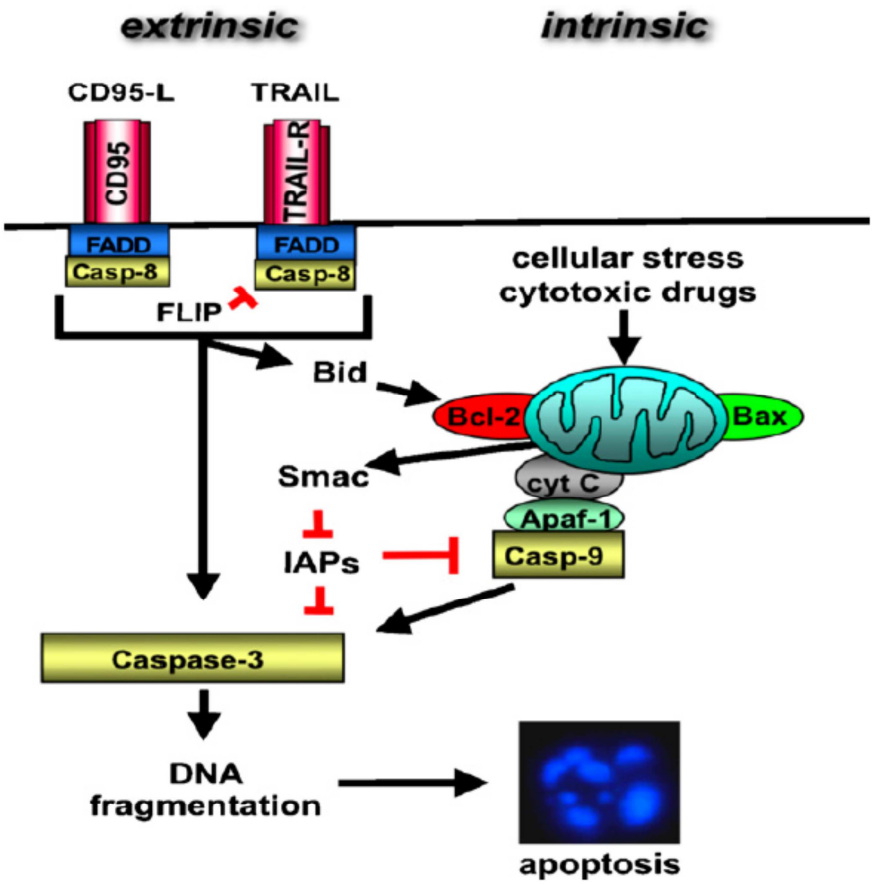

Figure 2. Apoptosis pathways.

Apoptosis pathways can be initiated by ligation of death receptors (DR) by their respective ligands, i.e. death-inducing ligands (DIL), followed by receptor trimerization, recruitment of adaptor molecules (FADD) and activation of caspase-8 (receptor pathway). The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c or Smac from mitochondria in the cytosol. Apoptosis pathways are regulated by pro-apoptotic Bcl-2 family proteins such as Bax and Bak, anti-apoptotic Bcl-2 family proteins, i.e. Bcl-2 and

Bcl-XL, and "Inhibitor of Apoptosis" (IAPs) proteins. Smac promotes apoptosis by neutralizing IAP-mediated inhibition of caspase-3 and -9 (Fulda & Pervaiz 2010).

1.2.1. Fat cell apoptosis

Apoptosis of adipocytes was shown in several disease states accompanied by loss of adipose tissue including tumor cachexia (Prins, Johannes et al. 1994) and autoimmune lipodystrophy (Fischer-Posovszky et al. 2006). Additionally, HIV patients under highly active anti-retroviral therapy showed fat cell apoptosis (Domingo, P., et al. 1999). Interestingly, our group already showed that despite expressing all components of the apoptosis pathways, fat cells are resistant to apoptosis under normal physiological conditions (Fischer-Posovszky & Wabitsch 2004). Furthermore, blocking protein synthesis which mimics starvation, render fat cells sensitive to apoptosis induction by death ligands (Fischer-Posovszky & Wabitsch 2004).

1.3. Obesity

Overweight and obesity are defined as abnormal or excessive storage of energy as fat, which has adverse effects on health (O'Rahilly & Farooqi 2008). A measure of obesity is the body mass index (BMI), a person's mass (in kilograms) divided by the square of his or her height (in meters). A person with a BMI of 30 or more is generally considered obese (WHO, 2014) (Figure 3). Obesity is major risk factor for a number of chronic diseases, including diabetes, cardiovascular diseases, and cancer. In physiological context, BMI is driven via the balance between the adipocyte's growth and removal. Adipose tissue can grow by either hyperplasia

or hypertrophy or both, whereas adipocyte removal is facilitated by apoptosis (Cristancho Ana G. 2011)(Lowe et al. 2011)(Ahima 2006).

Figure 3. Body mass index.

WHO global estimates from 2008 showed, that 1.4 billion adults were overweight, of these over 200 million men and nearly 300 million women were obese. Overall, more than 10% of the world's adult population was obese. This number is rising every year (WHO, 2014). Obesity reduces life expectancy (Symonds 2010) and at least 2.8 million people die each year as a result of being overweight or obese (WHO, 2014). Although obesity is preventable, it is worldwide a leading cause of death, it is regarded as the most serious public health problem of this century (Wilkin & Voss 2004). The cause for obesity besides medical or psychiatric (Barness et al. 2007) reasons might be a genetic predisposition. Twin studies, for instance, estimated the average heritability of obesity in the range between 40-75% (O'Rahilly & Farooqi 2008). Additionally, genome-wide association studies revealed several genes with single nucleotide polymorphisms (SNPs) that associate with obesity (Tang et al. 2014). However, results are only consistent for some genes, or fat mass and obesity (FTO) (Frayling et al. 2007). The fundamental cause of obesity is an overconsumption of energy together with decreased energy expenditure. Our modern lifestyle, a combination of having access to high

energy food with a lack of physical activity therefore creates the perfect basis. The adipose tissue is the first tissue ever affected by these changes.

1.4. Chronic low grade inflammation in obesity

With weight gain in obesity a chronic low-grade inflammation develops associated with several metabolic diseases, such as type 2 diabetes mellitus, atherosclerosis and liver steatosis. This low-grade inflammatory response is mainly mediated by adipocytes regulating the release of adipocytokines, such as IL-6, TNF-α and MCP-1 which stimulates the infiltration of macrophages into adipose tissue and their activation (Shoelson et al. 2007). Adipocyte hypertrophy and local hypoxia are also implicated in macrophage recruitment, since both conditions can mediate increased production of inflammatory cytokines and chemo-attractants (Wood et al. 2009) (Han & Levings 2013) (Figure 4).

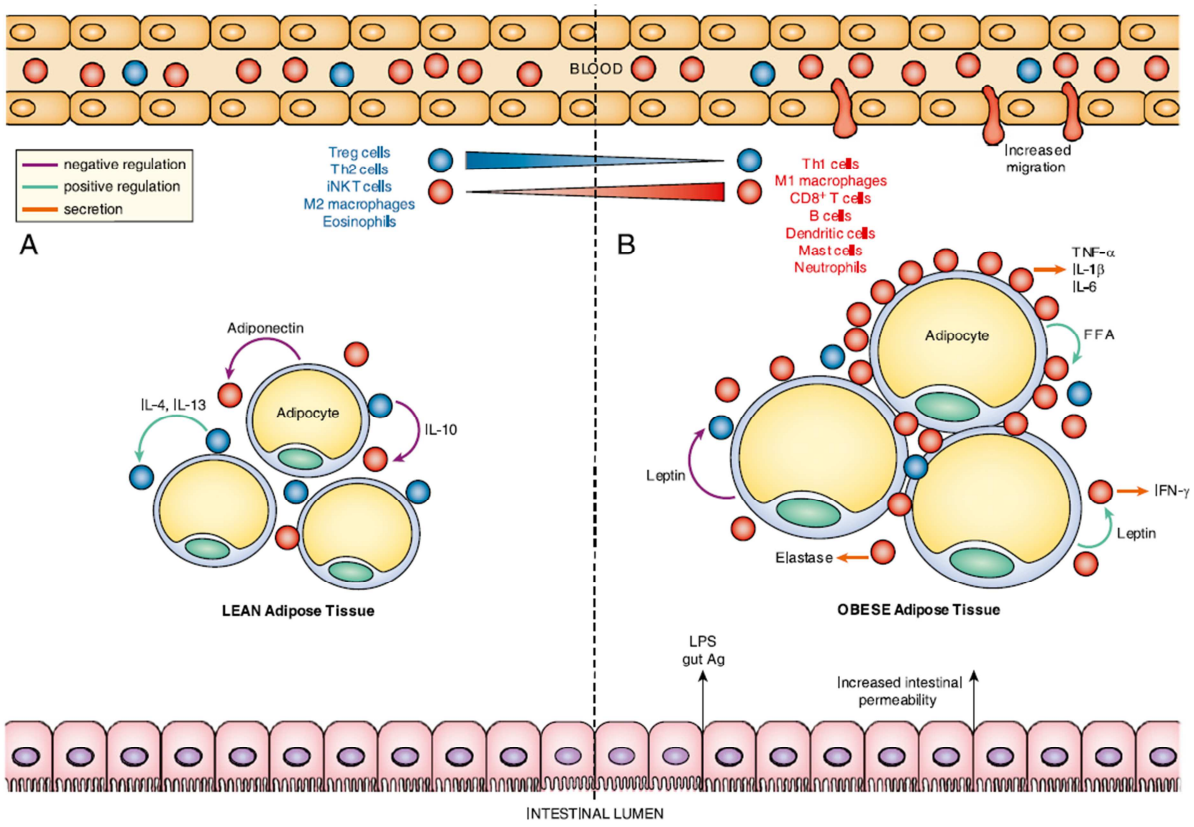

Figure 4. The loss of immune regulation in obesity-associated adipose tissue inflammation.

(A) Lean adipose tissue contains regulatory immune cells (blue) that suppress pro-inflammatory immune cells (red) and sustain alternative activation of macrophages via Th2-associated cytokines. Adipocytes in lean adipose tissue are of normal size and produce adiponectin, which has anti-inflammatory properties. (B) In contrast, obese adipose tissue is infiltrated with pro-inflammatory immune cells that produce high amounts of inflammatory cytokines and chemokines. M1 macrophages accumulate in crown-like structures around hypertrophic adipocytes that have increased rate of lipolysis, and secrete free fatty acids (FFA) that can serve as endogenous danger signals to stimulate production of inflammatory cytokines, such as TNF-α. Adipocytes in obese adipose tissue also have increased leptin production, which promotes Th1 cells and inhibits Treg expansion. The gut barrier is disrupted in obesity, causing gut Ags and PAMPs such as lipopolysaccharide (LPS) to enter

the adipose tissue and stimulate inflammation. Furthermore, immune cells in the blood migrate into the adipose tissue in response to heightened chemokine production (Han & Levings 2013).

1.5. The role of atypical antipsychotics in the development of obesity

Atypical antipsychotics (AAPs), or second-generation antipsychotics (SGAs), are widely prescribed for the treatment of several psychiatric disorders. However, these drugs are associated with many mild and serious side effects. The major side effects of AAPs are weight gain and its associated metabolic disorders, such as type II diabetes and dyslipidemia (Newocomer 2005)(Paredes et al. 2014). The increase in obesity-related adipose tissue mass may derive from both increased adipocyte size due to lipid accumulation in differentiated adipocytes, and increased adipocyte number due to the differentiation of adipose- derived stem cells (PAs) present in adipose tissue (Tan et al. 2010). Studies in cultured rodent adipocytes suggest that certain AAPs can facilitate lipid storage and stimulate adipogenesis (Vestri et al. 2007)(Yang et al. 2007).

2. AIM OF THE STUDY

Adipogenesis, when adipocytes develop from mesenchymal stem cells, is a fairly well known process. **Our aim was 1) to quantitate the transition of human preadipocytes to adipocytes and 2) to measure morphological and viability changes of differentiating adipocytes on a cell-by-cell basis with statistical relevance.**

Although there is a direct contact between adipocytes and macrophages due to the crown like structure formation in obese adipose tissue, not much is known about the consequences of these interactions. Most of the studies in the field rely on *in vivo* mouse model experiments, only a few cell culture-based observations were reported (Keuper et al. 2011). These studies were reflecting on the importance of cell-cell contact of adipocyte-macrophage interaction, which leads to pro-inflammatory cytokine secretion, such as IL-1β, TNFα and IL-6 (De Boer et al. 2014), (Xie et al. 2010), (Lumeng, Deyoung, et al. 2007). These data, however, still represent murine cell experiments. Knowing that mouse and human macrophages differ with respect to activation profiles (Mantovani et al. 2004), we developed a human *in vitro* experimental system to study co-cultures of adipocytes and macrophages and learn what the outcome of their interaction is. We hypothesized that interaction between macrophages and adipocytes may lead to phagocytosis of the latter with significant consequences in the balance of pro- and anti-inflammatory factors in obese adipose tissue. **We intended 3) to confirm this hypothesis and 4) to investigate the inflammatory consequences of interaction of macrophages with adipocytes.**

There is limited information regarding the effect of atypical antipsychotic drugs on human preadipocytes (Sertié et al. 2011). It is not yet known how AAPs affect the differentiation process of resident preadipocytes or the terminally differentiated adipocytes, or whether

increased lipid storage could cause a level of cellular stress high enough to trigger a cell death pathway at the gene expression level in adipocytes. Since to date, only a few studies have examined the effect of the AAP drugs used for the treatment of psychiatric disorders at the gene expression level (Yang et al. 2007)(Sertié et al. 2011)(Minet-Ringuet et al. 2007)(Vik-Mo et al. 2009)(Hemmrich et al. 2006), **an additional aim of the present study was 5) to investigate the effect of atypical antipsychotic (AAP) drug treatment on differentiated adipocytes**.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Cell culture media

- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) - Sigma
- Fetal bovine serum (FBS) - Gibco
- Human AB serum - Sigma
- Macrophage colony-stimulating factor (MCSF) - Bioscience/Promega
- Modified Dulbecco's Media (IMDM) – Sigma

3.1.2. Cell dyes

- Annexin V-Fitc/Propidium iodide-Apoptosis detection kit - MBL
- Cell tracker green (CMFDA) - Invitrogen
- Cell tracker orange (CMTMR) - Life Technologies
- Hoechst 33342 (Sigma, 50 µg/ml)
- IKK-2 Inhibitor, SC-514 (Calbiochem)
- NF-κB SN50 Cell-Permeable Inhibitor Peptide (Calbiochem)
- Nile blue – Sigma
- Nile red – Sigma
- NucRed – Suigma

3.1.3. Antibodies/Inhibitors

- Primary antibody, goat polyclonal anti-human IL-6 IgG - R&D Systems
- Secondary antibody Anti-Goat IgG–FITC, produced in rabbit - Sigma
- TNFα – PeproTech
- (IL)-6, IL-1β, IL-8, TNFα, MCP-1 ELISA DuoSet - R&D Systems

3.1.4. Reagents

- BrefeldinA (BrefA) – Sigma
- Cycloheximide (CHX) - Sigma
- CytochalasinD (CytD)- Sigma
- Lipopolysaccharides (LPS) - Sigma
- Trypsin – Sigma

3.2. Methods

In current studies, multiparametric model systems have been developed, where adipocyte differentiation, its nuclear, cytoskeletal and sub-organellar morphological changes can be monitored together with different drug applications in an automated manner. Among these works, Lee had introduced a noticeable flow cytometric assay to assess differentiation of preadipocytes, based on cytoplasmic granularity changes corresponding to lipid droplet accumulation (Lee et al. 2004). However, application of flow cytometry, which requires cell suspensions, strongly violates physiological optima of solid tissues. This technique also omits the possibility of *in situ* visualization and reanalysis of samples attached to culture dish. These latter obstacles were solved by applying image cytometry in solid tissue quantifications, as in Lin's work, where laser scanning cytometry was utilized to study adipocytes and measure capacity of drugs to induce adipocyte apoptosis (Lin et al. 2005)(Lin et al. 2004). Yet, cell recognition of attached cells was not solved or at least not clearly explained whether single cell contouring or phantom contouring was used in their study. When attached cells get more confluent during their culturing, cell-by-cell segmentation becomes challenging and requires sophisticated algorithms and tools. There have been a few attempts to enable cell-by-cell analyses, e.g. McDonough et al. (McDonough et al. 2009)(McDonough et al. 2011) introduced an algorithm to recognize cells in confluent cultures and a later study applies this algorithm (Linehan et al. 2012). However, their assumptions that preadipocytes or adipocytes cover all the surface of culture disk, and nuclei are located in center of cells, seems inapplicable for human fat cells. Till now there was no quantitative study about human adipogenesis that focuses on single-cell imaging cytometry and would have been efficiently adaptable for high throughput applications.

3.2.1. Isolation of adipose tissue-derived stem cells

Human adipose tissue was obtained from the subcutaneous adipose depot of volunteers undergoing herniectomy without other medical condition. Selection was made based on body mass index (BMI < 30), but not on age or gender. Informed consent was obtained from the subjects before the surgical procedure. The study protocol was approved by the Ethics Committee of the University of Debrecen, Hungary (No. 3186-2010/DEOEC RKEB/IKEB). The adipose tissue samples (1-10 ml) were immediately transported to the laboratory after being removed. The adipose tissue specimens were dissected from fibrous material and blood vessels, minced into small pieces and digested in phosphate buffer saline (PBS) with 120 U/ml collagenase (Sigma) for 60 min in a 37°C water bath with gentle agitation. The completely disaggregated tissue was filtered (pore size 140 µm) (Sigma) to remove any remaining tissue. The cell suspension was centrifuged for 10 min at 1300 rpm, and the pellet of stromal cells, including the adipose tissue-derived stem cells, were re-suspended in complete medium, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) containing 10% fetal bovine serum (FBS), 100U/ml penicillin/streptomycin, 33/17 µM biotin/panthothen and centrifuged again for 10 min at 1300 rpm. Cells were seeded into 6-well plates at a density of ~3 x 10^4 cells/cm^2 and cultured in complete DMEM-F12 medium at 37°C in 5% CO_2 for 24 h to attach. Floating cells were washed away with PBS, and the remaining cells were cultured until they became confluent.

3.2.2. Characterization of adipose tissue-derived stem cells

Table 1. *Characterization of surface antigen expression on adipose tissue-derived stem cells.*

		Percentage of positive cells (Mean±SD)
Hematopoietic/ Monocyte markers	CD14	10.92 ± 4.87
	CD34	3.23 ± 4.56
	CD45	0.00 ± 0.00
	CD47	99.31 ± 0.74
	CD133	0.00 ± 0.00
	CD117/c-kit	89.70 ± 5.58
	HLA-DR	3.73 ± 2.30
Endothelial markers	CD31/PECAM	6.69 ± 3.61
	CD144/ VE-Cadherine	85.32 ± 7.88
	VEGFR2/KDR	0.00 ± 0.00
	CD104/Integrin β4	4.14 ± 1.85
MSC/ Fibroblast markers	CD73	98.37 ± 1.03
	CD90/Thy-1	95.05 ± 0.13
	CD105/Endoglin	93.20 ± 8.73
	CD147/Neurothelin	98.49 ± 1.51
	PDGF Rβ	92.91 ± 7.91
Integrins and CAMs	CD29/Integrin β1	99.81 ± 0.05
	CD44/H-CAM	78.79 ± 23.36
	CD49a/Integrin α1	99.52 ± 0.19
	CD49b/Integrin α2	21.31 ± 4.28
	CD49f/Integrin α6	0.00 ± 0.00
	CD56/NCAM	4.66 ± 6.59
	CD146/MCAM	65.27 ± 12.87
	CD166/ALCAM	98.19 ± 1.70

To investigate the phenotype of the preadipocytes used in experiments, a multiparametric analysis of surface antigen expression was performed by three-color flow cytometry using the following fluorochrome-conjugated antibodies: CD34, CD44, CD45, CD49f, CD73, CD144, CD147 (all from BD Biosciences, San Jose, CA, USA); CD49a (Biolegend, San Diego, CA, USA), CD29, CD31, CD47 CD49b, CD56, CD90, CD104, CD105, CD117, CD146, CD166,

PDGFRb, VEGFR2 (all from R&D Systems, Minneapolis, MN, USA) and CD133 (Miltenyi Biotech, Gladbach, Germany) with isotype matching controls (Table 1). After cells were harvested with 0.025% trypsin-EDTA, they were washed once with normal medium and twice thereafter with FACS buffer. Cells were incubated with antibodies on ice for 30 min according to the manufacturers' protocols, washed again with FACS buffer, fixed in 1% PFA/PBS and analyzed within 1 day. Samples were measured using a FACS Calibur BD flow cytometer, and data were analyzed using WinMDI2.8 software.

3.2.3. Induction of adipocyte differentiation

Adipose tissue-derived stem cells and Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes have been used as adipocytes sources for our experiments. SGBS preadipocytes are a recently developed model for studying human adipocytes with subcutaneous (Fischer-Posovszky et al. 2008). These cells have high capacity for adipose differentiation displaying a gene expression pattern similar to mature human fat cells (Heinze et al. 2001). Preadipocytes were seeded in ibidi 8-chamber slides, or 24 well plates at a density of $1.5x10^4$ cells/cm^2 in DMEM/F12 (Sigma) medium containing 100 U/ml penicillin/streptomycin, 33 µM biotin, 17 µM pantothenate and 10% FBS at 37°C in 5% CO_2 until they reached confluency. Differentiation was induced for four days using serum-free differentiation medium [DMEM/F12 supplemented with 2 µM rosiglitazone (Caymen Chemicals), 25 nM dexamethasone (Sigma), and 500 µM 3-isobutyl-1-methylxantine (Sigma), 10 µg/ml human apo-transferrin (Sigma), 20 nM human insulin (Sigma), 100 nM cortisol (Sigma), and 200 pM triiodothyronine (Sigma). From the fifth day, cells were further cultured in DMEM/F12 supplemented with 10 µg/ml human apo-transferrin, 20 nM human insulin, 100 nM cortisol, and 200 pM triiodothyronine (Fischer-Posovszky et al. 2008).

3.2.4. Induction of macrophage differentiation

Human PBMCs were isolated by density gradient centrifugation on Ficoll-Paque Plus (Amersham Bioscience) from “buffy coats” obtained from healthy blood donors. $CD14^+$ cells were separated by magnetic sorting with Vario-MACS (Miltenyi Biotech), followed by washing with PBS containing 0.5% BSA and 2 mM EDTA. Freshly isolated monocytes were seeded into 24 well plates at a density of 10^6 cells/ml and cultured in Modified Dulbecco's Media (IMDM) medium (SIGMA) suplemented with 10% of human AB serum (Sigma) and 5 nM macrophage colony-stimulating factor (MCSF) (Bioscience/Promega) for 5 days to differentiate them to macrophages, the medium was refreshed after three days.

3.2.5. Phagocytosis assay

Primary adipocytes (PA) differentiated for ten days and SGBS adipocytes (SA) were stained with Hoechst 33342 (Sigma, 50 µg/ml) and 1 µg/ml Nile red for 30 minutes. To decrease nonspecific accumulation of Hoechst and Nile red by macrophages during the phagocytosis process, cells were carefully washed 2 times in PBS. Macrophages were stained with fluorescent cell tracer green cell tracker green (CMFDA) (Invitrogen) according to the manufacturer’s protocol. Macrophages were layered on the top of adipocytes in a ratio of 5:1 and were co-cultured for 24 hours at 37°C in a 5% CO_2 atmosphere. Phagocytic ratio was determined counting the macrophages containing lipid droplets applying laser-scanning cytometry (LSC). For flow cytometric (FACS) measurements, cells were trypsinized (Sigma) and centrifuged at 1800 rpm for 10 minutes; when macrophages sedimented to a pellet, while adipocytes, due to their lipid content, remained in the supernatant. Cells in the pellet were

examined by a FACSCalibur BD flow cytometer and list mode data were analyzed by WinMDI2.8 software.

3.2.6. Apoptosis induction

Every third day of the differentiation period, apoptosis induction was conducted, in which SGBS cells were treated with a mixture of 10 nM human TNFα (PeproTech) and 10 ng/ml of cycloheximide (CHX) (Sigma) for 12 hours.

3.2.7. Experimental design for imaging cytometry measurements

Imaging cytometry measurements were performed along two parallel courses of study. In sequential study, a similarly induced group of fresh SGBS cells was divided into subgroups, which were then placed on separate slides; each subgroup was allowed to grow in an incubator until its scheduled daily measurement. In longitudinal follow-ups, a particular sample was studied in a cohort pattern where cellular changes were tracked on cell-by-cell basis.

Analyses of same cells in cohort type protocols were also carried out by scanning the sample before and after apoptotic induction, and after the lysis of halo assay. Subsequently, “before” and “after” image layers were merged, i.e. computationally stacked over each other, to allow analyses of correlated information from exactly the same cells at different experimental phases.

3.2.7.1. Cell staining

On the day of measurement, cells were stained with Hoechst 33342 (50 µg/ml) for 60 minutes, Nile Red (25 µg/ml) and Nile Blue (750 µg/ml) for 20 minutes. Cell death was detected by propidium iodide and FITC-conjugated Annexin V with concentration indicated in Apoptosis Detection Kit (MBL) manuscript. Samples were washed once with PBS and then kept in fresh medium.

3.2.8. Image acquisition

Images were obtained by using iCys Research Imaging Cytometer (iCys^{TM}, CompuCyte Corporation, Westwood, MA, USA). Sample slides were mounted on a computer-controlled stepper-motor driven stage. Area with optimal confluence was determined in low-resolution scout scan with X10 magnification objective (NA 0.30) and 10-micrometer scanning step. High-resolution images were consequently obtained by using X40 objective (NA 0.75) and 0.25-micrometer step. Size of a pixel was set to 0.25 by 0.245 micrometer at X40-magnification.

Laser lines were separately operated, namely 405-nm Violet diode laser was used to excite Hoechst 33342, 488-nm Argon-ion laser was used for FITC, Nile Red and propidium iodide, 633-nm HeNe gas laser for Nile Blue. Emission was collected by 4 photomultiplier tubes (PMT); Hoechst was detected at 463±20 nm, FITC at 530±15 nm, Nile Red at 580±15 nm, propidium iodide and Nile Blue at 675±25 nm. Transmitted laser light was captured by diode photodetectors in which light loss and shaded relief signals were measured to gain information about light absorption and light scattering of the objects.

3.2.9. Assessment of lipid accumulation

The combination of the Nile Red and texture signal was used to quantitate lipid accumulation, and cells that contained lipid above a preset threshold value were considered differentiated adipocyte. The number of adipocytes over the total count of nuclei gave rise to differentiation ratio. This ratio was calculated in a region of at least 100 field images where each field covered an area of 1024 by 768 pixels. Consequently, data of at least 1000 gated cells were collected and analyzed in cell-by-cell as well as cell-population bases using statistical softwares (GraphPad Prism and Microsoft Excel). All steps of gating were confirmed by relocation and visual identification of cellular events.

3.2.10. Laser-scanning cytometry (LSC)

Imaging cytometry measurements were performed by using iCys Research Imaging Cytometer (iCysTM, Thorlabs Imaging Systems, Sterling, VA, USA) equipped with 405-nm 488-nm, and 633-nm solid-state lasers, photodiode forward scatter detectors and photomultiplier tubes with three filters in front. Sample slides were mounted on the computer-controlled stepper-motor driven stage. An area of optimal confluency was selected in low-resolution scout scan with 10× magnification objective (NA 0.30) and 10-micrometer scanning step. High-resolution images of selected areas were obtained by using 40× objective (NA 0.75) and 0.25-micrometer step. Size of a pixel was set to 0.25 by 0.245 micrometer at 40× magnification. Laser lines were separately operated, namely 405-nm violet was used to excite Hoechst 33342 to recognize nuclei, and 488-nm blue line was used for CMFDA to recognize whole macrophages, and Nile red to identify lipid droplets. Emission of Hoechst was detected at 463±20 nm, CMFDA at 530±15 nm and Nile red at 580±15 nm. Images were processed and analyzed by an automatic cell recognition protocol developed by us utilizing

iCys software (iNovator Application Development Toolkit, CompuCyte Corporation, Westwood, MA, USA), Image J (National Institute of Health, MD, USA) (Schneider et al. 2012), and CellProfiler (The Broad Institute of MIT, MA, USA) (Carpenter et al. 2006)'(Kamentsky et al. 2011), as described earlier (Doan-xuan et al. 2013). Briefly, nuclei from both cell types were identified first and marked as primary objects. Based on parent nuclei, the secondary objects, e.g. whole macrophages and adipocytes, were subsequently recognized according to CMFDA or Nile red signals. When macrophages have been segmented, image regions occupied by macrophages were excluded from further search for adipocytes. Phagocytotic ratio was determined counting macrophages containing Nile red stained lipid droplets.

3.2.11. Time-lapse imaging microscopy

Adipocytes were stained with Nile red and Hoechst 33342, macrophages were layered on top of the adipocytes in a ratio of 5:1. The co-culture was placed in a temperature-, humidity- and CO_2-controlled, motorized IX81 Olympus inverted microscope, which was equipped with a cooled Hamamatsu ORCA-R2 high resolution monochrome CCD camera and a DP21-CU 2-megapixel digital color camera. Cells were monitored for 15 hours and in every five minutes an image was taken. Data were converted into a video file with the use of the xcellence software.

3.2.12. Determination of cytokine release

Differentiated adipocytes (PA, SA) were co-cultured with macrophages for 12 hours, and culture supernatants were harvested and stored for cytokine measurements. In some experiments macrophages were treated with 100μM IKK-2 Inhibitor, SC-514 (Calbiochem), or with 50μg/ml NF-κB SN50 Cell-Permeable Inhibitor Peptide (Calbiochem) and co-cultured with PA or SA. In further experiments macrophages were pretreated with 50 ng/ml crude LPS (sigma) for 30 minutes, or 20 μM CytochalasinD (CytD) (Sigma)for 45 min, and added to PA or SA; supernatant was collected after 12hours. The concentration of interleukin (IL)-6, IL-1β, IL-8, TNFα, MCP-1 was measured from the collected cell culture media using ELISA DuoSet (R&D Systems).

3.2.13. Immunostaining of intracellular IL-6 cytokine

Macrophages were prestained overnight with the cell tracer, cell tracker orange (CMTMR) (Life Technologies). PA or SA and macrophages were co-cultured with macrophages for 12 hours in the presence of 100 ng/ml BrefeldinA (BrefA) (Sigma), a protein transport inhibitor. After co-incubation, cells were fixed in 4% paraformaldehyde (Sigma), permeabilized with 0.1% triton X-100 (Sigma), blocked for 1 hour with 5% horse serum (Gibco) containing milk powder, dissolved in PBS containing 0.005% Tween-20 (Sigma). As a primary antibody, goat polyclonal anti-human IL-6 IgG (R&D Systems) was used in 1:200 dilution for 2 hours. As secondary antibody Anti-Goat IgG–FITC, produced in rabbit (Sigma) was applied in a 1:500 dilution for 1 hour. Olympus FluoView 1000 Confocal microscope was used to detect the localization of IL-6 (FITC 488 nm), macrophages (CMTMR 546 nm) and the nucleus (NucRed 647 nm). For the excitation of FITC labeled secondary antibody the 488 nm line of an Argon ion laser; for CMTMR a 543-nm He-Ne laser; for NucRed a 633-nm He-Ne laser

was used. Fluorescence emissions were detected through 500- to 530-nm, 555- to 625-nm and 655- to 755-nm band-pass filters, respectively.

3.2.14. Real-time Q-PCR

Macrophages were pretreated for 30 minutes with 50 ng/ml crude LPS, then co-incubated with PA and SA. Cells were collected after 1, 2, 3, 4, 5, 6 and 12 hours in 1 ml Tri Reagent (Invitrogen) for total RNA isolation and reverse transcribed to cDNA by High-Capacity Reverse Transcription kit (Applied Biosystem) according to the manufacturers' instructions. Transcript levels of IL-6 were determined by real time Q-PCR using TaqMan Gene Expression Assay (Applied Biosystems). Samples were measured in 3 technical parallels. Genes were normalized for GAPDH housekeeping gene.

Total RNAs from adipocytes derived from four individuals separately were extracted by Trizol Reagent (Invitrogen) and transcribed to cDNA using the High-Capacity Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. PCR array: CAPH09329 Custom Human RT^2 Profiler^{TM} PCR Arrays (SABiosciences) containing premanufactured reactions for 26 customized genes in triplicate and five housekeeping genes were performed according to the SABiosciences' instructions; real-time monitoring was carried out using an ABI Prism 7900 instrument (Applied Biosystems) (Table 2). Genes were selected on the basis of Metabolic Diseases PCR array (SABiosciences) to study obesity and inflammatory response. Each adipocyte RNA sample was analyzed for the expression of the 26 selected genes in triplicate. Delta cycle threshold (Ct) method was used to correct the differences between RNA samples in which the Ct of target genes were normalized to the Ct of cyclophilin D. As all genes were measured at the same time in the same assay for each RNA sample, relative quantification was utilized. Using the Delta Ct values, the comparative

cycle threshold method was used to calculate the fold-change of the different genes (AAP-treated) relative to untreated control used as the calibrator. Calibrator is designated a value of 1.

The fold changes for target genes presented in Figures were calculated as the ratio of expression levels of the (untreated) control and AAP-treated differentiated adipocytes.

To detect changes in gene expression most closely correlated, the fold-changes of relative expression levels were log2-transformed and clustered by complete linkage of Euclidian distances using the Gene Cluster 3.0 software and visualized with heat maps using TreeView (Eisen lab, UC Berkeley).

3.2.15. Drug treatment

The seven schizophrenia drugs were dissolved in DMSO (Sigma) and used in the following final concentrations: olanzapine 50 ng/ml, ziprasidone 50 ng/ml, clozapine 100 ng/ml, quetiapine 50 ng/ml, aripiprazole 100 ng/ml, haloperidol 10 ng/ml, risperidone 50 ng/ml. Drugs were added at the first day of differentiation of adipocytes and then subsequently every day until day 11. Every third day the cell culture media were collected and replaced.

3.2.16. Statistical analysis

For the statistical analyses, two-tailed paired t-TEST (*), and two way ANOVA test (#) was applied.

4. RESULTS

4.1. Monitoring adipocyte differentiation in cell culture conditions

The study of cell differentiation was conducted for 15 days on both cellular (Figure 5 A) and histocytometric aspects (Figure 5 B). The preadipocyte commitment was found to occur on day 2-3 with compacting cytoplasm revealed by increasing light-loss signal (Figure 5A). At this point, forming lipid structures still did not stain with Nile Red (triglyceride) but with Nile Blue (phospholipid). Shortly after that transient mono-signal stage, minor but measurable Nile Red signal appeared (Figure 5 A, Day 4-5). Relatively few small separated lipid droplets and enhanced nuclear condensation were shown in committed preadipocytes on day 2-5. This was in contrast to undifferentiated cells, which had more cytoplasm, round giant and faintly stained nuclei with no Nile Red fluorescence (Figure 5 A, Day 0-1). From day 6 to day 12 most of the adipocytes reflected typical morphological signs of *in vitro*-differentiated adipose cells, such as round distending shape, a cytoplasm filled with lipid droplets as well as strong Nile Red fluorescence and light-loss signals. Shrinking and more brightly stained nuclei, signs of nuclear condensation, became even more pronounced (Figure 5 A, Day 8-9, 10-14). At day 9-12, when ratio of fully differentiated cells reached 40% at the region of optimal confluence (two-dimensional *in vitro* confluence), the differentiation curve was considered saturated (Figure 5 B). Indeed, from day 12 until the completion of the experimental regime (18 days), no further formation of lipid droplets was seen, rather the lipid droplets fused and became distorted (Figure 5 A, Day 14-15).

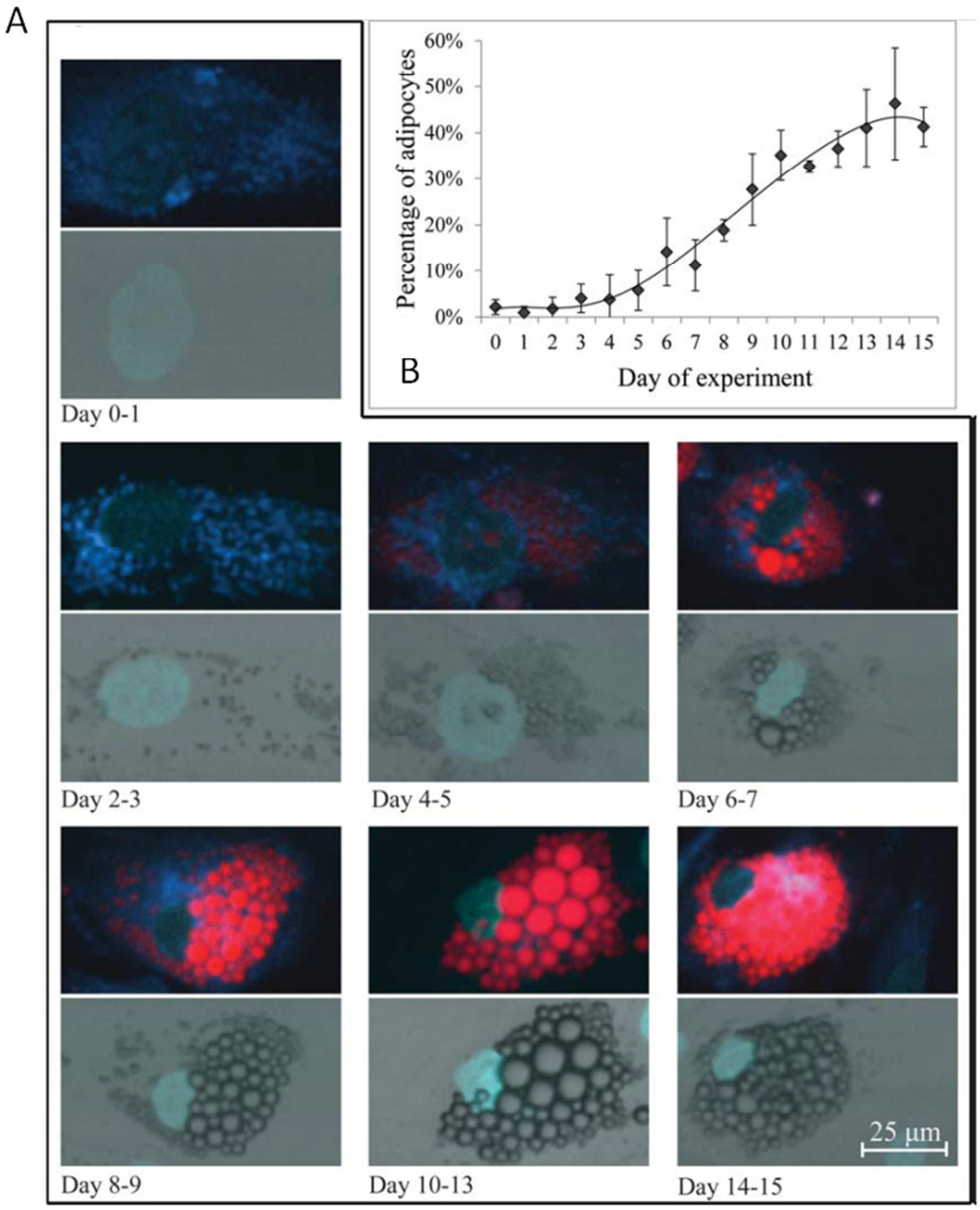

Figure 5. Inspection of adipocyte differentiation.

(A) Representative SGBS cells at different cell ages. Upper panels show phospholipid (blue: Nile Blue) and neutral lipid droplet staining (red: Nile Red), while their lower counterparts are merged images of light loss and nuclear signals (cyan: Hoechst 33342). During the transformation from preadipocytes to adipocytes, the cells underwent morphological changes including shrinkage of nuclei, increase of chromatin compactness and gradually forming lipid droplets. (B) Kinetics of ratio of adipocytes matured over total number of cells. Data were taken from sequential measurements (mean±SD, n=7).

4.2. Detection and quantification of apoptosis in pre-adipocytes and adipocytes

Individual information of each particular cell on how it progressed through the apoptotic induction was gained by merging images taken before and after TNFα treatment. Annexin V labeled cells in the former image layer represented spontaneous apoptosis. AV+ cells in the image layer recorded after the induction (Figure 6 A-F) indicated the inducing effect. A stable spontaneous apoptotic rate was seen in both adipocytes and their precursors (Figure 6 E,F, dark data points), ranging from 7.05 % to 17.91 %. After apoptotic induction, significant increase of Annexin V and propidium iodine positivity were seen in both cell types at every time point of differentiation (Figure 6 E,F, light data points). Particularly, preadipocytes showed more sensitivity to the apoptotic induction, as from 26.06 % to 50.09 % of preadipocytes, with a rising tendency, showed Annexin V positivity (Figure 6 E) compared to a plateau at 30 % average in the adipocyte population (Figure 6 F).

Additionally, the apoptotic induction brought on apoptotic bodies, which showed highly fragmented DNA, and strong fluorescence of Hoechst intensity. Typical membrane blebs in close association with condensed nuclei were observed in the majority of apoptotic cells (Figure 6 C). There was remarkable cell loss, which was proportional to the percentage of cells responding to the apoptotic induction: 24.12 % ± 10.5 % of cells was lost in induced samples compared to 10.67 % ± 4.52 % in controls (from data comparing nuclei counts before and after treatment in day-12 samples).

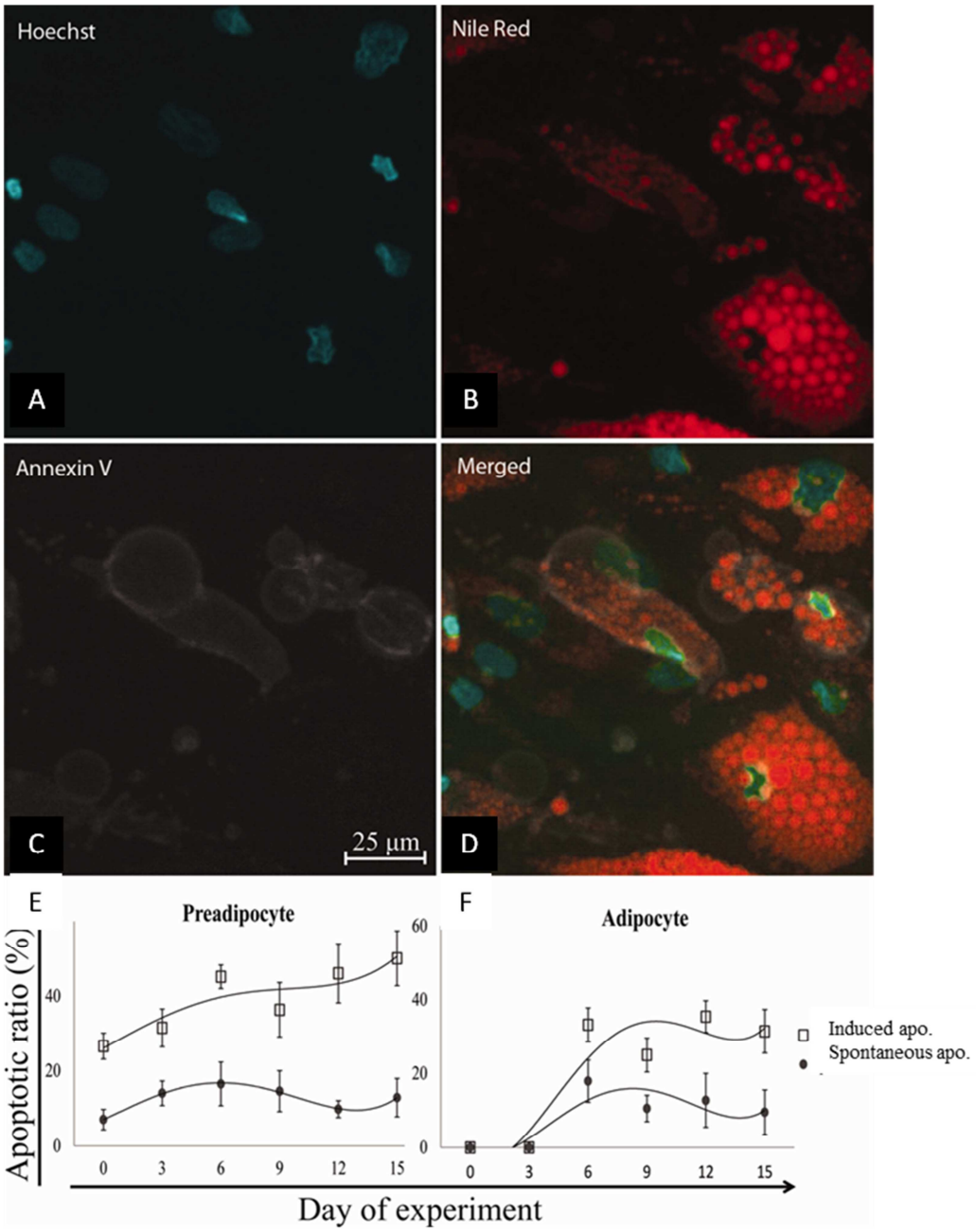

Figure 6. Preadipocytes are more sensitive to TNFα induced apoptosis than adipocytes. *Multi-fluorescent signals exhibited different cellular components after subjecting cells to apoptotic induction: (A) nuclei with Hoechst 33342 staining (cyan), (B) lipid droplets stained with Nile Red (red), (C) apoptotic membrane with Annexin V labeling (white), (D) merged images of Hoechst, Nile Red and Nile Blue (phospholipid stain, shown in blue). (E, F) Response to apoptotic induction of preadipocytes and adipocytes was compared at each time*

point of differentiation. Apoptotic cells were recognized by their Annexin V labeling (mean±SD, n=4).

4.3. Effect of apoptotic adipocytes on macrophages

We have co-incubated the TNF-α + CHX treated dying adipocytes with macrophages for 3 and 24 hours. After co-incubation, the lipid droplet containing macrophages were counted by flow or laser-scanning cytometry. Both analyses confirmed that macrophages efficiently engulfed portions of TNF-α + CHX treated adipocytes: after 3 hours of co-incubation 25-40% of macrophages already contained lipid droplets, which increased up to 60 % after 24 hours (Figure 7).

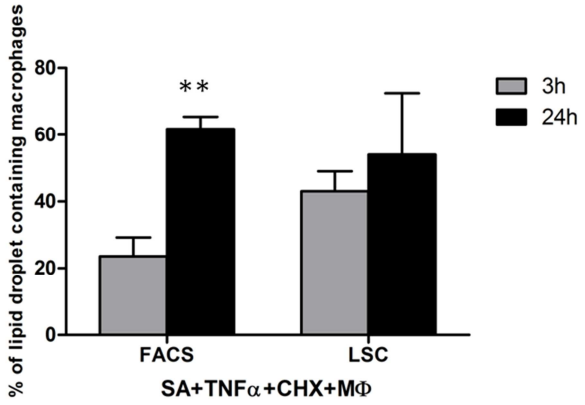

Figure 7. Detection of phagocytosis of TNFα and CHX treated dying adipocytes by macrophages (MΦ).

Adipocytes were pre-treated with 10 nM TNF-α and 10 ng/ml CHX, then stained with 1 µg/ml Nile red. MΦs were pre-stained with 5 µM CMFDA. Cells were co-incubated for 3 and 24 hours. Lipid droplets containing stained MΦs were counted in whole cell co-cultures using laser-scanning cytometry. The phagocytosis ratio was determined by counting the percentage of lipid containing MΦs by LSC and FACS. Data are expressed as mean ±SD of 3 independent experiments.

To support the apoptotic nature of TNFα and CHX treated adipocytes, we have co-incubated these dying cells with LPS treated pro-inflammatory macrophages and observed a significant decrease in the level of IL6 and IL1-β cytokines in the presence of dying adipocytes (Figure 8).

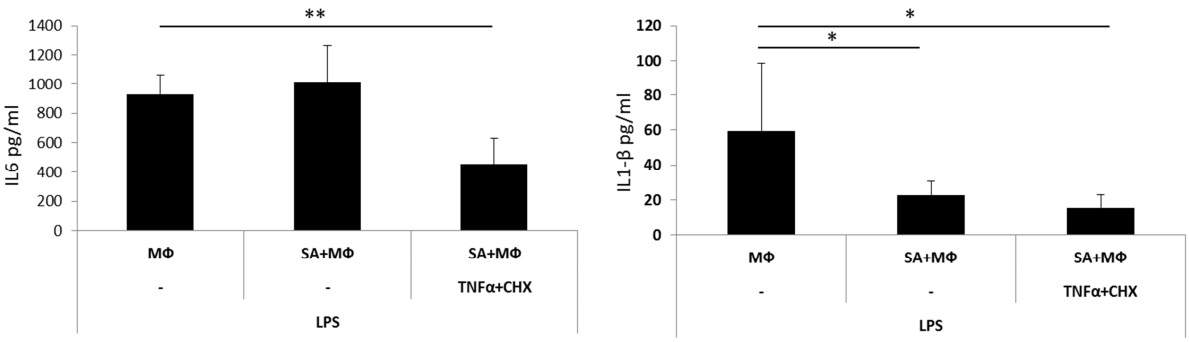

Figure 8. Anti-inflammatory effect of TNFα and CHX induced apoptotic cells

*MΦs were pretreated for 30 min with 0.5 µg/ml crude LPS, and then the activated macrophages were placed onto the culture plates or added to adipocytes (SA) for 12 hours. Supernatants were collected and the level of secreted cytokines was measured by ELISA method. Data are expressed as mean ±SD of 5 independent experiments. p values are as follows; * $p<0.05$, ** $p<0.01$.*

4.4. Pieces of differentiated adipocytes are phagocytosed by macrophages

Besides the basic level of spontaneous apoptosis occurring during adipocyte differentiation (Figure 6E, F), we have also shown that spontaneous DNA fragmentation occurs during adipocyte differentiation in the absence of apoptosis (Doan-xuan et al. 2013). We wanted to investigate whether macrophages could phagocytose some of the differentiated adipocytes, we added macrophages to *in vitro* differentiated adipocytes. After their co-incubation high proportion of macrophages contained lipid droplets (Figure 9 A). The lipid containing macrophages were counted by flow or laser-scanning cytometry. Both analyses confirmed that macrophages efficiently engulf portions of adipocytes: after 3 hours of co-incubation ~15% of macrophages already contained lipid droplets, which increased up to 25-30% after 24 hours (Figure 9 B). Time-lapse microscopic images were taken to follow the process of phagocytosis; we could see several macrophages attacking one adipocyte (a much larger cell type) at a time and phagocytose pieces of the target cells (Supplementary video on CD).

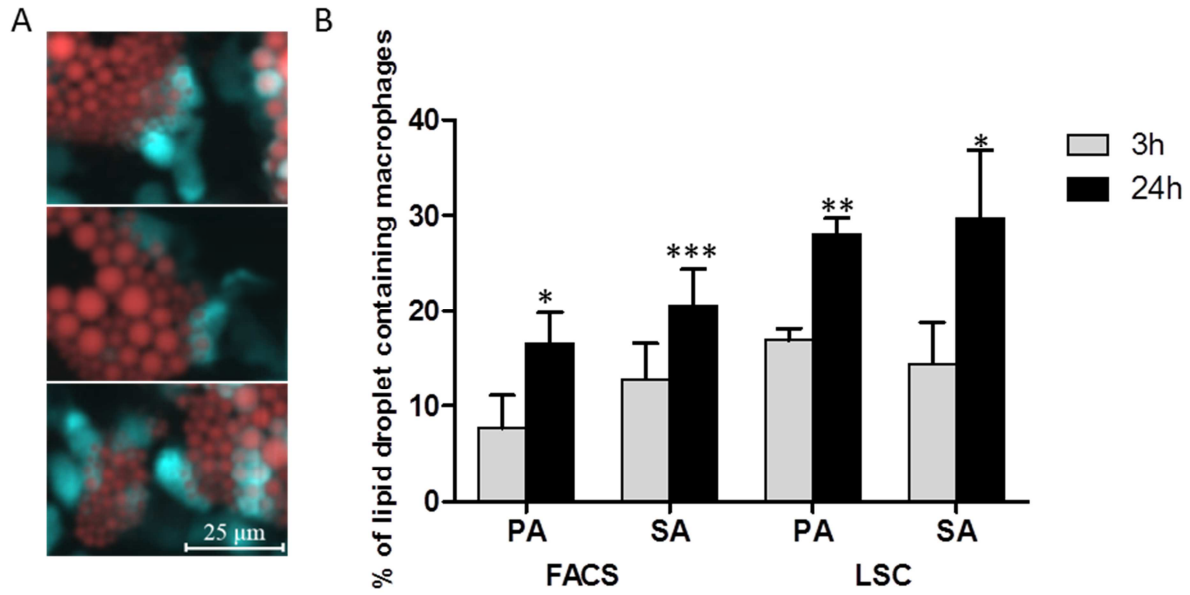

Figure 9. Detection of phagocytosis of living adipocytes by macrophages (MΦ). *Adipocytes were pre-stained with 1 µg/ml Nile red, MΦs were pre-stained with 5 µM CMFDA and cells were co-incubated for 3 and 24 hours. (A) Fluorescent microscopic images show MΦs stained with CMFDA (cyan) which contain adipocyte derived lipids stained with Nile red (red). (B) Red lipid droplets containing stained MΦs were counted in whole cell co-cultures using laser-scanning cytometry. The phagocytosis ratio was determined by counting the percentage of lipid containing MΦs by LSC. Data are expressed as mean ±SD of 3 independent experiments, p values are as follows; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

4.5.Interaction of macrophages and adipocytes leads to selective IL-6 secretion

Significant increase in IL-6 was detected in the culture media upon adipocyte-macrophage co-incubation as compared to the basal level secreted by macrophages or adipocytes alone (Figure 10 A). To prove that the adipocyte induced IL-6 production is a unique property of adipocyte-macrophage communication and not a response from the macrophage to not being allowed to attach to the surface of the plate, macrophages were plated onto another adherent cell type, namely HEK cells; this did not result in induction of IL-6 secretion (Figure 10 B). The level of secreted IL-8 and IL-1β did not change (the latter was below the detection limit of the ELISA kit) (Figure 10 C). MCP1 secretion was increased but it reached a significant level only in presence of SGBS adipocytes (Figure 10 D). Secretion of TNFα was not significantly induced during the co-incubation (Figure 10 E).

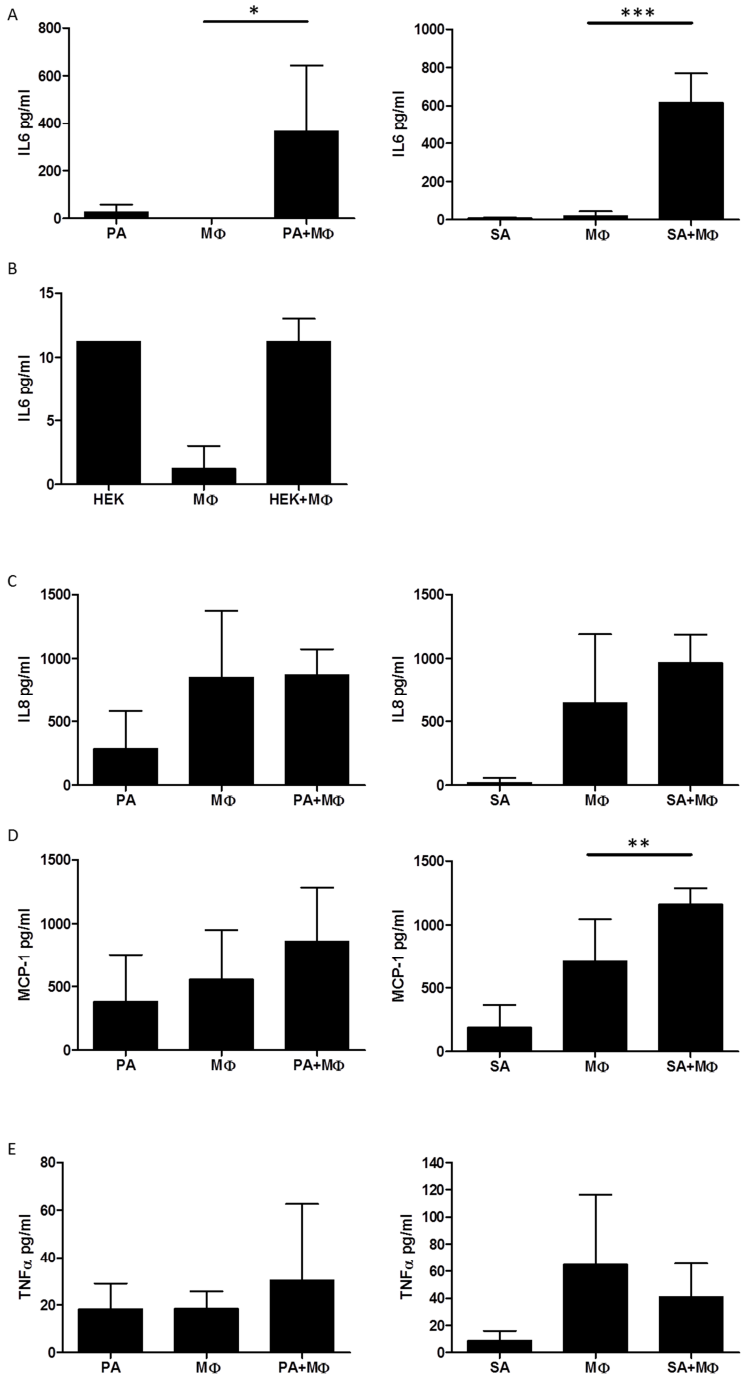

Figure 10. Interaction of macrophages and adipocytes leads to selective IL-6 secretion. *Adipocytes (PA, SA) or HEK cells were co-incubated with MΦs for 12 hours, then supernatants were collected and cytokine concentrations were measured by ELISA. Macrophages (MΦ) were added to the adipocytes or placed onto empty culture plates. Measurement of IL-6 secretion during co-incubation of MΦ with PA or SA (A) and HEK cells*

*(B). Secretion of IL-8 (C), MCP-1 (D) and TNF-α (E) during co-incubation of PA or SA and MΦ.Data are expressed as mean ±SD of 5 independent experiments. p values are as follows; * p<0.05, ** p<0.01, *** p<0.001.*

4.6.IL-6 production in co-cultures of adipocytes and macrophages depends on phagocytosis and is mediated by macrophages.

To learn whether there are any secreted molecules originating from the adipocytes which induce the IL-6 secretion during co-incubation, we cultured macrophages in adipocyte conditioned medium. This did not lead to an increased IL-6 secretion (Figure 11 A, B) suggesting that the induction of IL-6 production is a consequence of interaction between adipocytes and macrophages. To see whether the IL-6 secretion depends upon phagocytosis of adipocyte content, we blocked phagocytosis with CytD. This agent attenuated IL-6 secretion, suggesting that cell-cell contact was not enough to induce the same level of IL-6 secretion as in non-treated controls. (Figure 11 A, B).

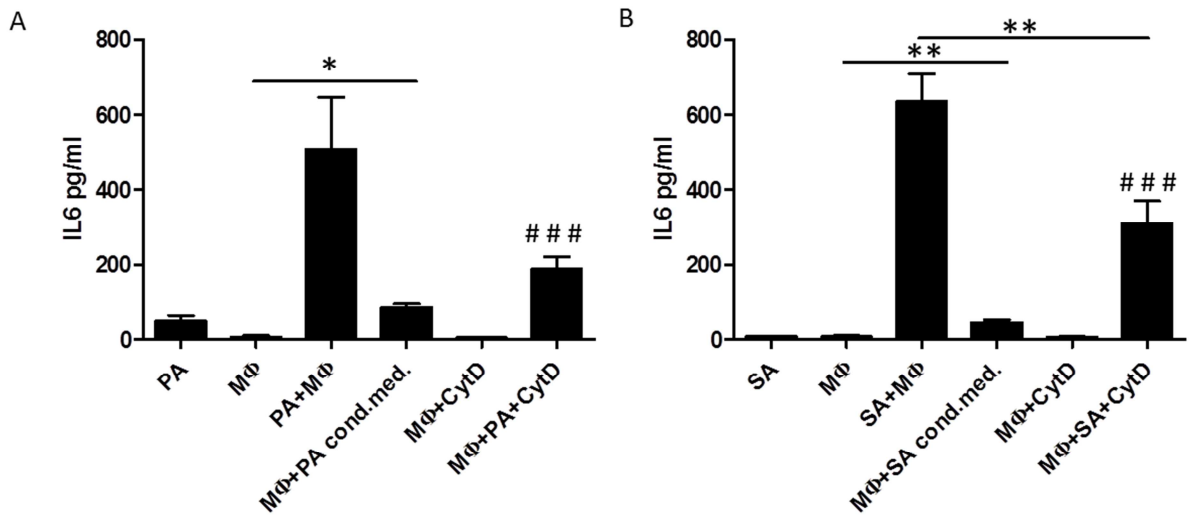

Figure 11. IL-6 secretion during adipocyte co-incubation with MΦs is phagocytosis dependent.

*MΦs were cultured in adipocyte conditioned media or pretreated for 45 minutes with 20µM cytochalasin-D (CytD) before co-culture with adipocytes. CytD concentration was maintained during co-incubation as well. After 12 hours supernatants of the cells were collected and the level of secreted IL-6 cytokine was measured by ELISA. Co-culture of MΦ with PA (A) and SA (B).Data are expressed as mean ±SD of 5 independent experiments. p values are as follows; two-tailed paired t-TEST * p<0.05, ** p<0.01; two way ANOVA test ###<0,001.*

Next, we compared the dynamics of the secretion and release of IL-6 after exposure of macrophages to either adipocytes or LPS. Time-dependent analysis of mRNA and secreted IL-6 shows the same pattern in the two cases (Figure 12 A, B) suggesting that IL-6 is *de novo* synthesized during adipocyte-macrophage co-incubation. Although the increase in the IL-6 mRNA level is delayed in SA samples compared to PA, which phenomenon can be due to the difference between the two cell types. The fact that, mRNA level stays lower in case of PA and SA, compared to the LPS treated macrophages could come because of the different mechanisms involved in IL-6 induction and production in the two different conditions.

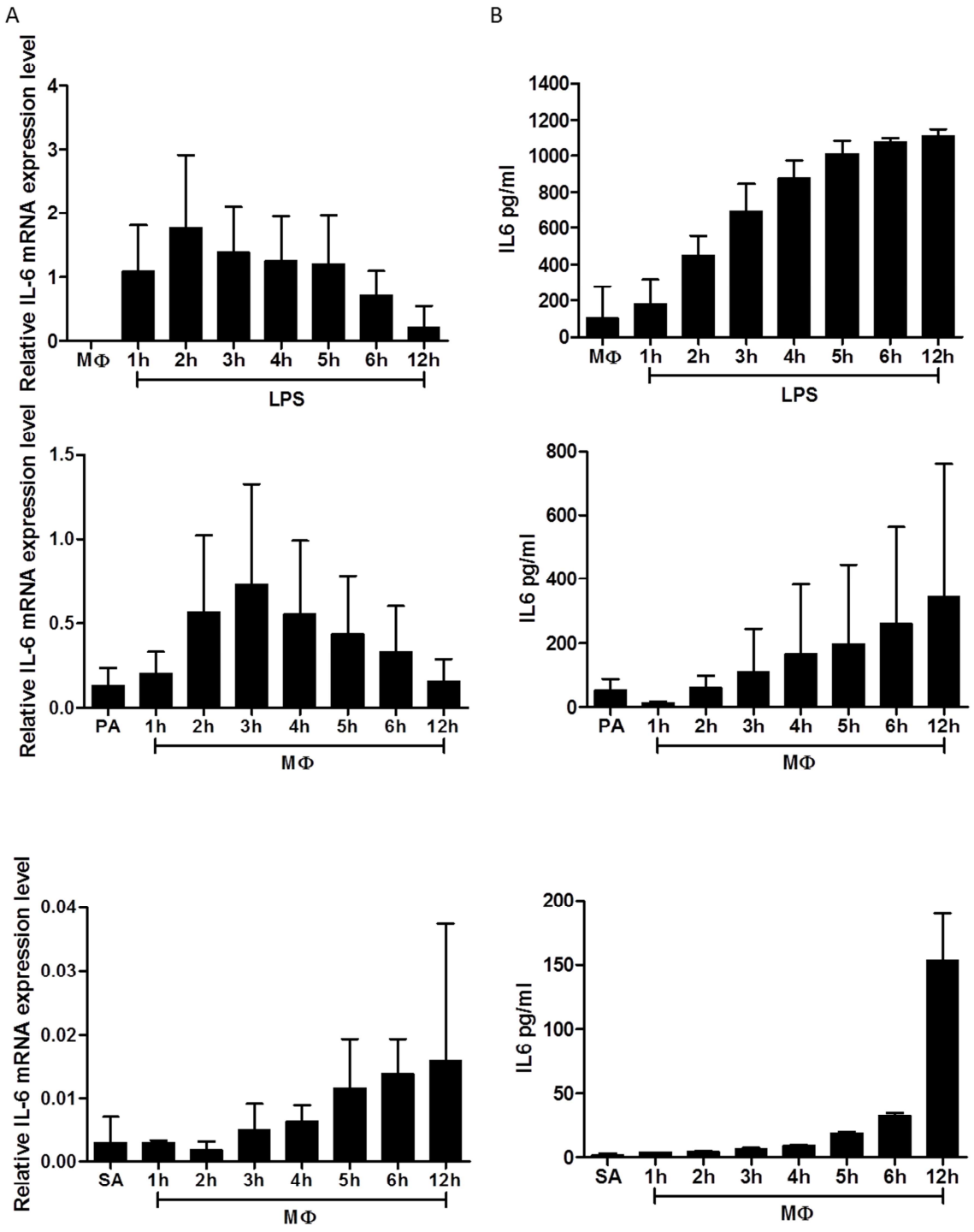

Figure 12. Relative gene expression and secreted protein levels in cultures of LPS treated MΦs and co-cultures of adipocytes and macrophages.

MΦs were pretreated for 30 minutes with 0.5 µg/ml crude LPS, then cells were cultured in fresh media for 12 hours. Adipocytes were co-incubated with MΦs for 12 hours. Relative

mRNA expression of IL-6 (A) and levels of secreted IL-6 protein (B). Data are expressed as mean ±SD of 3 independent experiments.

4.7. IL-6 is produced by macrophages during adipocyte-macrophage interaction in an NF-κB dependent way

To further investigate which cells synthesized and secreted IL-6, brefeldin A (BrefA) was added to the co-cultures of adipocytes and MΦs, and then cells were immuno-stained for IL-6. BrefA blocks protein transport through the Golgi, as well as, the endoplasmic reticulum and the produced IL-6 cytokine should accumulate in the cell.

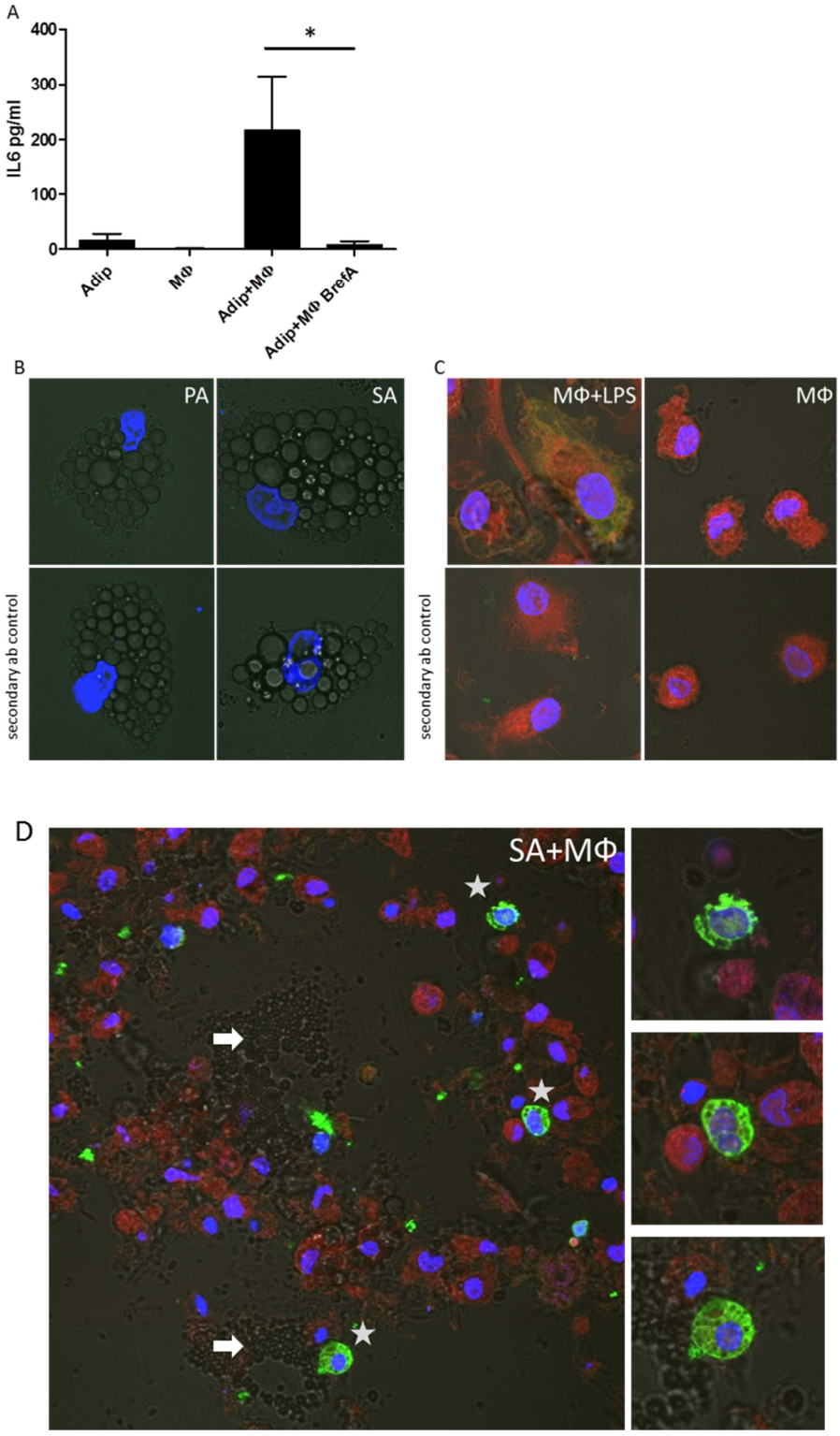

Figure 13. Immuno-staining of IL-6 in MΦ and the effect of Brefeldin A on IL-6 secretion.

Cells were treated with 100 ng/ml brefeldin A (BrefA) to block the secretion of IL-6 during co-incubation for 12 hours. (A) BrefA treatment blocked IL-6 secretion during adipocyte and

MΦ co-incubation. Fluorescent confocal microscopic (B, C, D) images of MΦs stained with CMTMR (red), of all cell types (MΦ as well as adipocytes) stained with NucRed (blue) and immune-stained for IL-6 (green). (B) Adipocytes alone; (C) LPS-treated and control macrophages were also treated with BrefA; (D) Adipocytes were co-incubated with MΦs and, after 12 hours cells were fixed and immuno-stained. Arrows indicate adipocytes; asterisks indicate IL-6 producing MΦs, images on the side show six time magnified details of the original image.

While there was no trace of IL-6 in BrefA treated adipocytes (Figure 13 A, B), IL-6 accumulated in macrophages when the co-cultures of adipocytes and macrophages were treated with BrefA (Figure 13 A, D) similarly to LPS and BrefA treated macrophages (Figure 13 C). Based on these data it can be concluded that in adipocyte-macrophage interaction IL-6 is produced mostly by macrophages.

To detect, whether the adipocyte induced IL-6 secretion is mediated through NF-κB signaling, NF-κB pathway inhibitors were applied. SC-514 is a selective and reversible cell-permeable inhibitor of IKKβ (IKK-2), SN50 is a cell-permeable peptide, which inhibits translocation of the NF-κB active complex into the nucleus.

Both SC-514 and SN50 could decrease the IL-6 secretion during co-incubation of PA or SA with macrophages (Figure 14) indicating that IL-6 secretion is mediated through NF-κB signaling.

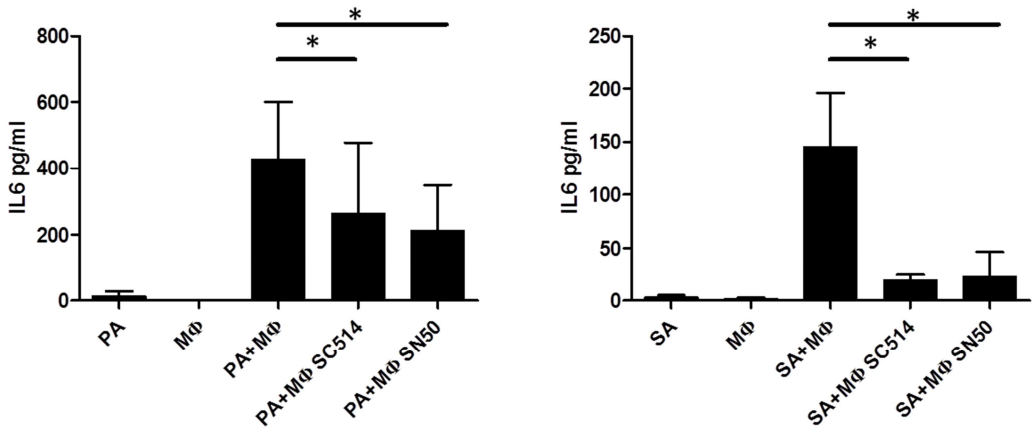

Figure 14. IL-6 secretion is mediated through NF-κB signaling

*Inhibitors of the NF-κB pathway blunt IL-6 secretion during adipocyte-MΦ co-incubation. 100 µM SC-514, or 50 mg/ml SN50 was applied to inhibit the NF-κB pathway. After 12 hours of co-incubation the level of secreted IL-6 protein was measured as described previously. Data are expressed as mean ±SD of 3 independent experiments. p values are as follows; * $p<0.05$.*

4.8.The influence of differentiated adipocytes on LPS-induced cytokine secretion of macrophages

To check how ingested adipocyte material influences macrophages in an inflammatory environment, we pretreated macrophages with LPS before adding them to adipocytes. After co-incubation, culture media were collected to measure the level of inflammatory cytokines (IL-6, IL-8, MCP1 and TNFα). While the level of secreted IL-6 did not change (Figure 15 A) in the presence of adipocytes, the amount of IL-8 has been decreased (Figure 15 B), and the concentration of MCP1 and TNFα have been significantly reduced, but in the case of SA and macrophage co-incubation, the level of MCP1 has significantly increased (Figure 15 D). This discrepancy can be due to the difference between the two adipocyte cell types we have been using for our experiments, and this phenomenon underlines the importance of the usage of human primary cells.

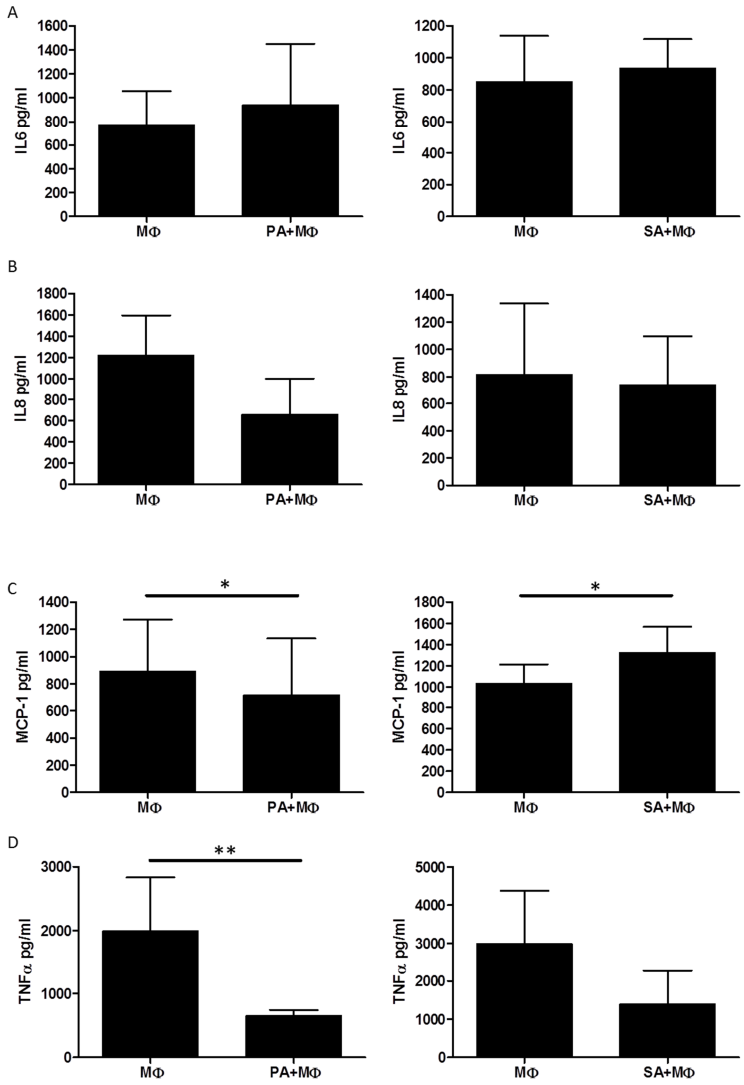

Figure 15. Influence of adipocytes on the secretion of inflammatory cytokines by LPS-treated macrophages.

*MΦs were pretreated for 30 min with 0.5 µg/ml crude LPS, then the activated macrophages were placed onto the culture plates or added to adipocytes (PA, SA) for 12 hours. Supernatants were collected and the level of secreted cytokines was measured by ELISA method. Secretion of (A) IL-6, (B) IL-8, (C) MCP-1 and (D) TNF-α during co-incubation PA or SA and LPS treated MΦ. Data are expressed as mean ±SD of 5 independent experiments. p values are as follows; * $p<0.05$, ** $p<0.01$.*

4.9.Analysis of gene expression patterns of differentiating adipocytes and the effect of the presence of antipsychotic drugs

The homogeneity of adipose tissue derived stem cells was characterized by FACS analysis, which revealed that more than 90% of the cell population expressed the mesenchymal stem cell-related markers CD73, CD90, CD105 and CD147. On day 1 of the differentiation process, we began administering the drugs to the cells at doses comparable to their therapeutic plasma concentrations. After 11 days, there were multiple small lipid droplets in the cytoplasm, detected by laser scanning cytometry, in both treated and untreated adipocytes.

Table 2. *Biochemical classification of genes studied upon antipsychotic drug treatment*

Studied genes		
Cell cycle	ANAPC2	anaphase promoting complex subunit 2
	CDK4	cyclin-dependent kinase 4
Apoptosis	BCL2	B-cell leukemia/lymphoma 2
	BAX	B-cell leukemia/lymphoma 2
Receptors and transporters	ABCA1	ATP-binding cassette, sub-family A member 1
	LEPR	leptin receptor
	INSR	insulin receptor
Signal transduction	GHR	growth hormone receptor
	IRS1	insulin receptor substrate 1
	PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
	SIRT1	sirtuin 1
Transcription factors	CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
	SREBF1	sterol regulatory element binding transcription factor 1
	NFκB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells
Nuclear receptors	PPARA	peroxisome proliferator activated receptor alpha
	PPARG	peroxisome proliferator-activated receptor gamma
Adipogenic differentiation markers	ADFP	adipose differentiation related protein
	FABPN	fatty acid bindin protein
Lipid metabolism enzymes	LPL	lipoprotein lipase
	ACSL1	acyl-CoA synthetase long-chain family member 1
Adipokines	ADIPOQ	adiponectin
	LEP	leptin
Cytokines and chemokines	TNF-alpha	tumor necrosis factor-alpha
	CCL2	chemokine (C-C motif) ligand 2
	IL1β	interleukin 1, beta
	IL8	interleukin 8

We analyzed the expression patterns of 26 genes (Table 2). The relative expression changes of the 26 genes measured for adipocyte stages are presented in Figure 16, showing significantly increased expression of the genes associated with adipogenesis and pro-inflammatory cytokine production.

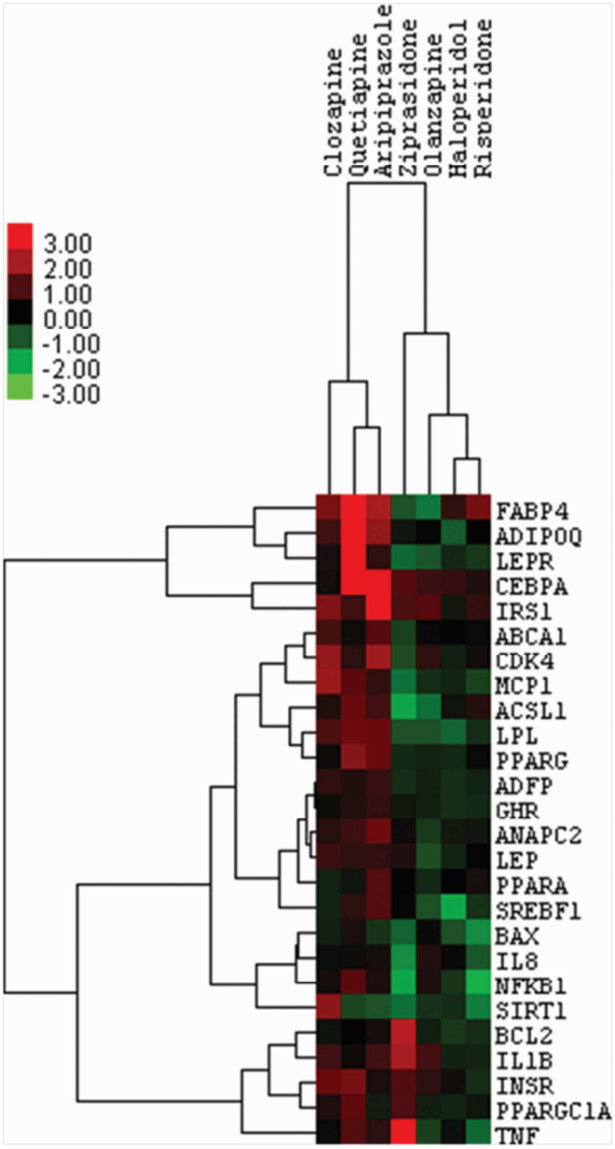

Figure 16. Heat map of relative gene expression changes induced by antipsychotics in differentiating adipocytes.

Hierarchical clustering of the 7 characterized anti-psychotic drugs according to their effects on the expression profiles of inflammatory and lipid metabolic genes. The colors in the heat map from green to red indicate the mean relative log-transformed inductions of normalized gene expression in differentiated primary human adipocytes measured by quantitative RT-PCR from 4 independent experiments.

4.10. Transcriptional effects of antipsychotic drugs on pro-inflammatory cytokine production

NF-κB1 is a transcription factor that plays a key role in the regulation of immune responses, such as the inflammatory response. Compared to controls, NF-κB1 was expressed at an increased level in differentiated adipocytes treated with antipsychotics. Most of the antipsychotics increased the gene expression of NF-κB1, like olanzapine, clozapine, quetiapine, aripiprazole, and haloperidol (Figure 17 A). In line with this, the expression of several pro-inflammatory genes, including the NF-κB1 target genes, as TNF-α, IL-1β, IL-8 and MCP-1, was measured in differentiated adipocytes. Increased TNF-α mRNA levels were induced by ziprasidone, clozapine, quetiapine and haloperidol (Figure 17 B). Among the studied pro-inflammatory genes, IL-1β and IL-8 were up-regulated the most during antipsychotic treatments. Almost all of the drugs enhanced IL-1β and IL-8 expression, but clozapine was the most effective (Figure 17 C–D). Expression of the chemokine monocyte chemotactic protein (MCP-1) was moderately increased by clozapine, quetiapine and aripiprazole, in comparison to the untreated control cells (Figure 17 E).

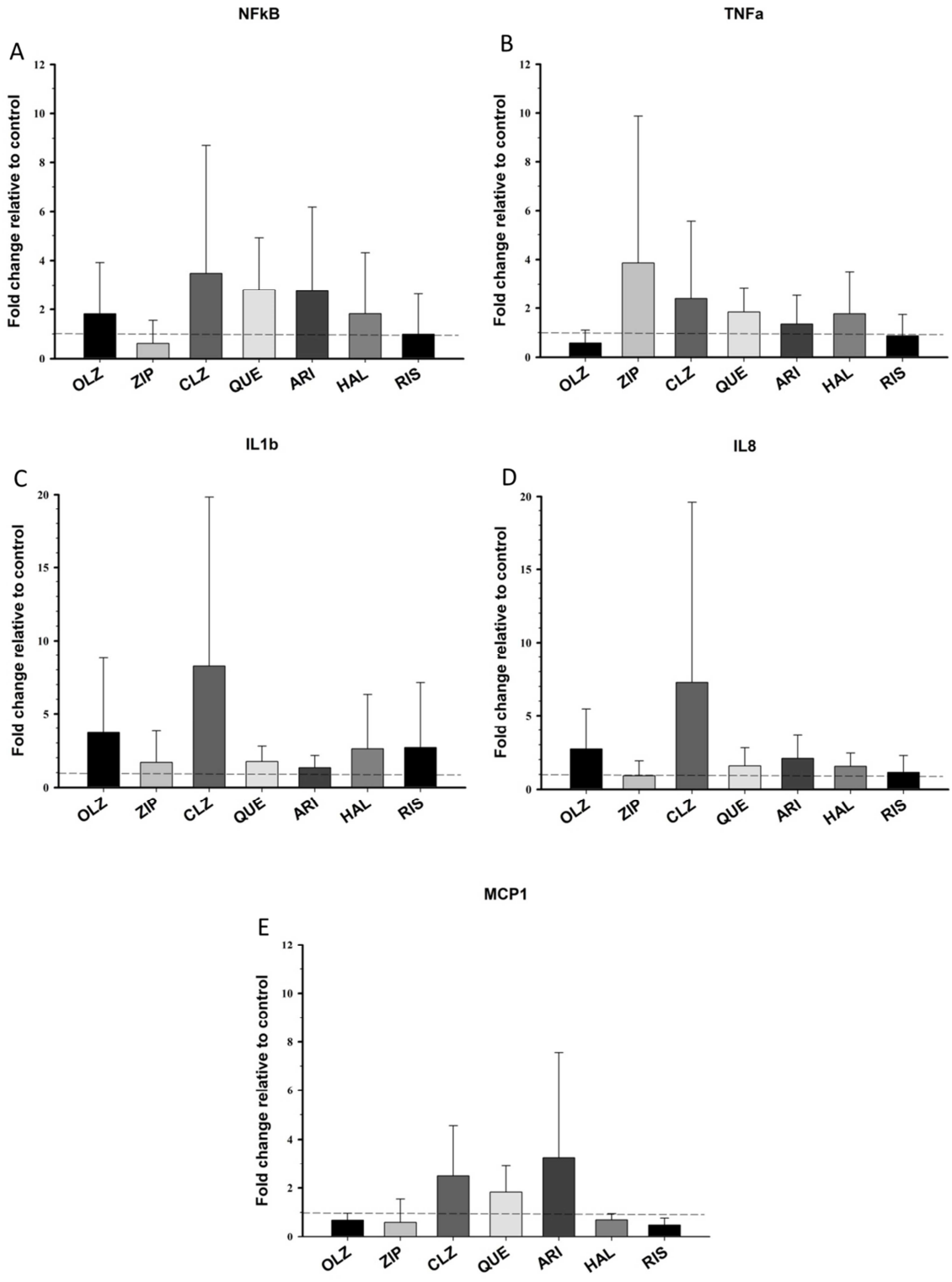

Figure 17. Proinflammatory gene expression in differentiated adipocytes in response to antipsychotic treatment.

Confluent PAs were differentiated into adipocytes in vitro in the presence of antipsychotics. Gene expression of NF-KB1 (A) and its target genes TNF-α (B), IL1β (C), IL8 (D) and MCP-

1 (E) were analyzed by qRT-PCR after 11 days of drug treatment. All data shown are means of fold changes and standard deviations calculated from relative changes in normalized expression levels measured in cells from 4 individuals. Abbreviations: OLZ, olanzapine; ZIP, ziprasidone; CLZ clozapine, QUE, quetiapine; ARI, aripiprazole; HAL, haloperidol; RIS, risperidone.

4.11. Cytokine production in culture supernatant of antipsychotic drug-treated differentiating PAs

To examine the effect of antipsychotic treatment on pro-inflammatory cytokine production, the level of TNF-α, IL-1β, IL-8 and MCP-1 was determined by ELISA from supernatant of adipocyte cell cultures. The level of TNF-α in the supernatant did not change significantly for any of the antipsychotic treatments, except ziprasidone (data not shown). The level of secreted IL-8 was enhanced upon the antipsychotic treatments, but only clozapine and aripiprazole treatment resulted a significant increase (Figure 18 A). The secretion of MCP-1 was enhanced significantly by clozapine, ziprasidone and olanzapine treatment (Figure 18 B). The level of IL-1β it is not shown here, was under the detectable concentration of the ELISA kit.

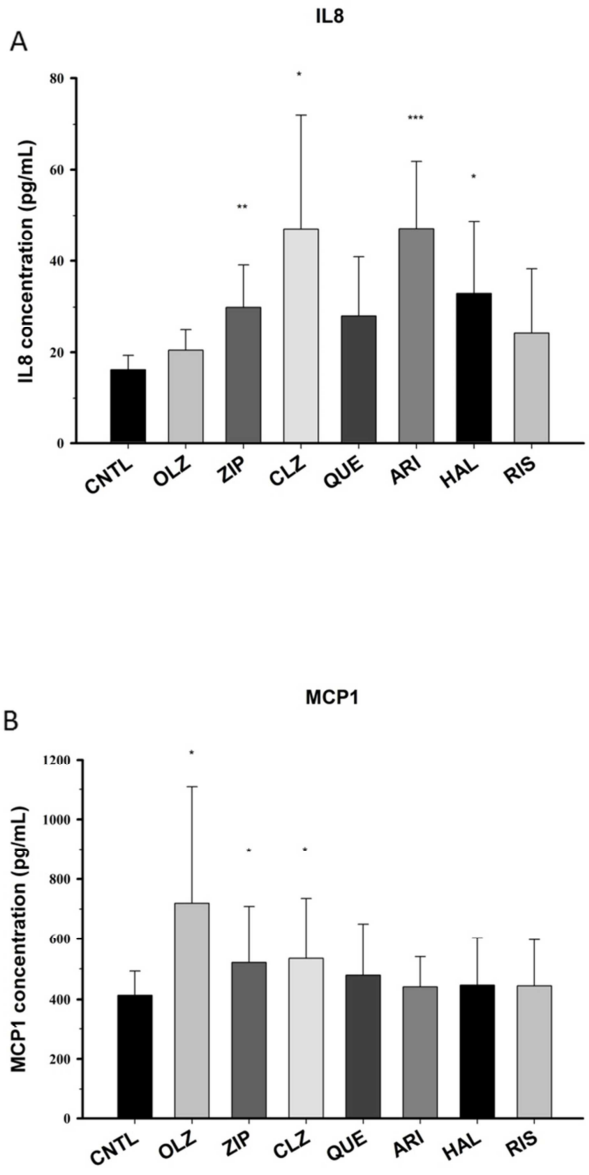

Figure 18. IL-8 and MCP-1 secretion of antipsychotic drug treated differentiating adipocytes.

Differentiating adipocytes were treated with the antipsychotic drugs every day for 11 days. Supernatant was collected every third day and stored for ELISA measurement. We pooled the supernatants from each sample and used them for the ELISA measurements (A) IL-8, (B) MCP-1. Abbreviations: CNLT, control; OLZ, olanzapine; ZIP, ziprasidone; QUE, quetiapine; ARI, aripiprazole; HAL, haloperidol; RIS, risperidone.

4.12. Effects of antipsychotic drugs on the gene expression patterns of cell cycle, apoptosis and adipogenesis regulators of differentiating adipose tissue derived stem cells

The heat map of gene expression data of individual antipsychotic drugs revealed that the most effective antipsychotic drug, in terms of the modulation of gene expression was clozapine (Figure 16). Figure 19 shows the mean fold changes in the expression level of non-inflammatory genes measured on clozapine treated *in vitro* differentiated adipocytes. The expression of cell cycle and apoptosis-related gene levels were between 2.8–7.2-fold (Figure 19 A); the expression of signal transduction components, receptors and transcription factors showed a 1.4–9.9-fold increase (Figure 19 B), while the level of adipogenic differentiation marker genes increased by 1.9–6.4-fold (Figure 19 C).

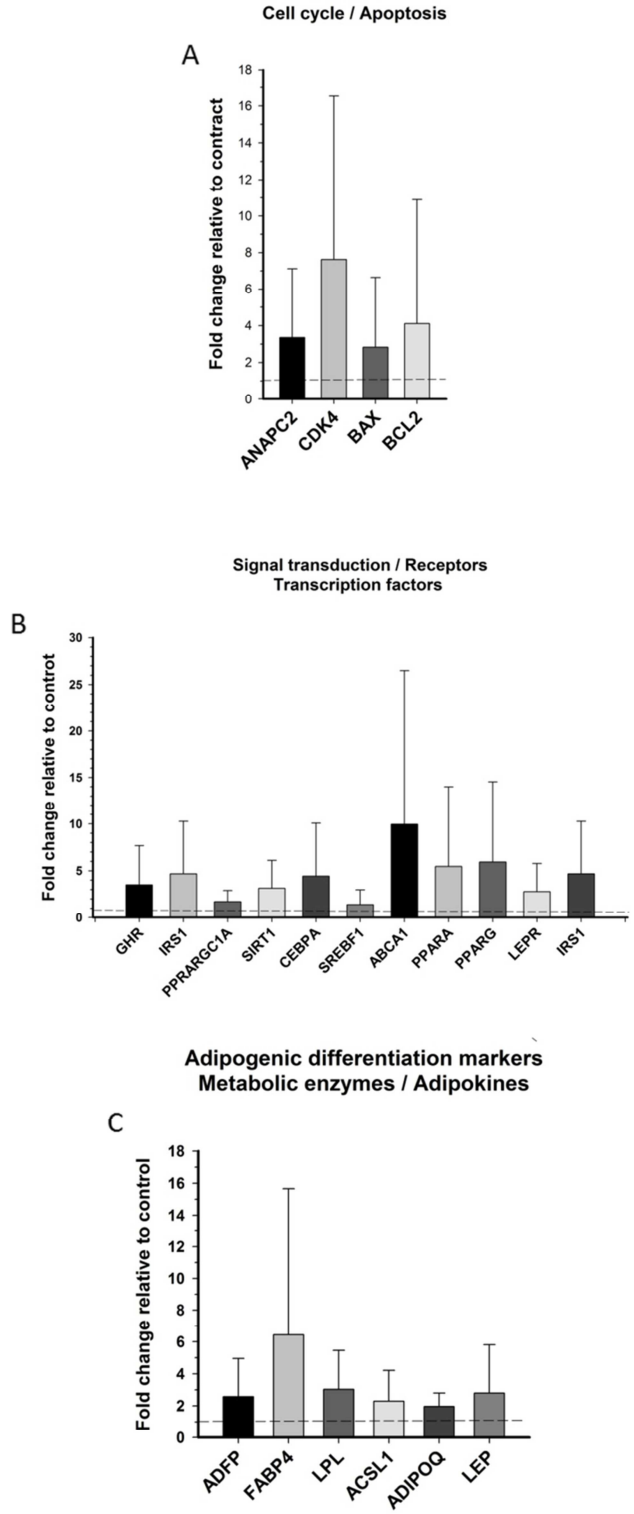

Figure 19. Clozapine-induced gene expression changes in differentiating adipocytes. *Differentiating PAs were treated with clozapine for 11 days. The expression of cell cycle/apoptosis genes (A), signal transduction genes/receptors/transcription factors (B), and*

adipogenic differentiation markers/metabolic enzymes/adipokines (C) were analyzed by qRT-PCR. All data shown are means of fold changes and standard deviations calculated from relative changes in normalized expression levels measured in cells from 4 individuals.

The adipogenic differentiation marker, fatty acid binding protein 4 (FABP4), the metabolic enzymes lipoprotein lipase (LPL) involved in the transfer of fatty acids from blood triacylglycerol to triacylglycerol stores of adipocytes, acyl-CoA synthetase-1 (ACSL1), and the adipokine (ADIPOQ) are peroxisome proliferator activated receptor (PPAR) target genes. The changes in the expression of FABP4, LPL and ACSL1 were coordinated with that of PPAR-γ. Upon treatment with olanzapine, quetiapine, aripiprazole, haloperidol and risperidone, the expression of FABP4 mRNA was increased. Treatment with olanzapine, quetiapine, aripiprazole and risperidone increased LPL expression by approximately 2.0-fold, while mRNA expression level of ACSL1 was elevated in the presence of olanzapine, quetiapine and aripiprazole. Olanzapine, quetiapine and aripiprazole had moderate effects on ADIPOQ expression. Leptin expression was increased by 1.7 ± 1.9- and 2.7 ± 0.2-fold respectively in the presence of quetiapine and aripiprazole in differentiated adipocytes. However, aripiprazole was the only AAP that enhanced adipose differentiation-related protein (ADFP) expression slightly, by 1.9 ± 1.5-fold (Figure 20).

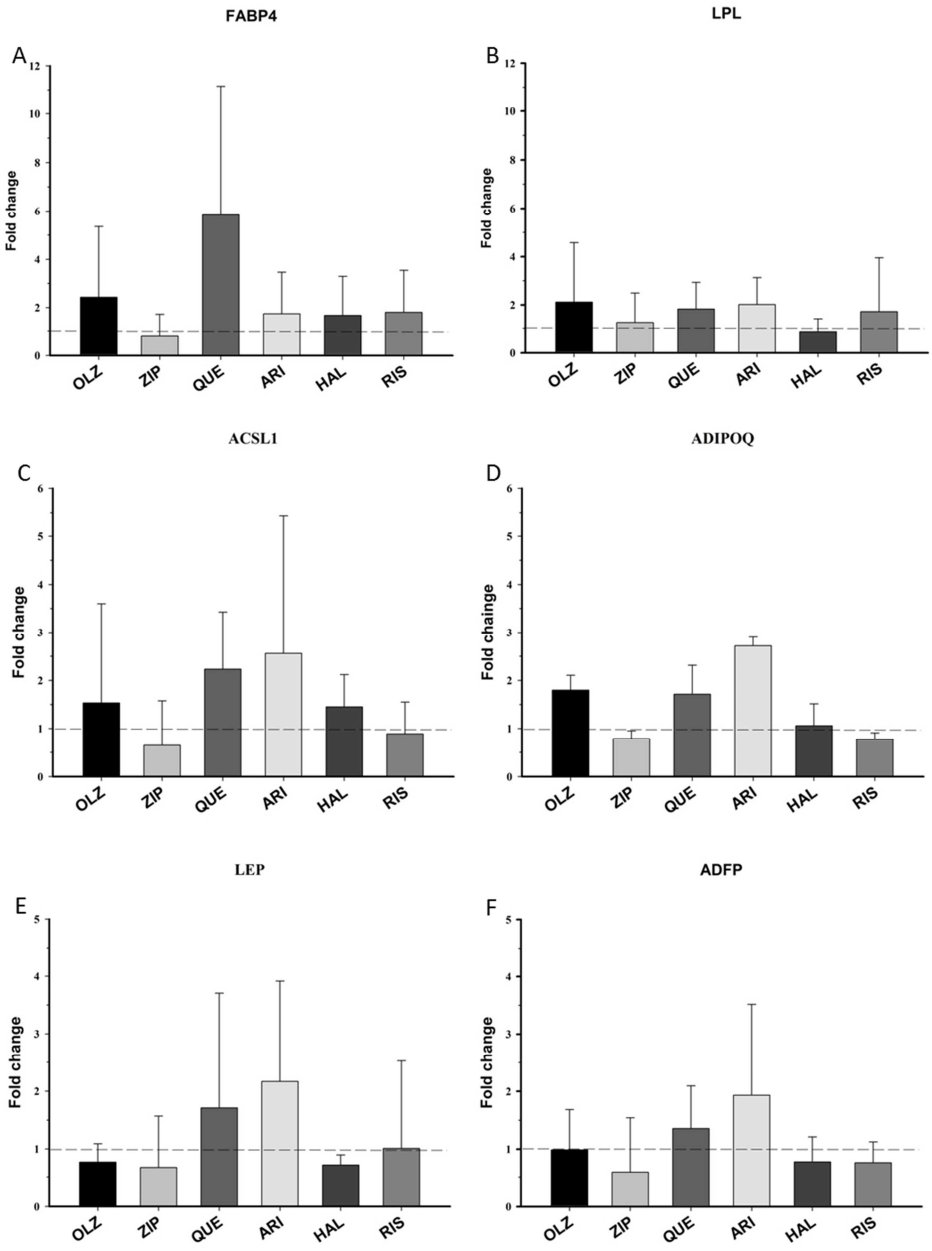

Figure 20. The expression levels of differentiation markers (ADFP, FABP4), metabolic enzymes (LPL, ACSL1) and adipokines (ADIPOQ, LEP) in differentiated adipocytes. *Gene expression was analyzed by qRT-PCR after 11 days of treatment with antipsychotics. All data shown are means of fold changes and standard deviations calculated from relative changes in normalized expression levels measured in cells from 4 individuals.*

5. DISCUSSION

INDUCED AND SPONTANEOUS APOPTOSIS OF CULTURED ADIPOCYTES

TNFα and CHX treatment induced apoptosis in both preadipocytes and adipocytes. Of note, the inducing effect was more pronounced in the preadipocyte population compared with their differentiated counterparts. In agreement with our results, different susceptibility of fat cell subpopulations to TNFα stimuli was also demonstrated in other studies, e.g. an intrinsic depot-specific susceptibility enhancement was described in human omental preadipocytes to programmed cell death compared with preadipocytes in the subcutaneous region (Niesler et al. 1998). Preadipocytes' prominent vulnerability to TNFα might be the result of the combined apoptotic and lipolytic effect of the TNFα.

Our LSC technique allowed us to detect and quantify apoptosis by Annexin V labeling of externalized phosphatidylserine at early apoptosis stage (Figure 6). An equal level of spontaneous apoptosis was found in both preadipocytes and adipocytes during each checkpoint of the differentiation. A plausible explanation for the prevalence of spontaneous apoptosis is that during the development process not all cells would evolve terminal differentiation; instead, a certain fraction of cells that was unable to adapt to any stages of the differentiation program, was thus led to self-destruction. Besides, it cannot be excluded that a non-ideal environment may contribute to this phenomenon and set a certain level of spontaneous apoptosis.

It has been recently shown that level of lamin A and B proteins decreased at peripheral regions of nuclei during adipocyte differentiation, while vimentin reorganized into cage-like structures near lipid droplets (Verstraeten et al. 2011). We observed that the transformation of preadipocytes to adipocytes involved significant nuclei size shrinkage along with intensifying

staining of DNA (Figure 5A). These observations might relate to each other, as in mitosis or apoptosis, where chromatin condensation is affiliated with the disintegration of nuclear lamina (Kihlmark et al. 2001)(Oberhammer et al. 1994).

TROGOCYTOSIS OF ADIPOCATES BY MACROPHAGES AND ITS CONSEQUENCES

While studying the interaction between human differentiated adipocytes and macrophages we observed efficient phagocytosis of pieces of the adipocytes leading to the appearance of lipid drops in the macrophages. Adipocytes prepared from either adipose tissue derived stem cells or an established preadipocyte cell line, were consumed by the macrophages. The observed phagocytic process is not a typical apoptotic cell clearance phenomenon in which apoptotic cells are rapidly and completely engulfed by phagocytes to preserve tissue integrity and prevent release of potentially noxious or immunogenic intracellular materials from the dying cells (J Savill & Fadok 2000). In our case the adipocytes did not seem to die while macrophages took pieces out of them, and we did not see adipocyte-derived DNA in the phagocytes (data not shown). The phenomenon looks like trogocytosis, a process in which one cell takes bites out of another; this has been previously described among immune cells and proposed to serve as a way for cells to acquire nourishment from other cells (Nancy 2014) (Joly & Hudrisier 2003). As we have previously reported, a significant portion of the differentiated human adipocytes have phosphatidylserine on their surface (Figure 6) and contained partially fragmented DNA (Doan-xuan et al. 2013). A possible mediator of this special phagocytosis is the milk fat globule-EGF-factor 8 (MFGE-8) which is a secreted glycoprotein produced by activated macrophages. It binds specifically to apoptotic cells by recognizing phosphatidylserine and attaches them to phagocytes for engulfment (Toda et al. 2012). The engulfment of apoptotic cells induces and activates PPAR-δ, which then further

enhances the expression of opsonins, such as MFGE-8 (Mukundan et al. 2009). The expression of MFGE-8 and the α_v and β_5 integrin subunits are increased in adipose tissue of obese humans (Henegar et al. 2008). A novel and possibly related role of MFGEf-8 has been recently revealed; it coordinates fatty acid uptake through $\alpha_v\beta_3$ integrin-and $\alpha_v\beta_5$ integrin-dependent phosphorylation of Akt by phosphatidylinositide-3 kinase and mechanistic target of rapamycin (mTOR) complex2, leading to translocation of CD36 and Fatp1 from cytoplasmic vesicles to the cell surface. MFGE-8 promotes the absorption of dietary triglycerides and cellular uptake of fatty acids from blood stream (Khalifeh-Soltani et al. 2014). These findings raise the possibility of the potential involvement of MFGE-8 in attraction of macrophages towards phosphatidylserine expressing differentiating adipocytes mediating engulfment of adipocyte pieces.

Engulfment of apoptotic cells brings large amount of cellular lipids including oxidized fatty acids and oxysterols into the macrophage and PPARs are the sensors of the native and oxidized fatty acids, and the derivatives of the free fatty acids serve as hormonal ligand for PPARγ. By sensing lipids from apoptotic cells, PPAR-δ functions as a molecular switch that discriminates between the pro-inflammatory and immunosuppressive actions of macrophages, it mediates the macrophage program of alternative activation (Mukundan et al. 2009). Liver X receptors (LXRs) can respond to phagocytosed lipids and modulate apoptotic cell clearance and maintain immune tolerance through transrepression of inflammation. In this context inhibition of inflammatory gene expression is linked to metabolism of lipids in apoptotic cells (A-Gonzalez et al. 2009). The engulfment and brake down of the lipid content of adipocytes by macrophages also may activate the PPAR and LXR transcriptional programs, further facilitating the phagocytosis and suppressing the pro-inflammatory reactions.

As an outcome of lipid engulfment during co-incubation of adipocytes and macrophages, macrophages produced a high amount of IL-6 which was not accompanied by the induction of

TNF-α and IL-1β. It has been previously demonstrated that IL-6 is released *in vivo* from human subcutaneous adipose tissue, while there was no TNFα secretion from this depot (Mohamed-Ali et al. 1997); in this study the cellular source of IL-6 was not determined. Our immuno-staining images obtained in an experimental system made of human cells revealed that the source of IL-6 is the lipid droplet containing macrophage. This corroborates other studies, where it was shown that the macrophages are responsible for almost all IL-6 expression in adipose tissue (Weisberg et al. 2003). It cannot be excluded that different adipose tissue depots release different combination of cytokines.

FFA and other lipids have been found to regulate the activation state and immune function of macrophages; saturated fatty acids activate classical inflammatory responses in macrophages and other immune cells through engagement of pattern recognition receptors, including Toll-like receptors (TLRs) (Lumeng, Deyoung, et al. 2007)(Lee et al. 2001). The induction of IL-6 expression in our system was not mediated by the conditioned medium of the cultured adipocytes which excluded the possibility that fatty acids released from the adipocytes may mediate this phenomenon. However, IL-6 induction and secretion required phagocytosis of the lipid content of adipocytes and the digestion of triacylglycerol in lysosome could lead to release of fatty acids to lipid sensing TLRs, such as TLR4 (LPS serves as ligand), TLR1-2 heterodimer (liganded by triacyl lipopeptide) and TLR2-6 (activated by diacyl lipopeptide) which are located on the surface of the cells and in the cell membrane (Alexopoulou et al. 2002)(Takeuchi et al. 2002). Saturated fatty acids are known to exert pro-inflammatory effects (Lumeng, Bodzin, et al. 2007); lauric acid (Lee et al. 2003) and palmitic acid (Suganami et al. 2007) released from dysregulated adipocytes can activate toll-like receptor (TLR)-2 and TLR4 signaling respectively, which ultimately triggers nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) mediated pro-inflammatory gene expression and subsequent cytokine secretion from macrophages. Macrophages activated through TLR2

(Lee et al. 2003) and TLR4 (Suganami et al. 2007) signaling have been shown to undergo polarization to a unique M1-like phenotype characterized by increased lipid content and secretion of the pro-inflammatory cytokines TNFα and IL-6 (Lumeng, Deyoung, et al. 2007). We could partially block the adipocyte induced IL-6 secretion using different NF-κB inhibitors, showing a possible involvement of TLR-dependent pathways in IL-6 secretion during co-culture of adipocytes and macrophages.

POSSIBLE ANTI-INFLAMMATORY EFFECT OF IL-6 RELEASED DURING TROGOCYTOSIS OF ADIPOCYTES

Although both IL-6 and TNFα are expressed by adipose tissue, it has been shown that there are important differences in their systemic release. TNFα is not released by subcutaneous depot; in contrast, IL-6 is released from this depot and is thereby able to signal systemically (Mohamed-Ali et al. 1997). The release of IL-6 from subcutaneous depots into the systemic circulation and the fact that this release is greater on obese subjects support a possible novel role for IL-6 as a systemic regulator of body weight and a regulator of lipid metabolism (Mohamed-Ali et al. 1997). Taking into consideration that leptin receptor shares homology with the gp130 signal-transducing component of the IL-6 receptor (Tartaglia et al. 1995) IL-6 may modulate even the actions of adipocyte secreted leptin, which binds to hypothalamic receptors and regulates energy balance by causing changes in food intake, physical activity, and thermogenesis (Mohamed-Ali et al. 1997).

While there is little doubt about the pro-inflammatory nature of TNF-α and IL-1β released during adipose tissue inflammation, IL-6 seems to be a pleiotropic cytokine, being able to act as pro- and anti-inflammatory regulator as well. During classical NF-κB inflammatory pathway activation, TNF-α and IL-1β is secreted together with IL-6, but our results show an

isolated IL-6 secretion when macrophages trogocytose pieces of adipocytes. It has been published that the endogenous IL-6 has a regulatory role in local acute inflammation and suppresses the pro-inflammatory cytokine synthesis, such as TNF-α and IL-1. IL-6 does not only negatively regulates the production of these cytokines, but it also induces the production of IL-1 and TNF antagonists in U937 cells (Tilg et al. 1994). Furthermore IL-6 can act to prime myeloid cells for IL-4 signaling during obesity in mice (Mauer et al. 2014). As obese adipose tissue is described to be in an inflammatory state, we have checked the effect of adipocytes on cytokine production in an inflammatory environment using LPS treated macrophages. The secretion of IL-6 was not influenced by adipocytes, but the levels of MCP-1 and TNF-α were significantly reduced during their co-incubation with macrophages. Previously, it had been shown that IL-6 could inhibit LPS induced TNF-α and IL-1β expression and secretion (Aderka et al. 1989), and IL-6 limits LPS-induced endotoxemia in mice (Mauer et al. 2014). Based on this data and our observation presented here one may presume that IL-6 secreted during interaction of adipocytes and macrophages might have an anti-inflammatory role in the inflamed adipose tissue down-regulating the induction and release of pro-inflammatory cytokines.

ATYPICAL ANTIPSYCHOTIC DRUGS AND INFLAMMATORY POTENTIAL OF ADIPOCYTES

Although AAPs are widely prescribed for the treatment of several psychiatric disorders, we have relatively little information about how these drugs affect gene expression in various tissues and whether this varies among individuals. It is also unknown whether changes in peripheral tissues, such as adipose tissue, could manifest undesirable side effects of SGAs,

such as weight gain and metabolic disorders, in which adipose tissue is a crucial site for inflammatory responses and mediators (Lee & Pratley 2005)(Hotamisligil 2006). To answer these questions, systematic qPCR analysis was used to measure several candidate genes of adipocyte-derived hormones, receptors and genes related to energy expenditure. One of the most remarkable findings from these gene expression studies was that we observed significant effects of some of these antipsychotics on key genes involved in the regulation of critical adipose biochemical processes, for example, signal transduction, mitochondrial biogenesis, adipogenesis and metabolism. In these subclasses, of the 26 genes measured, clozapine enhanced the expression of 21 genes, aripiprazole 20, quetiapine 18, olanzapine 13, ziprasidone 7 and risperidone 6 genes. In contrast, the first-generation antipsychotic haloperidol induced a minor increase in the expression of only one gene. Several studies have shown that aripiprazole treatment may have positive metabolic effects in patients treated with other atypical antipsychotics (Wang et al. 2013)(Stroup TS et al. 2014). Our experiments show that in adipocytes aripiprazole induced gene expression of INSR, IRS1, PPARA, LPL, LEP, ADIPOQ and SIRT, which are all key regulators of energy storage, expenditure and mitochondrial biogenesis, and may have a remarkable effect on energy metabolism and lead to a healthier body weight, lower triglyceride levels in the plasma and improved insulin sensitivity. While ziprasidone enhanced the expression of seven genes to a moderate degree, quetiapine induced a greater increase in the expression of 18 genes. It has been reported that patients who switched from quetiapine to ziprasidone showed improvement in clinical symptoms, weight loss and lipid profiles. While ziprasidone decreased GHR expression, quetiapine enhanced it, which may result in the inhibition of insulin-stimulated glucose uptake in adipocytes (Sasaki-Suzuki et al. 2009). One of the most notable related findings is that mice harboring a disrupted GHR gene show extreme insulin sensitivity in the presence of obesity (List et al. 2011). The expression of 19 genes out of the 26 remained unchanged,

consistent with ziprasidone's main advantage in terms of a low propensity to induce weight gain and associated adverse effects (Paredes et al. 2014)(Komossa et al. 2014).

Inflammatory abnormalities may be involved in the pathophysiology of schizophrenia, although some inflammatory processes may emerge epiphenomenally during treatment. It is also known and widely accepted that macrophages account for almost all obesity-related pro-inflammatory cytokine production (Weisberg et al. 2003). In olanzapine-treated rats, TNF-α expression increased significantly in adipose tissues with widespread macrophage infiltration, suggesting that macrophages were the source of the overexpression of TNF-α (Victoriano et al. 2010). In our human *in vitro* differentiated adipocytes treated with antipsychotics we observed a concerted increase in the mRNA levels of the transcription factor NF-KB1 and its target genes, the pro-inflammatory cytokines TNF-α, IL-1β and IL-8, and the MCP-1. This suggests that chronic treatment with antipsychotics that induce weight gain may cause a low-level pro-inflammatory state in patients that is initiated by adipocytes. When the adipocytes were treated for up to 34 days, each antipsychotic induced NF-KB1 expression to various extents between day 11 and day 34. This increase in NF-KB1 expression was associated with a coordinated increase in the expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-8, and the chemokine MCP-1 was also elevated by day 34 in almost every case (data not shown). An elevated level of MCP-1 could potentially contribute to the infiltration of monocytes/macrophages into adipose tissues, which could further increase the inflammatory properties of adipose tissues. More importantly, high levels of TNF-α have been shown to reduce the function of both IRS1 and glucose transporter 4, and elevated IL-8 expression may inhibit insulin-induced AKT phosphorylation in adipocytes. Together, these changes could cause a critical level of inhibition of insulin activity, leading to insulin resistance and metabolic disorders (Gustafson et al. 2007)(Aguirre et al. 2002)(Kobashi et al. 2009)(Guilherme et al. 2008). Clozapine treatment has been associated with elevated weight

gain and TNF-α plasma levels (Lett et al. 2012)(Hauner H, Röhrig K, Hebebrand J 2003)(Røge et al. 2012). According to our clozapine gene induction data and previously published results, although TNF-α mRNA expression was elevated, clozapine concurrently greatly enhanced the expression of both PPARG and adipocyte hormones (Figure 14 and 16). PPARG is necessary for both adipocyte differentiation and the normal lipid metabolism. Adipocyte hormones such as leptin and adiponectin regulate both glucose and fat metabolism, including food uptake and energy expenditure (Hemmrich et al. 2006). While leptin can limit food intake and increase energy expenditure, via which it can regulate the overall body weight, adiponectin plays an important role in insulin sensitization and maintaining energy homeostasis (Schwartz et al. 2000)(Lee & Shao 2014).

In addition to these biologically important trends, our data revealed a high donor-dependent variability in the effects of antipsychotic treatment, especially in the case of clozapine. This may be explained, in part, by many factors including age, gender, a limited number of donors and the number of neurotransmitter receptors of differentiated adipocytes in cell cultures. The unique feature of this study is the use of primary human cells for these investigations, but this advantage also represents a limitation in data interpretation.

It was important to create a stable human *in vitro* experimental system, in which we could characterize the interaction between adipocytes and macrophages. This will help us to get more information occurring during obesity. Our experimental setup makes possible to test the effect of different drugs on human cells, which can be very useful in the future.

6. SUMMARY

- We could follow adipocyte differentiation in physiological conditions at different time points, could quantitate single cells in high-content manner, and could study both adipocytes and preadipocytes simultaneously in a slide-based platform.
- Spontaneous apoptosis was found to exist in both preadipocytes and adipocytes during differentiation; preadipocytes show higher sensitivity to TNF-α and CHX combined death stimuli compared to mature adipocytes.
- Using a newly designed human *in vitro* experimental system, we could capture the interaction of macrophages and adipocytes in co-culture. During the co-incubation, trogocytosis of living adipocytes by macrophages has been revealed by time-lapse microscopy and quantified by laser scanning cytometry and flow cytometry.
- High level of *de novo* IL-6 secretion was detected by macrophages as a result of engulfment of the lipid content of adipocytes by macrophages.
- The absence of the secretion of the typical pro-inflammatory TNF-α and IL-1β and the selective appearance of the pleiotropic IL-6 shed a different light on the role of IL-6 in this interaction. Our data suggest that IL-6 might act in an anti-inflammatory way in suppressing the level of TNF-α and MCP-1, in maintaining adipose tissue homeostasis and in preventing the consequences of high pro-inflammatory cytokine levels, as insulin resistance and other elements of the metabolic syndrome.
- We found a concerted induction of pro-inflammatory genes and upregulation of pro-inflammatory mediators in response to a versatile group of antipsychotic drugs.
- Three of the most potent agents, clozapine, quetiapine and aripiprazole, demonstrated a clear propensity to induce adipogenic genes. These data suggest that independently of the primary inflammatory process of the illness, a secondary adipocyte-dependent

inflammatory abnormality can occur, which could support the monocyte-macrophage accumulation due to MCP-1 expression, and thus the infiltrating macrophages would be the third source of the pro-inflammatory cytokine production in adipose tissue, which may further contribute to the development of metabolic syndrome associated with second-generation antipsychotic treatment.

7. ÖSSZEFOGLALÁS

- Munkánk során, nyomon követtük a zsírsejtek érési folyamatát, sejtenkénti méréseket végeztünk és egyazon idő alatt tudtuk vizsgálni mind az érett és érettlen zsírsejteket.
- Spontán apoptózis jelenlétét észleltük, mind a zsírsejtek, mind pedig az előzsírsejtek esetében, az érési folyamat alatt, valamint az előzsírsejtek nagyobb érzékenységet mutattak a TNFα és a CHX kombinált kezelés hatására.
- Egy új *in vitro* kísérleti rendszert alkalmazva, nyomon tudtuk követni, a zsírsejtek és makrofágok hatását. Együtttenyésztésük során az élő sejtek makrofágok általi trogocitózisát észleltük time-lapse mikroszkópos felvételek segítségével, mely jelenséget lézerpásztázó mikroszkópiával, valamint áramlási citométerrel kvantifikáltunk.
- A makrofágok nagy mennyiségű, *de novo* termelődött IL-6 szekrécióját detektáltuk, mely a zsírsejtek lipid tartalmának bekebelezése révén keletkezett.
- A tipikus gyulladásos citokinek, mint a TNFα és az IL-1β hiánya és a több funkciós IL-6 szeparált megjelenése új szerepben tünteti fel az IL-6 citokint ebben az interakcióban. Eredményeink az IL-6 gyulladáscsökkentő szerepét támasztják alá, gátolva a TNFα és MCP-1 szintjének növekedését, fenntartva a zsírszövet nyugalmi állapotát, valamint megakadályozva a gyulladásos citokinek szintjének növekedése következtében fellépő inzulin rezisztencia és egyéb metabolikus betegségek megjelenését.
- A gyulladásos gének szintjének összehangolt növekedését figyeltük meg egy csoport antipszichotikus gyógyszer alkalmazásának hatására.
- Három, a vizsgált gyógyszerek közül, mégpedig a clozapine, quetiapine és aripiprazole, egyértelmű hajlamosságot mutattak a zsírsejt specifikus markergének

szintjének indukálására. Ezen adatok azt jelzik, hogy a betegség következtében, elsődlegesen fellépő gyulladástól függetlenül, kialakulhat egy másodlagos, zsírsejt függő, gyulladásos elváltozás, mely hozzájárulhat a monocita-makrofág felhalmozódás, az MCP-1 expresszió növelésével, ezáltal a bevándorló makrofágok válnak a gyulladásos citokinek harmadik forrásává, mely tovább fokozza a második generációs antipszichotikumok által okozott metabolikus szindrómák kialakulásának lehetőségét.

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List of publications related to the dissertation

1. **Sárvári, A.K.**, Doan-Xuan, Q., Bacsó, Z., Csomós, I., Balajthy, Z., Fésüs, L.: Interaction of differentiated human adipocytes with macrophages leads to trogocytosis and selective IL-6 secretion.
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List of other publications

4. Bánfalvi, G., **Sárvári, A.**, Nagy, G.: Chromatin changes induced by Pb and Cd in human cells. *Toxicol. Vitro. 26* (6), 1064-1071, 2012.
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9. KEYWORDS

Human preadipocyte, SGBS adipocyte, differentiation, laser scanning cytometry, human macrophage, trogocytosis, IL-6, anti-inflammatory effect, obesity, atypical antipsychotics; inflammation; clozapine.

10. TÁRGYSZAVAK

Humán előzsírsejt, SGBS zsírsejt, differenciálódás, lézerpásztázó mikroszkóp, human makrofág, trogocitózis, IL-6, gyulladáscsökkentő hatás, elhízás, atipikus antipszichotikumok, gyulladás. clozapine.

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