Charles University in Prague

Third Faculty of Medicine

Summary of Ph.D. thesis

Role of immune and adipose cells in the development of adipose tissue inflammation induced by stress associated with obesity

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Prague 2015

This work was performed at the laboratories of the Department of Sports Medicine, Third Faculty of Medicine, Charles University in Prague within the scope of doctoral study of biomedicine under the Division Council of Molecular and Cell Biology, Genetics and Virology.

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Date of distribution of the Summary of PhD thesis: 4th June, 2015

Date of the Ph.D. Thesis defence: 25th June 2015. Time: 14:30

Place of the Ph.D. Thesis defence: Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University in Prague – conference room

Albertov 4, Praha 2, 128 00

This dissertation is available at the dean´s office of the Third Faculty of Medicine, Charles University of Prague.

CONTENT

LIST OF ABBREVIATIONS

ACP5	acid phosphatase 5
AdipoQ	adiponectin
AT	adipose tissue
ATF	activating transcription factor
CCL	chemokine (C-C Motif) ligand
CTRP	C1q/Tumor Necrosis Factor Related Protein
DI	dietary intervention
DPP4	dipeptidyl-peptidase 4
ER	endoplasmic reticulum
ERS	endoplasmic reticulum stress
FA	fatty acid
FoxP3	forkhead box P3
GATA3	GATA binding protein 3
GDR	glucose disposal rate
HFM	high fat meal
HG	hyperglycemia
HOMA-IR	homeostasis model assessment of insulin resistance
ICAM	intercellular adhesion molecule
IL	interleukin
IL1Ra	intracellular interleukin1 receptor antagonist
IR	insulin resistance
LCD	low calorie diet
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAI1	plasminogen activator inhibitor 1
PBMC	peripheral blood mononuclear cells
RBP4	retinol binding protein 4
qRT-PCR	quantitative real time polymerase chain reaction
RORC	RAR-related orphan receptor C
SAAT	subcutaneous abdominal adipose tissue
SGAT	subcutaneous gluteal adipose tissue
SFRP5	secreted frizzled-related protein 5
SVF	stromal vascular fraction
T2DM	diabetes mellitus type II
TACE	TNFα-converting enzyme
TAG	triacylglycerol
TBX21	T-Box 21
T_C	cytotoxic T lymphocytes
TGF	transforming growth factor
T_H	helper T lymphocytes
TIMP3	tissue inhibitor of metalloproteinases 3
TLR	toll-like receptor
T_{REG}	regulatory T lymphocytes
UPR	unfolded protein response
VAT	visceral adipose tissue
VCAM	vascular cell adhesion molecule
VEGF-A	vascular endothelial growth factor
VLCD	very low calorie diet
WM	weight maintenance

1. INTRODUCTION

Obesity is characterized as an excessive accumulation of adipose tissue (AT) due to the imbalance between calorie intake and energy expenditure. It is defined as body mass index (BMI, i.e. ratio of weight in kg divided by the square of height in meters) above 30 kg/m^2. In western countries, the upsurge of obesity is presumably driven by high availability of food abundant in fat and sugar in connection with a dramatic shift from physical to sedentary work/leisure activities. Nowadays similar changes in lifestyle are observed also in developing countries [1]. Importantly, overweight and obesity are associated with the development of metabolic comorbities and additional disorders, for example 44% of cases of type II diabetes mellitus (T2DM), 23% of ischaemic heart disease and 7–41% of certain cancers are globally attributable to overweight and obesity (http://www.who.int/topics/obesity/en/).

1.1 Adipose tissue

A major and primary function of AT is to accumulate lipids upon energy excess and to release energy-rich substrates in response to energy needs. Nevertheless, AT is now recognized as a truly multifunctional organ with important immune, endocrine and paracrine, regenerative, mechanical, and thermal function [2]. This multifunctionality is based on AT specific cellular composition and also various anatomical localizations. Accordingly to its complex properties and also size, AT greatly contributes to the whole body metabolic homeostasis. AT is composed mainly of its functional units, i.e. adipose cells or adipocytes, and mixture of other cells together called stromal vascular fraction (SVF). SVF contains preadipocytes, mesenchymal stem cells, endothelial cell and various immune cells [3].

1.1.1 Distribution of adipose tissue

AT is one of the largest organ of the body (it represents 10-20% of body weight in lean up to 70% of body weight in obese [4]). The major anatomical AT depots are: 1. upper-body subcutaneous depot; 2. intra-abdominal (omental and mesenteric depots, also termed visceral fat); 3. lower-body (gluteal, subcutaneous leg or gluteo-femoral depot) and 4. ectopic fat deposited in atypical locations. The distribution of AT is affected by race, gender, aging and disease states, or physiological condition (e. g. starvation) and in response to drugs and hormones. Importantly, different anatomical depots of AT exhibit diverse ability to respond to external signal (e. g. insulin, lipolytic agents), secretory profile, as well as different composition of SVF [2]. These variances result in distinct metabolic properties of the depots.

An accumulation of AT in abdominal (upper) region is associated with an increased risk of displacement of AT into visceral region and ectopic depots and thus with an increased risk of cardiovascular and metabolic disorders development, as well as liver steatosis [5-7]. On the other hand, a higher accumulation of AT in gluteo-femoral depot was shown to be linked with the reduction of obesity associated complications [8-10] and with lower morbidity and mortality [11, 12]. Recently, it was hypothesized that the differences in metabolic impact of gluteal when compared to abdominal AT depot are related to the differences in the inflammation-related characteristics[13]. Hence, the characterization of the immune status of these two different subcutaneous AT depots (abdominal and gluteal) is one of the issues of this thesis.

1.1.2 Adipose tissue as endocrine organ

Since the discovery of AT endocrine potential, this aspect of AT has been under huge scientific interest that resulted in an assembly of an extensive list of AT produced molecules important for metabolic and immune homeostasis. These cytokines and/or adipokines affect several organs responsible for lipid handling on central (brain) and peripheral (liver, muscle) level as well as immunologically active cells and AT itself [14]. Summary of AT secretory products is presented in Table 1.

Table 1: Major factors secreted by adipose tissue and its predominant effect on immune system

Category	Factors
Anti-inflammatory	AdipoQ, apelin, CD163, IL1Ra, IL10, IL13, Omentin, Vaspin, CTRP
Pro-inflammatory	Leptin, adipsin, Visfatin, Resistin, TNFα, IL1β, IL6, IL8, PAI1, CCL2, CCL5, TGFβ, RBP4, DPP4, VEGF-A
Ambivalent effect	SFRP5

Adiponectin (AdipoQ); chemokine (C-C Motif) ligand (CCL); cluster of differentiation (CD); C1q/Tumor Necrosis Factor Related Protein (CTRP); dipeptidyl-peptidase 4 (DPP4); interleukin (IL); intracellular interleukin1 receptor antagonist (IL1Ra); plasminogen activator inhibitor 1 (PAI1); retinol binding protein 4 (RBP4); secreted frizzled-related protein 5 (SFRP5); transforming growing factor β (TGFβ); tumor necrosis factor α (TNFα); vascular endothelial growth factor A (VEGF-A)

1.1.3 Adipose tissue as immune organ

AT is infiltrated with a panoply of immune cells including both innate and adaptive components [15]. Phenotype and activity of them is affected by cytokines that are direct products of adipocytes and SVF cells.
AT immune cells from lean subjects exhibit anti-inflammatory phenotype, as mainly alternative M2 macrophages [16], eosinophils [17], T helper (T_H) 2 and regulatory (T_{REG}) lymphocytes are presented [18]. On the other hand in obese/metabolically unhealthy subjects a switch to pro-inflammatory classical M1 macrophages and T_H1 phenotype occurs and numbers of cytotoxic T (T_C) lymphocytes are amplified. For a long time it was assumed that the main and first players in AT ”colonization” by immune cells in obesity are macrophages that are attracted by dysfunctional or dying adipocytes [19, 20]. Contrary to expectations, Duffaut et al. [21] showed that the accumulation of macrophages in AT is preceded by T lymphocytes infiltration in response to high fat diet. Lymphocytes react to metabolic disturbances earlier than macrophages and thus they may regulate subsequent macrophage infiltration and activity [21-23].

1.1.4 Adipose tissue dysfunction in obesity

During energy surplus, there are two strategies used by AT to handle excess nutrients. Hyperplastic expansion is characterized by enlargement of AT depot via recruitment of new preadipocytes with high potential to store lipids. This type of expansion is not associated with disrupted AT functionality and metabolic complications but is rather limited in adulthood [24]. The most common type of AT expansion in adults is therefore based on hypertrophic growth of adipocytes. Hypertrophied adipocytes are however exposed to stressful conditions; e. g. increased

mechanical tension, local hypoxia or increased needs for synthesis of lipids. Consequently, hypertrophied adipocytes exhibit signs of stress, such as expression of markers of endoplasmic reticulum stress (ERS) and higher activation of pathways that may contribute to inflammation [25, 26]. These stress-activated pathways probably trigger worsening of adipocyte, and consequently AT function and thus prime adverse changes of the whole body metabolism.

1.1.4.1 Insulin resistance

Insulin is a hormone produced solely by pancreatic β cells and its major function is the regulation of carbohydrates metabolism. Postprandially increased levels of glucose induce a production/secretion of insulin. The action of insulin in the cells is mediated via its receptor with intrinsic tyrosine kinase activity [27].

Insulin resistance (IR) is defined as a lower capacity of cells to respond to insulin than expected for a given insulin concentration[28]. IR is thus frequently associated with increased plasma insulin levels and longer postprandial hyperglycemias (HG). Because of less efficient import of glucose, cells utilize other energy-rich molecules as a fuel and glucose remains longer in the circulation. Due to higher glucose values, β cells produce more insulin and prolongation of this state can lead to β cells damage and subsequently to T2DM development [28]. Several mechanisms why obesity frequently leads to whole body IR were suggested: dietary fluctuations, elevated fattu acid (FA) levels and inflammatory changes [29].

1.1.4.2 Endoplasmic reticulum stress

In healthy adipocytes, endoplasmic reticulum (ER) is responsible for protein folding, maturation, quality control, trafficking and importantly also for lipid synthesis. In hypertrophic adipocytes, there is an augmented demand on ER synthesis of proteins and lipids. Overwhelming of ER capacity leads to ERS that is characteristic by the activation of unfolded protein response (UPR) [30].

As suggested previously, ERS in adipocytes and immune cells could be caused by exposure of cells to saturated lipids and a high concentration of glucose [26, 31]. Nevertheless, putative effects of high postprandial levels of nutrients on UPR activation have not been elucidated *in vivo* in humans yet. Therefore one of the subjects of this thesis is to follow up the relationship between postprandial inflammation in both myeloid and lymphoid lineages of human peripheral blood mononuclear cells (PBMC) and ERS.

1.1.4.3 Role of elevated glucose and lipid metabolites in obesity associated inflammation

High levels glucose and FA affect various types of cells including immune cells, adipocytes and pancreatic β cells [31-33]. Detrimental effects of hyperglycemia, in humans defined as fasting blood levels of glucose above 5.5 mmol/l or 100 mg/dl [34], might be mediated through induction of oxidative stress and through the activation of inflammatory pathways resulting in increased secretion of pro-inflammatory cytokines [32, 35]. Still, only a few reports addressed responses of cells of adaptive and innate immunity to this metabolic stimulus *in vivo* in obese individuals [36, 37].

Role of FA in the development of pro-inflammatory state and macrophage accumulation have been investigated predominantly in experimental animal models or in cell cultures *in vitro*. *In vitro*, saturated FA induce increased mRNA expression and secretion of pro-inflammatory cytokines and chemokines (CCL2, IL6, IL8) in adipocytes,

macrophages and other cell types [38, 39]. Saturated FA were found to activate classical inflammatory responses in immune cells and to regulate secretion of pro-inflammatory cytokines in both, immune cells and adipocytes. Furthermore, long saturated FA induce ERS stress in β cells and thus mediate their apoptotic death *in vivo* and *in vitro* [38, 40, 41].

Studies in human subjects are rare and monitor mainly the impact of glucose and FA on adipokines levels in plasma. Therefore *Part one* of this thesis is based on *in vivo* human experiments focused on influence of acutely altered levels of nutrients on pro-inflammatory status and immune system response in blood and AT.

1.2 Treatment of obesity

Health problems related to obesity are closely linked with the impaired function of hypertrophied AT. Therefore, weight loss (based on the reduction of AT) is an obvious strategy to treat obesity-related metabolic disturbances. Modification of life style represents a physiological approach with the lowest health risks compared to medical or surgical intervention and therefore it is usually a first-choice method to reduce weight. Indeed, it was observed that even moderate diet-induced weight loss (5-10%) has beneficial effects on metabolic parameters [42-44]. This can be achieved by various types of diets (for overview see Table 2). *Part two* of this thesis is focused on the immune response of AT to the modest weight loss induced by multiphase dietary interventions (DI) in obese women.

Table 2: Types of dietary interventions (according to Tsigos 2008 [45])

Type of dietary intervention	Daily energy intake
Hypocaloric balanced diet	≥1200 kcal/day
Low calorie diet (LCD)	800 – 1200 kcal/day
Very low calorie diet (VLCD)	≤800 kcal/day
Multiphase diet	Combination of the above listed diets

2. AIMS

General aim of this thesis was to elucidate the connection among impaired levels of nutrients/metabolites and pro-inflammatory state, immune system activation and metabolic status in healthy (obese and lean) subjects. In the *Part one*, acute effects of experimentally increased levels of nutrients on content and phenotype of immune cells in both circulation and AT were elucidated. *Part two* of thesis is focused on the effects of weight reduction on secretory state of adipocytes and immune cells in AT in relation to the improvement of insulin sensitivity.

Specific aims:

PART ONE

- To analyze the inflammation induced by a single high fat meal (HFM) in peripheral blood mononuclear cells including cells of innate and adaptive immunity and to test whether this HFM-induced inflammation is linked with ERS
- To elucidate the effect of short term interventions simulating levels of nutrients and lipid metabolism products (i. e. hyperglycemia, hypertriglyceridemia) seen in metabolically unhealthy obese on inflammation and immune system activation in blood and AT in healthy obese subjects

PART TWO

- To compare expression of pro-inflammatory markers in subcutaneous abdominal and gluteal adipose tissue in steady state and during weight reducing dietary intervention
- To clarify the relationship between serum concentrations of soluble form and adipose tissue mRNA levels of macrophage marker CD163 and to evaluate its possible utilization as a marker of insulin resistance in cross-sectional design and during weight reducing dietary intervention
- To compare the secretory profile of adipocyte precursors before and after weight reducing dietary intervention

3. RESULTS AND DISCUSSION

List of original publications

PART ONE

1. **Postprandial inflammation is not associated with endoplasmic reticulum stress in PBMC from healthy lean men**

Jana Kračmerová, Eva Czudková, Michal Koc, Lucia Mališová, Michaela Šiklová, Vladimír Štich and Lenka Rossmeislová

British Journal of Nutrition, 2013, August, 112(4):573-582. IF 3.3

2. **Experimental hyperglycemia induces an increase of monocyte and T-lymphocyte content in adipose tissue of healthy obese women**

*Michaela Tencerová, *Jana Kračmerová, Eva Krauzová, Lucia Mališová, Zuzana Kováčová, Zuzana Wedellová, Michaela Šiklová, Vladimir Štich and Lenka Rossmeislová

Accepted to Plos One, 2015. IF 3.5

*These authors contributed equally to this work.

3. **Acute hyperlipidemia initiates pro-inflammatory and proatherogenic reaction in obese women**

Eva Krauzová, Jana Kračmerová, Michaela Tencerová, Lucia Mališová, Zuzana Kováčová, Lenka Rossmeislová, Vladimir Štich and Michaela Šiklová

Submitted to Arteriosclerosis, Thrombosis, and Vascular Biology, IF 5.6

PART TWO

4. **Expression of inflammation-related genes in gluteal and abdominal subcutaneous adipose tissue during weight-reducing dietary intervention in obese women**

Lucia Mališová, Lenka Rossmeislová, Zuzana Kováčová, Jana Kračmerová, Michaela Tencerová, Dominique Langin, Michaela Šiklová-Vítková and Vladimír Štich

Physiological Research, 2014, March, 63(1): 73-82. IF 1.5

5. **Soluble CD163 is associated with CD163 mRNA expression in adipose tissue and with insulin sensitivity in steady-state condition but not in response to calorie restriction**

Jana Kračmerová, Lenka Rossmeislová, Lucia Mališová, Zuzana Kováčová, Michaela Tencerová, Eva Klimčáková, Jan Polák, Vladimír Štich, Dominique Langin and Michaela Šiklová

Journal of Clinical Endocrinology and Metabolism, 2014, March, 99(3):528-535. IF 6.3

6. **Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile**

Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová, Michaela Tencerová, Zuzana Kováčová, Michal Koc, Michaela Šiklová-Vítková, Nathalie Viquerie, Dominique Langin and Vladimír Štich

Diabetes, 2013, June, 62(6):1990-1995. IF 7.9

4. COMMENTS TO THE RESULTS AND DISCUSSION

Since obesity and its associated metabolic comorbidities are one of the major health problems of 21st century, research of links leading from impaired function of AT in obese patients to IR development is in the center of interest of many research groups. It was shown that hypertrophied adipocytes release higher amounts of fatty acids as well as pro-inflammatory cytokines and other molecules that disrupt sensitivity of cells to insulin. In contrast, amount of released insulin sensitizing molecules, such as AdipoQ, is reduced. In studies on cell cultures and rodent models, negative effect of high levels of glucose and FA [46] on immune status of AT cells was indicated, but exact influence of their action in humans was not elucidated yet. Therefore, the aim of the first part of my thesis was to investigate effects of experimentally increased levels of glucose and lipid metabolites on immune cells in blood and AT and systemic markers of inflammation in human volunteers.

PART ONE

Obese subjects have impaired function of AT manifested by its insufficient ability to store energy that leads to increased levels of nutrients, such as glucose and lipid compounds (free FA and glycerol) in blood stream. Nevertheless, even in lean subjects, plasma levels of glucose and FA, triacylglycerol (TAG) etc. increase after meal consumption. This rise is associated with inflammatory state (so called postprandial inflammation), that is manifested by increased plasma levels of inflammatory cytokines and leukocyte activation [47, 48]. This effect of meal, especially of meal with high levels of nutrients, is however protracted in obese subjects and may contribute to aberrant immune system activation. Indeed, prolonged exposure to nutrients can cause ERS that may activate classic inflammatory regulatory molecules such as NFκB and Jun N-terminal kinase [49]. Nevertheless, it remains unknown whether ERS is prerequisite for the development of postprandial inflammation. Therefore, **in the first study**, effect of HFM on peripheral blood mononuclear cells and inflammatory state was examined, with emphasis on association between ERS and postprandial inflammation.

10 lean men consumed a high energy, high-fat meal (McDonalds, Prague, Czech Republic, 6151 kJ (1469 kcal), 32.8% carbohydrates, 47.4% lipids, 11.3% proteins) within 15 minutes. Blood samples were drawn each hour up to the 4th hour. Activation of immune system was monitored by flow cytometry of peripheral blood and by quantitative real time polymerase chain reaction (qRT-PCR) of mRNA from CD14+ cells (monocytes). These cells were separated from PBMC isolated by Histopaque/Accuspin density system.

In line with previous studies [48, 50] HFM intake induced postprandial increase of all main leukocyte groups - granulocytes, monocytes and lymphocytes – in blood. Moreover we confirmed the finding by Gower et al.[51] showing increased CD11c expression on the surface of monocytes after ingestion of the HFM by healthy volunteers. CD11c is considered as an activation marker of monocytes. Importantly, high-fat diet feeding results in the infiltration of CD11c+ monocytes into AT in mice [20, 52], and these monocytes/macrophages exhibit a pro-inflammatory M1 phenotype. CD11c expression has also been found to increase in blood monocytes of obese subjects and to positively correlate with HOMA-IR [53] . We then focused on gene expression in CD14+ monocytes from peripheral blood i.e. cells that are intimately exposed to metabolite fluctuations and upon activation may contribute to the development of AT inflammation. Remarkably, the mRNA expression of all tested pro-inflammatory cytokines was enhanced after the HFM challenge. As noted already for CD11c expression, postprandial changes in the expression pro-inflammatory cytokines were similar to the changes in their expression

associated with obesity [54, 55]. Therefore, a single HFM may activate monocytes in a similar direction to a long-term overfeeding or obesity.

Following the HFM challenge, mRNA expression of a majority of ERS markers representing all three arms of UPR was not altered in PBMC. Thus, the classic activation of UPR does not seem to be the driver of the postprandial increase in the expression levels of inflammatory cytokines in CD14+ monocytes. The only ERS marker whose expression was postprandially elevated was ATF3. ATF3 is, however, activated not only by ERS but also by other various stresses [56], and the absence of the upregulation of ATF4 (classic UPR pathway, which in turns induces expression of ATF3 [57]) in the analyzed CD14+ cells suggests that the up-regulation of ATF3 is not associated with the activation of UPR.

In conclusion, we demonstrated that inflammation induced by the HFM challenge in CD14+ monocytes was not accompanied by an activation of classic UPR.

It was shown that acute hyperglycemia can activate inflammatory pathways in various cells resulting in increased secretion of pro-inflammatory cytokines [58, 59]. Therefore, the objective of **the second study** was to investigate whether acute experimental HG, imitating increased glycaemia found in obese with metabolic syndrome, has an impact on phenotype and relative content of monocytes/macrophages and lymphocytes in circulation and the subcutaneous abdominal AT (SAAT).

30 healthy obese premenopausal women without signs of metabolic syndorme were recruited and divided into 3 groups (n=10 per group): one was exposed to hyperglycemic- euinsulinemic clamp (where the endogenous insulin release was blocked by octreotide infusion) and two control groups were exposed to the infusion of octreotide or saline. SAAT was obtained using needle biopsy. Blood and SAAT samples were collected before and after the 3-hours lasting intervention and used for flow cytometry analysis. Moreover, SAAT was used to examination of mRNA levels of chemokines, markers of macrophages and T lymphocytes subtypes by qRT-PCR.

We documented that HG induced an increase in CD45+/14+ monocyte/macrophage population in SAAT. It was shown previously that HG treatment of monocytes in vitro increases expression of Toll-like receptors [60] and also monocytes from patients with T2DM show a higher expression of toll-like receptor (TLR) 2 and TLR4 compared to healthy subjects [37], thus the expression of these two receptors was investigated. In SAAT, only TLR4+ monocyte population was increased. This selective effect of HG on TLR4+ monocyte population could point to a specific physiological function of this subtype of monocytes in HG-affected SAAT. Indeed, recent findings suggest that TLR4 and TLR2 activation in macrophages results in the differential expression and secretion of pro-inflammatory cytokines [61]. In accordance, HG clamp induced increase of mRNA expression of TLR4 along with TNFα, which has been shown to be up-regulated after TLR4 but not TLR2 stimulation in macrophages [61].

Contrary to monocyte population, a population of resident AT macrophages did not show any changes in response to HG in terms of relative content and TLRs expression (i.e. content of CD45+/14+/TLR2+ and TLR4+). Therefore, it seems that SAAT microenvironment, changed by HG, activated only monocytic cells that are not fully differentiated into macrophages. Such a population of CD206- monocytic cells was described by Wentworth et al. [62] and was shown to be elevated in human obesity. It is plausible that these monocytes represent "the newest arrivals" into AT but then later can mature into CD206+ macrophages. Nevertheless, CD206 marker used to identify resident AT macrophages was previously suggested to be preferentially expressed by M2 macrophages [63], and thus it is also possible that observed increase in CD45+/14+/206- population could be attributed to M1 macrophages.

Lymphocytes play a key role in infiltration of immune cells into AT [64, 65]. We found an increased content of total T lymphocytes and both major subpopulations of T lymphocytes, i.e. T_H CD4+ and T_C CD8+ in SAAT of obese women in response to short-term HG. In animal studies, CD8+ T cells direct macrophage infiltration into AT [66] and CD4+ T cells have both anti- and pro-inflammatory roles based on their further specialization [67]. In line with previous data showing that HG modulates expression of genes related to immune response in SAAT of lean subjects [36, 68], we observed that mRNA levels of CD3g, CD4 and CD8a increased in the experimental condition of HG in obese women, which nicely supports the FACS results. Furthermore, we found the up-regulation of T-Box 21 (TBX21), GATA binding protein 3 (GATA3) and forkhead box P3 (FoxP3) mRNA levels in SAAT (corresponding to T_H1, T_H2 and T_{REG} subtypes) after HG condition in obese women. It has been shown that T_H1, T_{REG} are increased and T_H2 subpopulation is decreased with obesity [69, 70]. Based on our results, one can hypothesize that HG enhanced infiltration of both pro- and anti-inflammatory T cells in order to maintain immune homeostasis in AT.

In summary, our results show that the short-term HG induces an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women and thus may contribute to the worsening of an immune status of AT in obese individuals.

Beside glucose, another possible contributor to inflammation development appears to be elevated levels of FA. In mice, FA blood concentration and increased mobilization of FA during fasting was associated with increased macrophages content in AT [46]. *In vitro*, saturated FA induce increased mRNA expression and secretion of pro-inflammatory cytokines and chemokines in adipocytes, macrophages and other cell types. Objective of **the third study** was to describe the impact of artificially increased circulating concentration of FA on immune system activation in blood and SAAT.

17 obese premenopausal women were recruited into the intervention: 10 subjects were included in the treatment group with 7 hours lasting infusion of 20 % Intralipid solution (lipid emulsion of soya-bean oil (20%) and 2.5% glycerol). 7 subjects participated in the control trial with infusion of glycerol (2.5%). To determine the effect of FA on relative content and phenotype of immune cells in blood and SAAT, blood and biopsied SAAT samples were collected before and after the interventions and analyzed by flow cytometry. Moreover, SAAT was used to examination of mRNA levels of chemokines, angiogenesis marker, markers of macrophages activation and T lymphocytes subtypes by qRT-PCR.

We found a trend to increased relative content of total T lymphocytes and an increase of T_H subpopulation in blood in response to lipid infusion. This observation is in agreement with previous studies showing that FA modulate T cells proliferation [71] and that lymphocyte counts increase postprandially in healthy as well as in hyperlipidemic subjects with coronary artery disease [72, 73]. Intralipid-induced increase of T_H lymphocyte content in blood is of particular interest as T_H cells appear to be essential players in the development of atherosclerosis [74]. We have observed an upregulation of SAAT mRNA expression of T_H17 marker (RAR-related orphan receptor C - RORC). T_H17 cells are pro-inflammatory and its increased numbers in AT of metabolically unhealthy obese subjects or in diet-induced obesity in mice were observed [75]. Together, our data suggest that acute hyperlipidemia induced by Intralipid infusion induce pro-inflammatory changes in lymphocyte populations.

In contrast to lymphocytes, the relative blood content of CD45+/CD14+ monocytes and the subpopulations of "non-classical" CD16+ activated monocytes was decreased. This trend could be possibly explained by the enhanced adherence of monocytes to the endothelial surface of vascular wall after the lipid infusion. Likewise, the

enhanced adhesion of monocytes was observed in response to postprandial hypertriglyceridemia in rats [76]. Indeed, levels of soluble adhesion molecules (sICAM, sVCAM) and angiogenic factor VEGF-A, expressed by endothelial cells [77], were increased in response to lipid infusion.

In SAAT, the relative content of monocytes was not changed, however the subset of CD45+/14+/206+/16+ resident macrophages was decreased. In some studies, CD206 is, similarly as CD16, considered as marker of non-classical –"M2" activated macrophages [16]. Therefore we can hypothesize that Intralipid infusion stimulated the switch of macrophages to "classical" activated pro-inflammatory phenotype. This switch could be supported by increased mRNA expression of CCL2 and IL6 cytokines.

In conclusion, the acute hyperlipidemia induced by Intralipid infusion was associated with pro-inflammatory and pro-atherogenic changes in monocyte and lymphocyte populations and soluble mediators in blood in obese women. Moreover, pro-inflammatory changes – represented by a decrease of M2 macrophages content and increased expression of inflammatory cytokines and marker of T_H17 cells - were observed in SAAT.

These results thus point at the processes that could contribute to the initiation of atherosclerosis and worsening of AT immune status in obese patients exposed to high circulating lipid levels.

PART TWO

In contrast to pro-inflammatory effects of overfeeding and high circulating levels of glucose and lipids, even moderate weight loss has beneficial impact on AT function and secretory profile, as well as on whole body immune status and insulin sensitivity, although the mechanisms of these benefits are not clear. Thus, the goal of the second part of this thesis was to improve our understanding of relationship between changes of immunity-related characteristics of AT and improvement of metabolic parameters triggered by weight loss.

The **fourth study** aimed to elucidate an impact of weight reduction on macrophage content and cytokines production in two different subcutaneous fat depots: subcutaneous gluteal AT (SGAT) and SAAT, and whether protective role of SGAT is attributable to different inflammation-related characteristics of this depot.

14 pre-menopausal women underwent 6 months DI consisting of 3 periods: 1 month of very low calorie diet (VLCD), 2 months of low calorie diet (LCD), followed by 3 months of weight maintenance (WM) phase. The paired samples of SAAT and GAT were obtained using needle biopsy in three phases of DI and used for RNA isolation. A gene expression of 17 genes related to immune status (cytokines and macrophage markers) of AT was analyzed by qRT-PCR.

Although protective role of AT accumulation in the lower body was suggested [8, 9, 78], our data, similar to findings of other groups [79, 80], did not show major difference in cytokines and macrophage markers between these depots in basal state, with the exception of two macrophage markers ACP5 and MRS1 and two cytokines IL10Rα and CCL2.

The main novelty of this study lies in the comparison of gene regulation in SAAT vs. SGAT during dynamic condition represented by two phases of a 6 months' DI. In both groups of genes (macrophage markers and cytokines), previously observed bi-phasic pattern [43, 44] of gene expression did not differ markedly in SAAT vs. SGAT, except for three cytokines – IL6, IL10 and CCL2. In light of our and others results demonstrating the absence of major differences between SGAT and SAAT it can be suggested that the deleterious effect of upper body obesity could be mediated by the excess of visceral adipose tissue (VAT) and not excess of SAAT. Furthermore, it should be noted that the present study compared SGAT and SAAT on transcriptional level and that the results of this study are limited to women.

In conclusion, we did not find major differences in mRNA levels of macrophage markers and cytokines between SAAT and SGAT at baseline condition or in the pattern of their regulation in response to two phases of hypocaloric weight-reducing DI. Therefore, our results do not bring evidence of an altered pro-inflammatory status or an altered "responsiveness" of immune cells in SGAT when compared with SAAT.

The fifth study was focused on macrophage marker CD163 and its soluble form sCD163. CD163 is predominantly expressed by tissue macrophages and it is cleaved and released to circulation by similar enzyme as TNFα – TNFα converting enzyme (TACE, also known as ADAM Metallopeptidase Domain 17) [81]. Levels of sCD163 were shown to be elevated in obese subjects and were found to represent a marker of IR due to its association with impaired insulin sensitivity [82, 83] and sCD163 concentration was predicted as a marker of macrophages infiltration to AT.

Two cohorts of subjects were examined in the study. Cohort 1 included 42 women with a wide range of BMI (17–48 kg/m2) divided into three groups according to their BMI and presence or absence of metabolic syndrome (lean, obese, obese with metabolic syndrome). Samples of VAT and SAAT were obtained during abdominal surgery. The values of glucose disposal rate (GDR; determinant/index of insulin sensitivity) were acquired from the euglycemic-hyperinsulinemic clamp method performed according to De Fronzo et al. [84].

Cohort 2 included 27 obese women who followed a DI consisting of 1 month of a VLCD and 5 months of a weight-stabilization period (consisted of 2 months of LCD and 3 months of a WM period). The biopsied samples of SAAT were obtained in three phases of DI. A gene expression of two macrophage markers (CD163, CD68), classical inflammatory marker TNFα and two genes responsible for sCD163 shedding (TACE, tissue inhibitor of metalloproteinases 3 - TIMP3) in SAAT was analyzed by qRT-PCR.

In a Cohort 1, our finding supported previous suggestion that plasma sCD163 levels are associated with mRNA expression in SAAT [82, 85] and furthermore a similar association of sCD163 and VAT was found. Moreover, the correlation of sCD163 levels with mRNA expression of macrophage marker CD68 suggests that serum sCD163 might be perceived as a possible indicator of macrophage activation in AT. Next, we documented a strong relationship between insulin sensitivity, expressed as GDR, and circulating sCD163. These results extend the previously reported findings of a close relationship between sCD163 and HOMA-IR [82, 86]. In our study, insulin sensitivity correlated also with CD163 mRNA expression in both SAAT and VAT depots. Thus, we confirmed a validity of sCD163 and CD163 expressed in AT as a biomarker of insulin sensitivity at steady-state condition.

However, in a dynamic condition represented by the weight-reducing hypocaloric diet in Cohort 2, the above-mentioned associations were not present: the diet-induced change of sCD163 showed different pattern and did not correlate with the change of CD163 mRNA levels in SAAT either during the initial dynamic phase of the DI (VLCD) or during the WM phase. The mRNA CD163 expression pattern was in line with magnitude of mRNA CD68 in AT and other macrophage markers analyzed in our previous publications [43, 44, 87]. The discrepancy between the dynamics of soluble and AT mRNA levels of CD163 could be based on the translational or posttranslational regulation of expression. Therefore, mRNA expression factors included in sCD163 shedding (TACE, TIMP3) was analyzed [88]. However, no relevant change of TACE or TIMP3 mRNA expression in AT throughout the DI was found. Therefore, the changes in the shedding of CD163 in AT during DI probably do not contribute to the changes of sCD163 in circulation. Other possible explanation of this discrepancy is that CD163 is expressed in several other tissues [83, 89], and also in blood monocytes [90]. Unfortunately, to our knowledge,

there are no studies that evaluate direct contribution of other tissues to circulating levels of sCD163 or investigating CD163 expression in other tissues or cells during DI.

In this study the evolution of sCD163 during weight-reducing DI paralleled that of the GDR measured by a hyperinsulinemic clamp. However, the direct correlations between the diet- induced changes of sCD163 and those of GDR were not found. Similarly, no correlation was found between the diet-induced changes of CD163 mRNA expression and insulin sensitivity. These findings suggest that circulating levels of sCD163 and AT mRNA expression of CD163 are probably not in a cause-effect relationship with insulin sensitivity.

In conclusion, in this study we demonstrated a quantitative association between the circulating levels of sCD163 and mRNA expression of macrophage markers CD163 and CD68 in SAAT and VAT in the steady-state condition. Furthermore, in the steady-state condition, we found a negative correlation between sCD163 levels and insulin sensitivity. However, in a dynamic condition represented by a weight-reducing DI, there is no such relationship between the diet-induced changes of the above-mentioned variables. Thus, there is no evidence that sCD163 might be used as a quantitative biomarker of the diet-induced changes of AT CD163 expression or changes of insulin sensitivity.

Worsening of metabolic state in obesity is associated with impaired endocrine function of adipocytes. The current knowledge on intrinsic endocrine potential of these cells is based on and limited to cross-sectional studies. We hypothesized that cell cultures of adipose precursors established from SAAT acquired before and after the diet-induced weight loss would reflect two distinct metabolic and nutritional stages of the donor and could provide information about the intrinsic endocrine potentials of obese and post-obese AT. Thus, **in the sixth study**, effect of moderate weight loss on the secretory profile of adipocyte precursors was examined.

23 premenopausal women underwent 5-6 lasting weight reducing intervention consisted of 3 months of LCD and subsequent 3 months of WM phase. Paired cell cultures of human preadipocytes were established from SAAT samples obtained by needle biopsy before and after the entire DI. To determine whether weight loss affects the intrinsic secretory potential of adipocytes, the secretion and mRNA expression of several cytokines and adipokines was measured in *in vitro* differentiated preadipocytes.

We showed that secretory capacity of *in vitro* cultured preadipocytes derived from adipocyte precursors is affected by moderate weight loss. This was documented by comparing secretion and expression of IL8, CCL2, leptin, and AdipoQ by cells isolated from paired subcutaneous AT biopsies from obese women undergoing long-term DI. In obesity, hypertrophied adipocytes produce prevalently pro-inflammatory cytokines and chemokines such as TNFα, IL6 and CCL2 [91]. These cytokines may affect the phenotype of the macrophages already residing in the AT and stimulate infiltration and activation of macrophages from circulation [92]. On the other hand, secretion of insulin sensitizing AdipoQ is diminished [93] in obese subjects.

We observed an increase of expression and secretion of AdipoQ and its HMW form in adipocytes after DI. Leptin mRNA levels were also elevated in adipocytes after weight loss. In contrast, CCL2 and IL8 mRNA levels in adipocytes obtained after DI were reduced compared to baseline.

Importantly, the secretion of adipokines with the exception of leptin by *in vitro* cultivated adipocytes reflected in general changes seen at the level of AT explants [44]. Lower secretion of CCL2 from adipocytes reprogrammed by weight loss could contribute to a lower infiltration of macrophages into AT described earlier [43, 94]. Selective increase of HMW AdipoQ secretion might underlie beneficial effects of weight loss on insulin sensitivity.

Studies performed on cell culture models may be influenced by culture conditions. Although we cannot completely exclude possible effects of sub-cultivation on secretory potential of cells, it has been shown that in vitro conditions preserve the original phenotype of a donor as shown previously for preadipocytes and adipocytes [95]. Moreover, sub-cultivation of stromal vascular cells eliminates contaminating cells like macrophages and results in more homogenous population than primary cells [96, 97]. It is also unlikely that the observed differences were based on dissimilar starting numbers of cells as there was no difference in the length of cultivation or yield of cells before and at the end of DI.

In conclusion, our study shows that weight loss alters secretory potential of preadipocytes. This effect may be associated with the improvement of the metabolic status of obese. We believe that the analysis of a distinct cellular population, such as preadipocytes subjected to uniform in vitro conditions, can offer a focused and unique image of an intrinsic adaptation of AT to weight loss.

5. CONCLUSIONS

This thesis analyzed immune status of AT and circulating leukocytes under various physiological and pathophysiological interventions in lean and obese humans. First part examined acute effects of elevated levels of nutrients on inflammation and representation of immune cells. Second part investigated beneficial effects of moderate weight reduction on immune attributes of AT, with respect to improvement of pro-inflammatory state and sensitivity to insulin action.

The major conclusions of this thesis are:

- Ingestion of high fat meal induced postprandial inflammation detectable on the level of gene expression in CD14+ PBMC. This inflammation was not associated with the concomitant increase in the expression levels of ERS markers.

- Acute short-term hyperglycemia induced an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women.

- Acute hyperlipidemia induced a pro-inflammatory response associated with alteration of relative content of immune cells in blood and SAAT and enhanced release of pro-atherogenic mediators.

- No major differences were found in mRNA levels of selected immunity related genes between SAAT and SGAT in basal conditions. During weight reduction, majority of genes changed with similar pattern thus refuting the hypothesis that protective role of SGAT is given by lower expression of pro-inflammatory/immune system related genes.

- sCD163 correlated with CD163 mRNA expression in SAAT and VAT and with whole-body insulin sensitivity in the steady-state condition. These associations were not observed with respect to the diet-induced changes during a weight-reducing hypocaloric diet.

- Secretory potential of human *in vitro* cultured pre/adipocytes was altered to the less inflammatory after the weight reduction.

6. SUMMARY

Obesity and overfeeding are associated not only with increased circulating levels of nutrients and metabolites, but also with increased risk of the development of additional disorders, such as cardiovascular diseases, cancer or insulin resistance. Plausible link between obesity and its comorbidities is inflammatory state, observed on the whole body level as well as in AT. As possible initiators of this inflammation, hypertrophied adipocytes were suggested. Adipocytes *per se* secrete a spectrum of heterogeneous molecules including cytokines. Under the stress conditions, adipocytes and subsequently AT resident immune cells switch to pro-inflammatory state and via secretory signaling attract additional immune cells. Furthermore, hypertrophic adipocytes release higher levels of metabolites that may also contribute to pro-inflammatory polarization of immune cells, mainly macrophages.

General aim of this thesis was to investigate connection between impaired levels of nutrients and pro-inflammatory statue and activation of immune cells in healthy (obese and lean) subjects.

In the Part one of this thesis, we analyzed acute reaction of immune cells in circulation and AT on artificially elevated levels of nutrients, imitating its increased values typical for metabolic syndrome. HFM ingestion led to inflammatory reaction detectable in circulating monocytes but not associated with ER stress. Similarly, short-term HG and hyperlipidemia induced a pro-inflammatory response associated with altered relative content of immune cells in blood and SAAT. Moreover, changes induced by acute hyperlipidemia were associated with enhanced release of pro-atherogenic mediators.

In the studies included in the Part two, we extended our knowledge about beneficial effects of weight reduction on pro-inflammatory and metabolic statue of obese patients. Moderate weight loss was accompanied by amelioration of levels of pro-inflammatory markers in circulation and in AT. The effect on mRNA levels of immunity-related markers was similar in abdominal and gluteal subcutaneous AT. Expression changes of one of these markers, CD163, which were induced by weight loss, were not associated with changes of insulin sensitivity. Furthermore, weight loss reprogrammed precursors of adipocytes and reduced their intrinsic inflammatory potential.

In conclusion, in short-term interventions we confirmed that impaired levels of glucose and lipid metabolites (FA, TAG) are associated with activation of immune cells in humans. On the other hand, weight reduction led to improvement of secretory function of adipocytes *per se* and inflammatory status of AT on mRNA level. Results of this thesis thus contribute to understanding of obesity and overfeeding associated inflammation, even so further investigation of the functional changes in AT by nutrients and obesity is warranted.

7. SHRNUTÍ

Obezita, charakterizovaná zvýšenou akumulací tukové tkáně (TT), i přejídání jako takové jsou spojeny nejen se zvýšenými plasmatickými hladinami živin a metabolitů, ale i s narůstajícím rizikem vzniku dalších chorob, např. chorob kardiovaskulárního systému, rakoviny nebo insulinové rezistence. Pravděpodobným pojítkem mezi obezitou a chorob s ní spojených je zánětlivý stav organismu, pozorovaný jak na systémové úrovni jako zvýšené hladiny plasmatických cytokinů, tak na úrovni TT. Příčinou tohoto zánětlivého stavu může být narušený metabolismus TT. Adipocyty sekretují širokou paletu různorodých molekul včetně cytokinů. Za stresujících podmínek (hypoxie, stres endoplasmatického retikula) začnou adipocyty a následně i rezidentní imunitní buňky produkovat prozánětlivé cytokiny, které atrahují další imunitní buňky. Adipocyty mohou navíc uvolňovat zvýšené množství metabolitů (mastné kyseliny, glycerol), které rovněž přispívají k polarizaci imunitních buněk, zejména makrofágů.

Cílem této práce bylo nalézt spojení mezi zvýšenými hladinami nutrientů (glukosa, mastné kyseliny) a zánětlivým stavem, resp. aktivací imunitních buněk u zdravých (obézních i štíhlých) jedinců.

V první části této práce byla sledována akutní reakce imunitních buněk na experimentálně zvýšené hladiny nutrientů. Pokrm s vysokým obsahem tuku a energie způsobil postprandiální leukocytózu a vedl k prozánětlivé reakci detekované v krevních monocytech. Obdobně krátkodobá hyperglykemie a hyperlipidemie indukovaly prozánětlivou odpověď, spojenou se změnou zastoupení imunitních buněk na úrovni krve i TT. Změny indukované akutní hyperlipidemií byly navíc spojeny s uvolněním aterogenních mediátorů.

Cílem druhé části bylo prohloubit znalosti o pozitivních efektech redukce hmotnosti na prozánětlivý a metabolický stav obézních pacientů. Mírný váhový úbytek byl provázen snížením hladin cytokinů v plasmě a TT. Efekt redukce hmotnosti na imunitní markery byl obdobný jak v abdominálním tak gluteálním tukovém depu. V průběhu dietní intervence nebyla pozorována spojitost mezi změnami v hladinách jednoho ze stanovovaných markerů, CD163, a insulinovou sensitivitou. Mimoto se vlivem váhové redukce upravil i sekreční potenciál samotných adipocytů.

Lze shrnout, že na základě analýz efektů krátkodobých intervencí byla potvrzena hypotéza, že zvýšené hladiny glukosy a mastných kyselin v krvi jsou asociovány s aktivací imunitního systému. Oproti tomu, redukce hmotnosti vedla ke zlepšení jak sekrečního profilu samotných adipocytů tak k snížení prozánětlivého stavu TT na úrovni mRNA, ale tyto změny nebyly přímo asociovány se zlepšením insulinové senzitivity. Výsledky této práce přispěly k pochopení prozánětlivých pochodů asociovaných s obezitou a přejídáním, i přesto je další výzkum funkčních změn na úrovni TT nezbytný.

8. ANNEX

List of co-authored articles not included in the thesis:

Stress of endoplasmic reticulum modulates differentiation and lipogenesis of human adipocytes
Michal Koc, Veronika Mayerová, Jana Kračmerová, Aline Mairal, Lucia Mališová, Vladimír Štich, Dominique Langin, Lenka Rossmeislová
Accepted Biochemical and Biophysical Research Communications. 2015. [Epub ahead of print]. IF 2.3

Ursodeoxycholic acid but not tauroursodeoxycholic acid inhibits proliferation and differentiation of human subcutaneous adipocytes.
Lucia Mališová, Zuzana Kováčová, Michal Koc, Jana Kračmerová, Vladimír Štich, Lenka Rossmeislová
PLoS One. 2013 Dec, 8(12): e82086. IF 3.5

Adaptation of human adipose tissue to hypocaloric diet.
Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová, Vladimír Štich
International Journal of Obesity (London). 2013 May, 37(5):640-50. IF 5.2

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