Immunometabolic Aspect of C5L2 and C5aR in Adiposity: Physical, Functional and Physiological Interactions

Thèse

Pegah Poursharifi

Doctorat en biologie cellulaire et moléculaire
Philosophiæ doctor (Ph.D.)

Québec, Canada

Résumé

L'obésité est maintenant reconnue comme présentant une inflammation chronique et est caractérisée par une augmentation de l'infiltration de macrophages dans le tissu adipeux. Cette infiltration est réflètée par l'activation du système du complément qui agit comme élément déclencheur et précurseur aux autres fonctions immunitaires. Le C5L2 (C5aR-like receptor 2) a récemment été identifié comme récepteur pour la protéine stimulant l'acylation (ASP) ainsi que pour le facteur inflammatoire C5a (capable quant à lui d'aussi se lier au C5aR). Cette thèse porte sur: (i) l'interaction entre C5L2 et C5aR suite à l'activation de ceux-ci par leurs ligands respectifs dans des lignées cellulaires adipocytaires (3T3-L1) et de macrophages (J774), (ii) la contribution du C5aR au métabolisme et à la réponse immunitaire d'adipocytes provenant de modèles murins et (iii) l'association entre C5L2 et C5aR avec des facteurs reliés à l'obésité chez l'humain.

Les récepteurs C5L2 et C5aR s'associent entre-eux de façon constitutive en homo- et hétérodimères et l'ajout de ligand à des cultures d'adipocytes 3T3-L1 ou de macrophages J774 augmente cette colocalisation. Autant l'ASP que le C5a ont réussi à induire une réponse fonctionnelle chez des adipocytes primaires. Lorsque des adipocytes primaires provenaient de souris invalidées pour le C5aR, les effets d'un traitement avec C5a étaient perturbés tandis que les effets médiés par un traitement à l'ASP étaient maintenus. De plus, l'addition de C5a bloquait la signalisation et la réponse fonctionnelle causées par un traitement à l'ASP chez les adipocytes primaires provenant de souris C5aRKO et de type sauvage. Finalement, l'expression des gènes C5L2 et C5aR dans le tissu adipeux de femmes obèses morbides était associée avec une adiposité croissante. De façon interessante, les ratios ASP/C5L2 et C5L2/C5aR augmentaient de façon significative avec l'obésité abdominale.

Lorsque pris ensemble, l'interaction fonctionelle, physiologique et la proximité physique des récepteurs C5L2 et C5aR chez les adipocytes suggèrent un rôle potentiel de ceux-ci sur l'immunométabolisme du tissu adipeux. De surcroit, cette interaction met en

valeur les liens qui existent entre le tissu adipeux et les protéines et récepteurs du complément tout en démontrant comment une réponse excessive au niveau immuno-métabolique pourrait exacerber le développement du niveau d'adiposité chez l'individu.

Abstract

From the convergence of metabolism and immune research has emerged a new research field, termed “Immunometabolism”. Obesity, an immunometabolic disease, is associated with a state of low-grade inflammation and is characterized by increased infiltration of macrophages into adipose tissue. Complement activation can act as an early trigger and precursor of other immune functions. C5aR-like receptor 2 (C5L2) has been identified as a receptor for Acylation Stimulating Protein (ASP) and the inflammatory factor C5a (which can also bind C5aR). This thesis sequentially evaluates (i) ligand-induced C5L2 and C5aR interaction in cultured 3T3-L1 adipocytes and J774 macrophages, (ii) the C5aR contribution in adipocyte metabolic and immune responses in mouse models, (iii) as well as C5L2 and C5aR association with obesity-related factors in humans.

The immunometabolic receptors, C5L2 and C5aR, constitutively self-associate into homo-/heterodimers and ligand treatment of 3T3-L1 adipocytes and J774 macrophages increased their colocalization. Both C5a and ASP directly induced primary adipocyte signaling and function. However, in C5aRKO primary adipocytes, C5a effects were disrupted, while stimulatory effects of ASP were mostly maintained. Moreover, addition of C5a completely blocked ASP signaling and activity in both C5aRKO and WT primary adipocytes. Finally, C5L2 and C5aR expression in adipose tissue from morbidly obese women was associated with increased adiposity. Interestingly, ASP/C5L2 and C5L2/C5aR ratio markedly increased with abdominal obesity.

Taken together, the closely linked physical, functional and physiological interaction between C5L2 and C5aR in adipocytes suggests a potential role in adipose tissue immunometabolism. This further highlights the important new links between adipose tissue and complement proteins/receptors and demonstrates how excessive immunometabolic responses may exacerbate adiposity.

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Abbreviations

ACM	adipocyte-conditioned medium
Akt	protein kinase B
ANOVA	analysis of variance
ApoA1	apolipoprotein A1
ApoB	apolipoprotein B
ASP	acylation stimulating protein
ATM	adipose tissue macrophages
BAT	brown adipose tissue
BCS	bovine calf serum
BSA	bovine serum albumin
BMI	body mass index
BPD	biliopancreatic diversion
BRET	bioluminescence resonance energy transfer
C5aR	C5a receptor; CD88
C5L2	C5aR-like receptor 2; GPR77
CACC	Canadian council of animal care
CoCM	co-culture conditioned medium
DAPI	4',6-diamidino-2-phenylindole
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GLUT	glucose transporter
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
HDL-C	high density lipoprotein cholesterol
HMGB1	high mobility group box protein-1
HSL	hormone sensitive lipase
IBMX	isobutylmethylxanthine
Icorr	correlation index
IFNγ	interferon gamma
IL	interleukin
IRS-1	insulin receptor substrate-1
JNK	Jun N-terminal kinase
KC	keratinocyte chemoattractant
KO	knockout
KRB	Krebs-Ringer buffer

LDL-C	low density lipoprotein cholesterol
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MBL	mannose binding lectin
MCP-1	monocyte chemoattractant protein-1
MCM	macrophage-conditioned medium
NEFA	nonesterified fatty acids
NFκB	κ-light-chain-enhancer of activated B cells
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositide 3-kinase
rASP	recombinant ASP
Rluc	Renilla luciferase
SF	serum free
SVF	stromal vascular fraction
T2DM	type 2 diabetes
TG	triglycerides
TH	T-cell helper
TLR	toll- like receptor
TNF	tumor necrosis factor
TRITC	tetramethylrhodamine
WAT	white adipose tissue
WHO	world health organization
WT	wild-type

Dedicated to my parents, my sister, and Amir.

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Avant-propos

This thesis titled "Immunometabolic Aspect of C5L2 and C5aR in Adiposity: Physical, Functional and Physiological Interactions" centers around elucidating fundamental relationships between innate immune responses and adipose tissue physiology, focuses on the immunometabolic roles of C5aR and C5L2 in adiposity. Regarding the setup of the thesis, CHAPTER 1 presents an overview of the basics relevant to understanding the work described in the thesis and the general objectives. This is an article-based thesis comprised of three published manuscripts presented as CHAPTERS 2-4. CHAPTER 5 discusses the study limitations and the significance of findings, offers interpretations, and further proposes new research directions. References used in CHAPTER 1 and 5 are indexed in the final bibliography.

CHAPTER 2 is composed of the article titled "C5L2 and C5aR Interaction in Adipocytes and Macrophages: Insights into Adipoimmunology", co-authored by Poursharifi, Lapointe, Pétrin, Devost, Gauvreau, Hébert, and Cianflone, and has been published in the journal of *Cellular Signalling* in 2013. I am the principal author and my contribution to this study includes: study design, implementation, experiments, data analysis, and manuscript writing/editing. Marc Lapointe performed fatty acid uptake assay and the related data analysis. Dr. Hebert's team performed Bioluminescence Resonance Energy Transfer (BRET) assays.

CHAPTER 3 contains the article "C5aR and C5L2 Act in Concert to Balance Immunometabolism in Adipose Tissue", co-authored by Poursharifi, Lapointe, Fisette, Lu, Roy, Munkonda, Fairlie, and Cianflone, and has been published in the journal of *Molecular and Cellular Endocrinology* in 2014. I am the principal author contributing to study design, implementation, experiments, data analysis, and manuscript writing/editing. Alexandre Fisette contributed to animal maintenance and dissection. Huiling Lu, Christian Roy, and

Mercedes Munkonda assisted in conducting the experiments, and David Fairlie provided the C5aR specific antagonist.

CHAPTER 4 contains the article "Association of Immune and Metabolic Receptors C5aR and C5L2 with Adiposity in Women", co-authored by Poursharifi, Rezvani, Gupta, Lapointe, Marceau, Tchernof, and Cianflone, accepted for publication in the *Mediators of Inflammation* in 2014. I am the principal author, with major roles in study design, implementation, experiments, statistical analysis, and manuscript writing/editing. Reza Rezvani and Abhishek Gupta conducted gene expression experiments. Samples were provided by Dr. Marceau and Dr. Tchernof.

In addition, during my doctorate studies, I have had the opportunity to participate in the following collaborative projects, which have resulted in publications as listed below. These publications are not included in the content of this thesis.

- Tom, F-Q., Gauvreau, D., Lapointe, M., Lu, H. L., **Poursharifi, P.**, Luo, X-P. & Cianflone, K. Differential Chemoattractant Response in Adipocytes and Macrophages to the Action of Acylation Stimulating Protein. *Eur. J. Cell Biol.* **92,** 61-9 (2013).
- Miegueu, P., St-Pierre, D., Lapointe, M., **Poursharifi, P.**, Lu, H., Gupta, A. & Cianflone, K. Substance P Decreases Fat Storage and Increases Adipocytokine Production in 3T3-L1 Adipocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **304,** G420-7 (2013).
- Roy, C., Gupta, A., Fisette, A., Lapointe, M., **Poursharifi, P.**, Richard, D., Lu, H., Lu, B., Gerard, N., Gerard, C. & Cianflone, K. C5a Receptor Deficiency Alters Energy Utilization and Fat Storage. *PLoS One*, **8,** e62531 (2013).
- Fisette, A., **Poursharifi, P.**, Oikonomopoulou, K., Munkonda, M. N., Lapointe, M. & Cianflone, K. Paradoxical Glucose-Sensitizing yet Proinflammatory Effects of Acute ASP Administration in Mice. *Mediators Inflamm.* **2013,** 713284 (2013).
- Gupta, A., Rezvani, R., Laponite, M., **Poursharifi, P.**, Marceau, P., Tiwari, S., Tchernof, A. & Cianflone, K. Downregulation of C3 and C3aR in Subcutaneous Adipose Tissue in Obese Women.. *PLoS One*, **9,** e95478 (2014).
- Rezvani, R., Gupta, A., Smith, J., **Poursharifi, P.**, Marceau, P., Perusse, L., Bouchard, C., Tchernof, A. & Cianflone, K. Ovarian Hormone Association with ASP and Related Receptor Gene Expression in Adipose Tissue Depots in Women. [Submitted] (2014).

CHAPTER 1

Introduction

1.1 Obesity and the metabolic syndrome – a growing epidemic

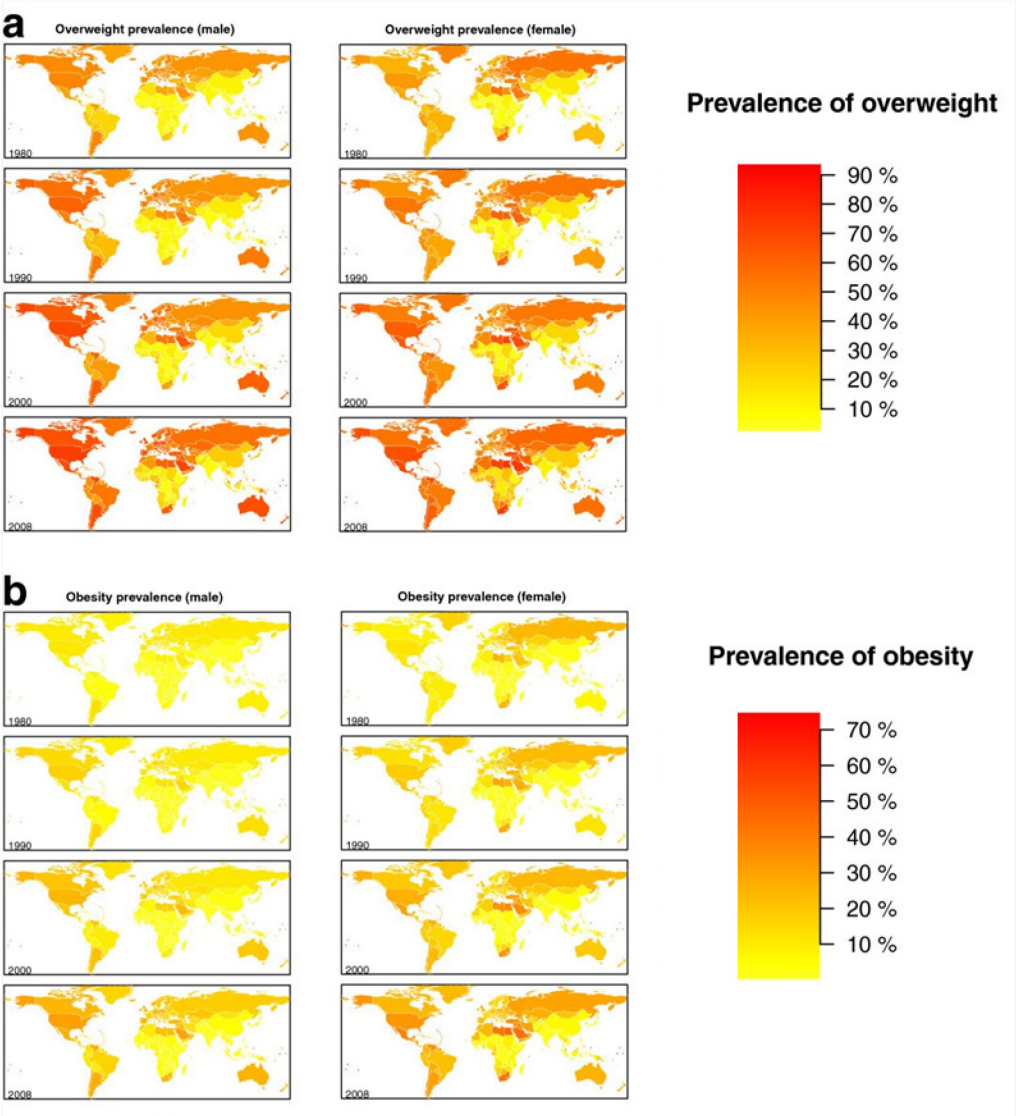
1.1.1 Definition and classification

Obesity is defined by the world health organization (WHO) as an "abnormal or excessive fat accumulation that may impair health" [1]. It is a rapidly growing threat to the global health and is recognized as one of the "greatest public health challenges of the twenty-first century" [1]. Obesity is associated with various comorbidities and a cluster of disorders termed "metabolic syndrome", including abdominal adiposity, dyslipidemia, hypertension, and glucose intolerance [2].

The classification of the weight status by body mass index (BMI) measurement enables clinicians to stratify an individual's health-risk and further to modify the level of intervention accordingly [3]. BMI has been frequently used as the most efficient clinical assessment of obesity and is calculated as weight (kilograms) divided by height (meters squared) [4]. Waist circumference on the other hand is a simple yet powerful index of body fat distribution [5]. Increasing BMI and waist circumference have been associated with an increasing risk of mortality from cardiovascular disease and all other causes [6]. According to WHO, obesity is classified as a BMI $\geq$30 kg/m^2, with further sub-classifications as follows; class I (BMI: 30-34.9 kg/m^2), class II (BMI: 35- 39.9 kg/m^2), and class III (BMI $\geq$40 kg/m^2) [7]. Individuals with BMI between 18.5 and 24.9 kg/m2 are classified as normal weight, while those with BMI 25-29.9 kg/m^2 as overweight [7].

1.1.2 Facts and numbers

During recent decades, the incidence of obesity has increased drastically among children and adults worldwide and is now considered to have reached pandemic proportions [8]. As presented in FIGURE 1.1, between 1980 and 2008, the prevalence of overweight and obesity has increased worldwide [9].



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POPULATION HEALTH METRICS

Stevens *et al. Population Health Metrics* 2012 10:22

FIGURE 1.1 – Prevalence of obesity and overweight from 1980-2008, for men and women.

In 2008, more than 1.4 billion adults were overweight and more than 500 million were obese [1]. WHO predicts there will be 2.3 billion overweight adults by 2015 and more than 700 million of them will be obese across the world. In addition, overweight and obese states are linked to more deaths worldwide than an underweight status; as proposed by WHO, overweight and obesity together are the fifth major risk factor of global death [1]. Furthermore, 44% of diabetes, 23% of ischemic heart disease and between 7% and 41% of various cancers have been attributed to obesity [1]. Notably, major efforts in current research are directed towards understanding the origins of obesity by studying the molecular mechanisms underlying the adiposity-related pathologies including type 2 diabetes (T2DM), hypertension, and atherogenic dyslipidemia.

1.2 Adipose tissue: A major player in metabolism

It is acknowledged that our ancestors needed stored fat in adipose tissue for survival during cold winters and in periods of food shortage [10]. However, in the modern world, with sedentary lifestyles and the abundant availability of food, excessive fat deposition contributes to various metabolic disorders [2,11]. During the last two decades, results from both basic and clinical studies have changed the scientific perspective of adipose tissue physiology [10]. Once adipose tissue was solely known as a reservoir of energy for storage and release of triglycerides (TG) [12], although it is now described as a complex multifunctional compartment, similar to an organ [13]. Novel data presented by Cook *et al.* in 1987 [14] and the discovery of leptin from the Friedman laboratory in the early 90s [15,16], has altered the dogma regarding adipose tissue biology by proposing a broader physiological role for fat depots in energy homeostasis regulation. Currently, it is well-accepted that a wide range of peptides and hormones secreted from fat depots are regulating diverse physiological functions, including immune responses, blood pressure control, bone mass, thyroid function, and reproductive function [10,17,18]. These novel aspects of adipose tissue biology have opened new possibilities to combat adiposity-related metabolic health problems, such as T2DM and atherosclerosis [19,20].

1.2.1 Trafficking of nutrients in the adipose tissue

It is well-appreciated that fat depots are more than just passive repositories for excess energy; even processing of metabolites (fatty acid and hexose uptake, storage into TG, and efflux of fatty acids) has been shown to be highly regulated [21]. Fatty acids derived from dietary chylomicrons, that are not immediately oxidized, are stored primarily in adipose tissue as TG. In turn, during prolonged fasting, adipose tissue TG lipolysis supplies free fatty acids (FFAs) as fuel to tissues/organs (such as muscle) that can oxidize fatty acids [22]. Catecholamines have been shown to promote lipolysis, whereas insulin is known to be a potent anti-lipolytic hormone [23].

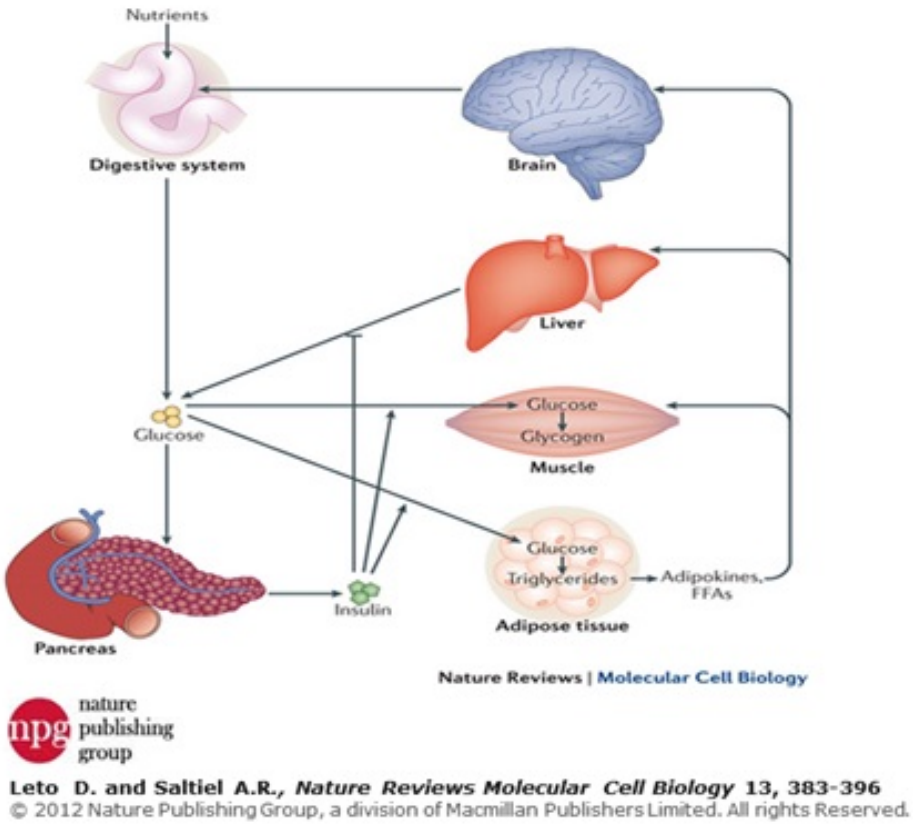


FIGURE 1.2 – Energy homeostasis depends on the communication between multiple organs.

Under normal dietary conditions, insulin regulates activity of hormone-sensitive lipase (HSL), reducing lipolysis, which in turn limits the supply of FFAs into the circulation. However, persistent positive energy balance, which is associated with increased storage of TG and increased adiposity, results in insulin-signaling disruption. Adipose tissue insulin resistance further leads to an increased supply of fatty acids in the circulation, potentiating ectopic fat deposition in liver and muscle tissues, where fatty acids will compete with glucose as a source of energy [24–26]. Maintenance of energy homeostasis due to energy demands and fuel availability rely on the interplay of multiple tissues (the digestive system, brain, pancreas, liver, muscle, and adipose tissue), through the release of metabolites and various hormones (FIGURE 1.2). Thus, insensitivity to the energy status results in disturbances of the glucose-fatty acid cycle, increases the availability of FFAs and their oxidation, reduces the utilization of glucose, and further exacerbates insulin resistance and hyperglycaemia [24–27].

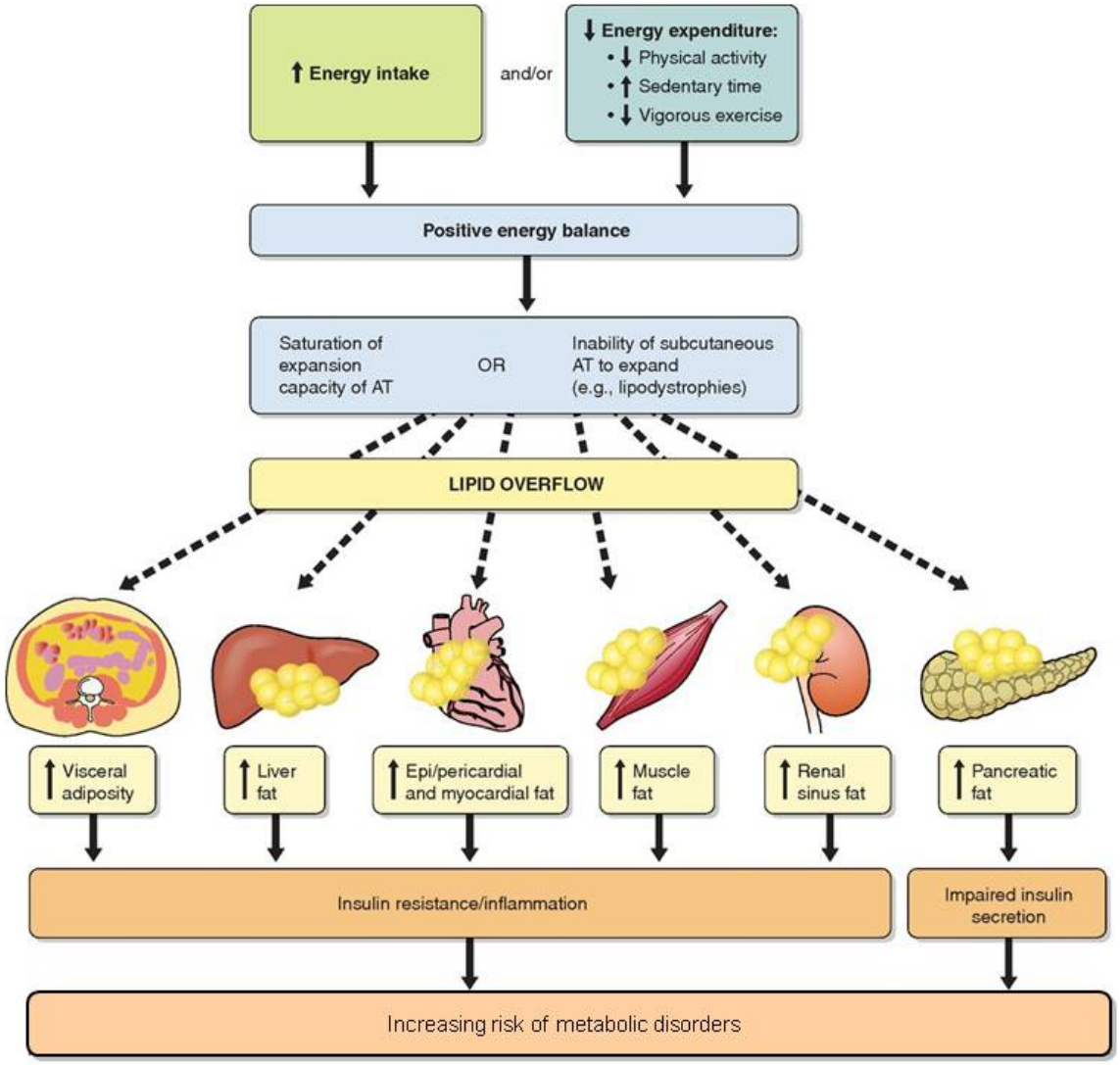
1.2.2 Adipose tissue distribution

Adipose tissue in mammals can be divided into white and brown adipose tissue (WAT and BAT, respectively), which are characterized by different anatomical locations, morphological structures, function, and regulation [28]. Both WAT and BAT are capable of storing energy as TG, although the main function of BAT is heat generation through expression of uncoupling-protein 1 [29,30]. Brown fat cells are characterized by an increased number of mitochondria and multiple cytoplasmic lipid droplets with a central nucleus, while spherical-shaped white adipocytes contain a single large lipid core, with shifting of the nucleus and organelles to the cellular periphery [10]. In humans, BAT is present in fetuses and newborns at axillary, cervical, perirenal, and periadrenal regions, but soon after birth BAT begins to diminish and is interspersed into the common white fat pads in adults [31]. The scattered brown fat cells within WAT, inducible by cold or hormones [31–33], have interchangeably been termed “beige” or “brite” [34]. On the other hand, human WAT represents the majority of body fat and, depending on the anatomical site, is termed either as subcutaneous or visceral (omental) adipose depot. As the terms imply, subcutaneous

adipose tissue is found under the skin layer throughout the body, whereas the omentum, intestines, and perirenal areas are surrounded by visceral or internal adipose tissue [35].

Central obesity, also referred to as either android or male-type obesity, is identified as an increase in intra-abdominal fat, particularly abdominal visceral and subcutaneous adipose tissue, resulting in enhanced waist size. The term gynoid obesity or lower-body obesity is characterized as preferential adipose tissue accumulation in the hips, thighs, and lower trunk, typically described as female obesity [36,37]. In the mid-1900s, the importance of upper-body obesity in conferring excess mortality was first proposed based on clinical observations [36,37]. This theory was later confirmed by Kissebah *et al.*, suggesting that the topography of adipose tissue contributes to metabolic complications (insulin resistance, hypertension, and dyslipidemia) [38]. Since then, numerous reports in the literature have reemphasized the notion put forward in 1947 by Vague [36], that obesity is not a homogeneous condition and that the pathological effects of excessive adiposity are associated not only with the quantity of fat, but also with the fat mass distribution [5,39–41].

Increased omental fat accumulation shows strong associations with an increased pool of circulating inflammatory adipokines and higher adipose lipolytic activity contributing to metabolic complications such as dyslipidemia, insulin resistance, T2DM, and increased risk of metabolic syndrome [6,42]. Studies have also addressed the health hazards of excess abdominal subcutaneous fat storage [43–45], however it is generally admitted that excess omental adiposity remains a more reliable predictor of metabolic disorders [5]. FIGURE 1.3 presents a proposed scenario explaining the link between omental adiposity and metabolic disorders [5]. Under this model, excess omental adiposity may be an indicator of disrupted subcutaneous fat storage in an energy surplus environment. Accordingly, fat accumulation, particularly in the peripheral subcutaneous depot could suppress daily lipid flux by increasing TG clearance [22]. Further, under excess caloric conditions, the reduced subcutaneous depot expandability results in elevated circulating FFAs and further leads to lipid drainage into the portal vein and, consequently, liver, which, with the secretion of pro-inflammatory adipokines, could further promote insulin resistance, inflammation, and ectopic fat deposition at undesired sites (FIGURE 1.3) [5,46].



Adapted from:

Physiological Reviews

Tchernof A. and Després J., *Physiol Rev* 93, 359-404

FIGURE 1.3 – Proposed mechanisms linking omental adiposity with metabolic complications.

1.2.3 Adipose tissue: An endocrine organ

Since the discovery of leptin [16], WAT has been considered an endocrine organ and an important source of bioactive peptides and proteins, collectively termed "adipokines", with local and/or systemic function [47–49]. Adipokines play multiple roles in metabolism and

energy balance, satiety, immunity, and also inflammation as they are fundamental to the pathogenesis of obesity-related disorders and metabolic complications [10,50] (TABLE 1.1).

TABLE 1.1 – Adipose tissue productions and secretions

Lipid and lipoprotein metabolism
Lipoprotein lipase
ASP
PGE_2, $PGF_{2\alpha}$, and PGI_2
Cholesterol ester transport protein
Retinol-binding protein-4
Food intake and activation of sympathetic nervous system
Leptin
Metabolism and energy homeostasis
Leptin, adiponectin, IL-6, IL-8, and resistin
Retinol-binding protein-4
Vessels and angiogenesis
VEGF and thrombopoietin
Monobutyrin
Leptin and apelin
Angiopoietin-2, angiotensinogen/angiotensin-2, adrenomedullin
Metabolism of extracellular matrix
Type VI collagen
Plasminogen activator inhibitor-1
Metalloproteases (gelatinases MMP-2 and MMP-9)
Immune system, acute-phase reactants, and inflammation
MCP-1, TNF-α, IL-1β, IL-6, IL-8, and IL-10
Adipsin and factors C3, B, and D of alternate complement system
α_1-Acid glycoprotein
Serum amyloid A3
Haptoglobin, pentraxin-3, and lipocalin 24p3
Metallothionein
Cathepsin S and L
Insulin sensitivity of muscle, hepatocyte, and adipocyte
Adipsin/ASP
Leptin, adiponectin, resistin, visfatin, omentin-1, vaspin, IL-6, and apelin
Growth factors influencing adipose tissue development
Insulin growth factor I
Nerve growth factor
VEGF and thrombopoietin

Adapted from:

Over 100 adipokines have been identified [51], many of which are secreted by adipocytes, such as leptin and adiponectin. However, particularly in obesity, additional adipose tissue cells can contribute to this secretome, such as immune cells present in the stromal vascular fraction (SVF), which contribute to the fat tissue-derived inflammatory cytokine production (eg, interleukin (IL)-6, and tumor necrosis factor (TNF)-α) [52,53]. Obesity, which is associated with increased macrophage infiltration in adipose tissue, could induce an imbalance in adipokine production. This has been shown to contribute to chronic low-grade inflammation and altered glucose and lipid metabolism [52]. Due to the sheer number of adipokines and their broad array of physiological effects, only a few of the many adipokines that are associated with adiposity and metabolic disorders will be briefly explored.

Leptin, a major role player in satiety and energy homeostasis [54], was the first obesity-related adipokine discovered and still remains the best studied [15,55,56]. This multifunctional protein, secreted primarily by adipocytes, functions by binding to its ubiquitously expressed receptor [57]. Of note, the highest levels of leptin receptor are found in hypothalamic regions known to play critical roles in regulating energy intake and body weight [57]. Leptin appears to be associated with total adiposity rather than with omental adiposity [58,59]. Moreover, its secretion was found to be higher in subcutaneous than omental adipocytes [60].

Another important adiposity-related adipokine is adiponectin, which exerts potent anti-inflammatory and insulin-sensitizing effects by binding to multiple receptors [61]. Interestingly, adiponectin circulating concentrations are extremely high, comprising approximately 0.01% of plasma proteins [62]. Adiponectin levels, unlike other adipokines, are inversely proportional to body fat content [63]. Its plasma levels have been reported to be reduced in obesity, particularly in subjects with visceral obesity [62,63]. Consistently, omental adiponectin release has been shown to be reduced markedly in viscerally obese women, compared to subcutaneous adipose tissue secretion [64,65]. Therefore, adiponectin circulating levels are proposed to be a predictable indicator for adiposity-associated metabolic abnormalities [61].

Additional factors such as TNF-α, plasminogen activator inhibitor-1, and IL-6 have all been identified as major metabolic role players in the regulation of inflammation and insulin sensitivity [50,66,67]. While the concept of adipocytokines has now been well-accepted, the pace of discovery of novel adipokines is rapidly growing. To the established list of adipokines there are additional, less well-described, but potentially important hormones, such as visfatin, omentin, vaspin, and others now being documented [49]. Hence, it remains to be seen how these new findings will transform the perspective of metabolic diseases and their related clinical manipulations.

1.2.4 Adipose tissue composition

Due to the dynamic characteristic of fat tissue, with various environmental and dietary stimuli, there will be an alteration in adipose tissue mass, as well as its cellular composition [35,68,69]. In addition to adipocytes, the most prominent cells, WAT also consists of a heterogeneous mixture of cells, collectively referred to as SVF, which includes pre-adipocytes, endothelial cells, monocytes/macrophages, and multipotent stem cells [35,70]. A continuous positive energy balance will initially induce hypertrophic adipocytes, which may also trigger a hyperplasia phenomenon (proliferation/differentiation of pre-adipocytes) [71,72]. WAT expansion during weight gain is also accompanied by dynamic changes in immune cell populations, mostly macrophages that are predominantly encircling necrotic adipocytes, forming crown-like structures within obese fat depots (FIGURE 1.4) [53,69].

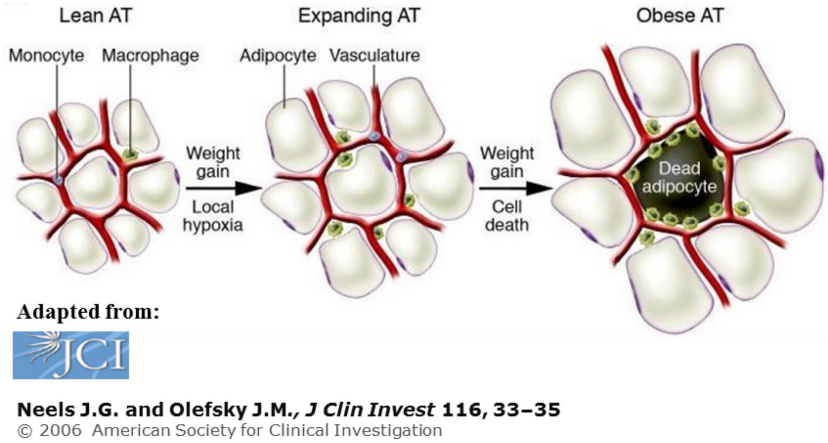


FIGURE 1.4 – Adipose tissue expansion.

Activation of adipose tissue macrophages (ATM) leads to the release of a variety of substances, which in turn recruit more macrophages, that further increases ATM content and aggravates the inflammatory state (FIGURE 1.5) [73]. Two different phenotypes for ATMs have been recognized: M1 macrophages referred to as pro-inflammatory or classically activated macrophages, and M2, alternatively activated macrophages which are primarily anti-inflammatory agents. Activated M1 macrophages secrete pro-inflammatory cytokines which exert paracrine effects to regulate inflammation and insulin sensitivity of the metabolic target tissues [73,74].

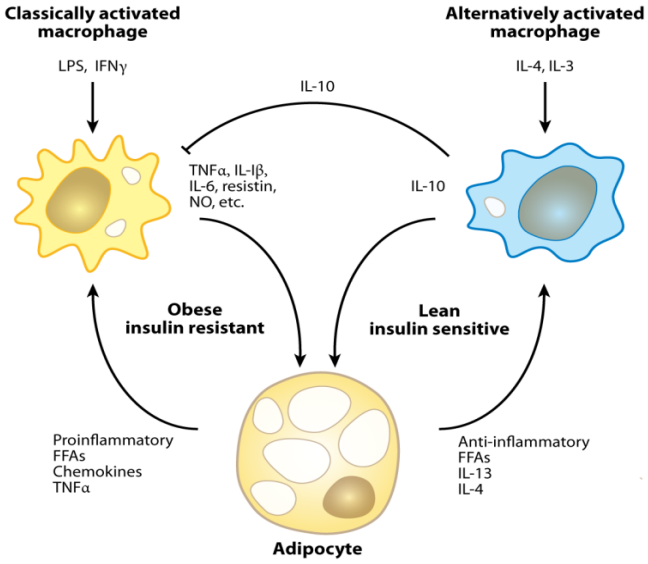


FIGURE 1.5 – Macrophage polarity and insulin resistance in adipose tissue.

In addition to the known crosstalk between macrophages and adipocytes within the obese fat depot, other immune cell types, particularly lymphocytes, also appear to participate in this network. Certain sub-populations of T cells have been proposed as the initiator of adipose tissue inflammation by regulating macrophage inflammatory responses [75,76]. Specifically, interferon gamma (IFNγ)-secreting T-cell helper (TH)-1 cells have been shown to enhance M1 macrophage inflammatory activation state and macrophage recruitment into fat tissue; conversely, anti-inflammatory IL-4 and IL-13-secreting TH-2

cells have been linked to macrophage differentiation into an anti-inflammatory M2 subset [77,78]. While present at lower levels than macrophages and T-cells, fat-resident neutrophils [79], dendritic cells [80], and mast cells [81] have been observed in obese animal models, although their pathophysiological relevance in human adipose tissue inflammation is less well explored.

Ultimately, high fat diet, expansion of fat mass, and dysregulated lipolysis, all lead to the recruitment of both innate and adaptive immune-derived cells with manipulation of various immune pathways [82]. This intersection of metabolic dysfunction and immunity, termed "immunometabolism" [82–84], is complex and continues to reveal sometimes unexpected interactions, but remains a novel, promising research area, which could offer new therapeutic targets for metabolic abnormalities. The immunometabolism and adiposity-associated inflammation features are discussed in the subsequent section.

1.3 Immunometabolism

It is now widely agreed that obesity, as with other chronic metabolic disorders, constitutes a state of low-grade chronic inflammation [11]. Multiple insulin target organs, adipose tissue featuring prominently among them, are affected by chronic inflammation with physiological alterations in nutrient metabolism, insulin sensitivity, and also inflammatory molecule production [73]. In the last few years, metabolism and immunity aspects have merged to become a novel research area termed immunometabolism, which better captures the pathogenesis of metabolic alterations that accompany obesity. This concept was initially proposed based on the discovery of increased levels of circulating inflammatory substances in obese individuals, such as C-reactive protein [85] and TNF-α [86]. Some of the components of the immune response which are implicated in the crosstalk between the immune system and fat metabolism are explained below.

The most notable example of immune impact on adipose tissue pathophysiology is the fat-resident immune cell populations. Beyond the classical adipocyte-derived pro-inflammatory molecules, the infiltrated immune cells are also considered as another source of cytokine secretion, which adds to the systematic inflammatory process and consequently

contributes to the metabolic alterations in obesity [74]. An imbalance in inflammatory agents further stimulate pro-inflammatory signaling pathways such as mitogen-activated protein kinase (MAPK), target of rapamycin (TOR), phosphoinositide 3-kinase (PI3K) and Jun N-terminal kinase (JNK), which in turn cause serine phosphorylation of insulin receptor substrate (IRS)-1 and eventually induce insulin resistance [87–90].

Another prominent example is the interaction between toll- like receptor (TLR)-4 and lipid metabolism, based on direct interaction of TLR with FFAs or the fatty acid carrier, fetuin-A [91,92]. Direct binding of FFAs to TLRs activates crucial inflammatory pathways, such as JNK and IκB/nuclear factor κ-light-chain-enhancer of activated B cells (NFκB), which further promotes adipose tissue inflammation and insulin resistance in diet-induced obesity [91–93].

Endoplasmic reticulum (ER) dysfunction as a result of chronic metabolic disease is another feature of immunometabolism, as it is linked to both metabolic and immune regulation. The ER is a cholesterol and nutrient sensor organelle that controls protein synthesis and folding [94]. Excessive adipocyte hypertrophy and the associated hypoxic condition disturbs ER homeostasis through metabolite surplus, which in turn activates the unfolded protein response and finally results in ER stress [11]. Stimulated ER stress leads to impaired insulin signaling and inflammation through phosphorylation of key inflammatory pathways in adipocytes, such as JNK and NFκB [95].

Hence, identifying immunometabolic pathways linking metabolism and immunity, specifically under conditions of nutrient excess, adiposity, and homeostasis dysregulation, is crucial to understand and control these metabolic pathologies. However, to date, relatively few features of immunometabolism have been described in the literature. For example, the immunometabolic aspect of the immune complement system and the interactions/interconnections with adipose tissue pathophysiology have not been well-explored [96,97]. This is of particular relevance, since early activation of the complement system is recognized as a crucial feature of immune response elsewhere in the body, and is a key initiating factor for immune cell infiltration and response [98,99]. The following sections

focus on the activation of different complement pathways, as well as specific complement components (proteins and receptors) and their pathophysiology and signaling outcomes.

1.4 Complement system

The complement system is a ubiquitous proteolytic cascade, constituting a vital component of innate immunity, and mediates various major immune responses as well as metabolic functions. The most well-known function of this humoral system, which consists of a cascade of proteases and soluble proteins, is in innate immunity and pathogen killing [100]. The series of proteolytic pathways is initiated by pathogenic recognition, lead to generation of anaphylatoxins (C3a, C4a, and C5a), and finally opsonization and the assembly of the membrane attack complex (MAC) (FIGURE 1.6) [101].

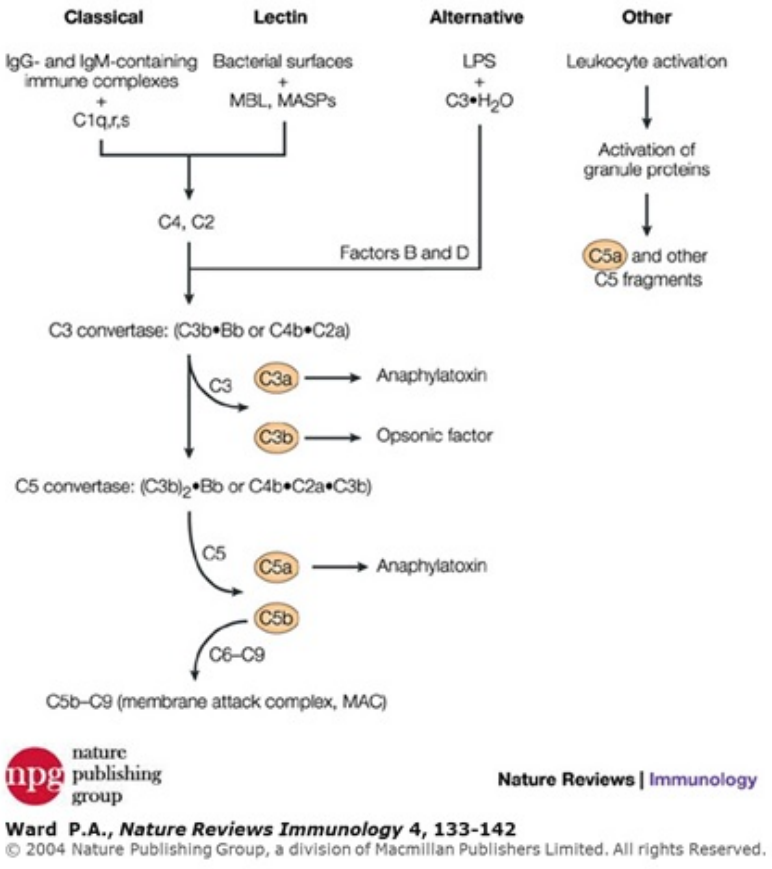


FIGURE 1.6 – The complement activation pathways.

There are three complement activation pathways: the classical, alternative, and lectin pathways (FIGURE 1.6) [100,101]. Initiation of the classical pathway occurs when C1q, in association with C1r and C1s (the C1 complex), binds to the Fc portion of IgG or IgM attached to pathogenic surfaces, followed by sequential cleavage of C4 and C2 to produce C3 convertase (C4bC2a), which further cleaves C3 into the anaphylatoxin C3a and the opsonin C3b and the formation of C5 convertase, which together with C3 convertase, are the central reactions in the complement pathways. C3b is involved in opsonization, while C3a and C5a are important anaphylatoxins mediating chemotaxis and inflammation. Further complement activation on and around the opsonized area through C3b binding to the microbial surface, continues with C5b and C6-C9, leading to assembly of the ultimate lysing pathogen construct (the MAC) [100,101].

The lectin pathway is immunoglobulin-independent but shares similarities to the classical pathway. The lectin cascade starting point is the recognition of non-self carbohydrate residues on the cell surfaces of microbes by mannose binding lectin (MBL) and ficolin, which activates the MBL-associated serine proteases to cleave C4 and C2 to form the C3 convertase (C4bC2a). MBL can bind to common carbohydrates on Gram-positive and Gram-negative bacteria, yeast, as well as on some viruses and parasites [100,101].

The alternative pathway is mechanistically different from the classical and lectin pathways (FIGURE 1.6) and is dependent on the continual low-level turnover of C3. It is initiated through spontaneous hydrolysis of C3 to C3(H2O) that allows the binding of plasma protein factor B (fB), which is then cleaved by factor D (FD) to Ba and Bb. The alternative pathway C3 convertase consists of Bb and C3b factors, forming an unstable compound (C3(H2O)Bb) that is stabilized by properdin, which amplifies the alternative cascade [102,103]. Because of the amplification potential of the complement system, several points within the cascade are tightly controlled by the regulatory proteins and inhibitors to maintain homeostatic balance and avoid inappropriate or exaggerated destructive responses [104,105].

Some studies have suggested additional mechanisms for the initiation of complement activation, differing from these three mentioned pathways. For example, it has been proposed that the activated neutral proteases, such as kallikrein, thrombin, or phagocyte-

derived proteases, can also cleave C3 or C5 [106]. Another mechanism involves properdin, the C3 convertase stabilizer, which has shown to be capable of *de novo* C3 convertase formation on the pathogen surface [107]. Taken together, this network of proteins can efficiently and rapidly recognize potential threats and response appropriately through different pathways, only if regulated effectively; otherwise the uncontrolled production of anaphylatoxins (particularly C5a) can lead to inflammatory pathologies, such as sepsis and inflammatory arthritis [108].

1.4.1 The complement system beyond pathogen killing: Immunometabolism aspects

Recently, the traditional view of complement as a potent arm of innate immunity and host defense has been broadened to a multilayer system with influences on tissue homeostasis, degeneration, and regeneration [109–111]. Interestingly, the complement components have been suggested to contribute to dysfunction of the metabolic endocrine organs (such as adipose tissue, liver, and pancreas), and, as a result, to associate with the development of the related-pathologies [99]. The first indications of potential adipose tissue and complement involvement were demonstrated several decades ago based on disease profiles: acquired lipodystrophy was found to parallel with hypocomplementemia [112], and familial C3 deficiency was associated with partial lipodystrophy [113]. Novel perspectives of this adipose tissue - complement system relationship are explained below.

Adipsin, a product of adipocytes, influences adipocyte differentiation [114], while also playing a potent role in innate immunity and the alternative complement pathway, in its alternate guise as factor D [115]. Besides adipsin (fD), other alternative complement components are also expressed in adipose tissue, including C3, fB, properdin, and regulator factors (fH and fI) [115–117]. A recent study has proposed an immunometabolic role for properdin in adipose tissue [118]. Moreover, some publications have hypothesized the involvement of classical complement pathway factors in adipose tissue biology [119,120]. As demonstrated by Peake *et al*., the adipose-tissue derived hormone adiponectin is capable of binding to complement protein C1q, initiating classical complement activity [120]. Furthermore, adipocytes have been shown to express complement factor C1 and its regulator decorin [119]. Interestingly, their expression levels are altered in insulin resistance

and obesity, suggesting a direct role for the complement system in adipose tissue inflammation [119].

Proximal activation of the complement pathway leads to production of C3a; subsequent distal activation leads to production of C5a, both of which interact with their respective receptors (C3aR and C5aR), as well as C5aR-like receptor 2 (C5L2) [121]. The interaction of C3, fB, and adipsin also leads to the formation of C3a. C3a is rapidly converted to $C3a^{desArg}$ (ASP; acylation stimulating protein) via carboxypeptidase cleavage of the C-terminal arginine [122]. Interestingly, all the components required for generation of ASP via the alternative complement pathway are produced in adipocytes and direct production of ASP is measurable [123]. Further, ASP is well-known for its potent anabolic effects on adipocytes, where it positively regulates fat storage and energy metabolism via binding to C5L2 [96,124–126]. In addition, animal knockout models of C3 (ASP precursor) and C5L2 (ASP receptor) share similar phenotypes that are characterized by alterations in lipid metabolism [127,128]. This phenotype is mimicked by treatment of wild-type (WT) mice with neutralizing anti-ASP and anti-C5L2 antibodies [129]. Further, C5a (via its receptors, C5aR and C5L2) has been recently proposed to have supplementary metabolic roles in adipose tissue [130], in addition to its well-known immune roles as a potent stimulator of anaphylaxis and inflammation [108,131–133]. Taken together, the effects of complement components ASP, C5a, and of their receptors are contributing to the immunometabolic aspect of adipose tissue.

1.4.2 ASP

Within the growing list of adipokines, ASP, a 76 amino acid protein, has been recognized as an autocrine/paracrine hormone playing a central role in the metabolism of fat-storing cells [96,134]. The ASP-regulating effects on fat storage are achieved by induction of fatty acid uptake [125] through stimulation of diacylglycerol acyltransferase activity [135], which relieves feedback inhibition by fatty acids on lipoprotein lipase [136] and decreases HSL activity [137]. Enhanced translocation of glucose transporters (GLUT1, GLUT3, and GLUT4) to the cell surface by ASP leads to increased storage of glucose and fatty acid-derived glycerol backbone into TG [124,135]. These effects of ASP are independent and

additive to those of insulin [137] and are transmitted through the only known ASP receptor, C5L2 [126,138]. Notably, the C5L2 role as a functional receptor for ASP is still considered controversial [138–140]. C5L2 (aka GPR77), a 337-amino acid protein, belongs to the large family of G-protein coupled receptors (GPCRs), and has closest homology with another seven-transmembrane receptor, C5aR (aka CD88) [133].

Typically, ligand binding causes conformational changes to the GPCR, leading to the activation of heterotrimeric (α, β, and γ) guanine nucleotide-binding proteins (G proteins). Sequentially, Gα-guanosine diphosphate (GDP) is exchanged for guanosine triphosphate (GTP) and, as a result, the Gβ and γ are dissociated from the complex. This initiates various intracellular signaling pathways, which are ultimately terminated by phosphorylation of the receptor, β-arrestin recruitment, and receptor desensitization/internalization [141].

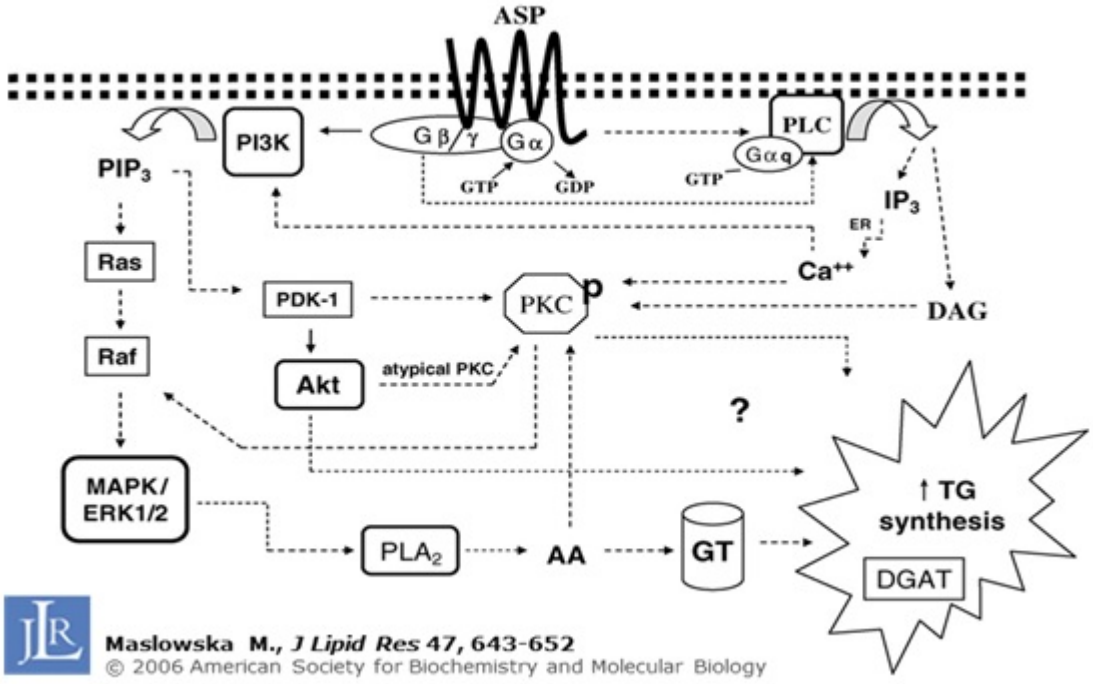


FIGURE 1.7 – Intracellular signaling pathway for ASP-stimulated TG synthesis.

Unlike Gαi-mediated signaling pathways for C5aR [142], C5L2 has been proposed to lack the ability to couple to intracellular G proteins due to an amino acid replacement of arginine by leucine in the DRY motif [132]. In receptors that have this DRY motif, located in the third intracellular loop of GPCRs, its presence is important to enable coupling of G proteins to GPCRs [143]. Accordingly, C5L2 has been suggested to belong to the group of non-signaling decoy receptors, negatively regulating the bioavailability of C5a [132,140]. On the other hand, there are some GPCRs that do not possess this DRY motif, yet still activate intracellular pathways [144]. C5L2 internalization is both ligand-dependent [121] and -independent [140] in transfected cells. Following ASP stimulation, intracellular signaling pathways involved in triglyceride synthesis include sequential activation of PI3K, phospholipase C, protein kinase C, protein kinase B (Akt), and MAPK (FIGURE 1.7) [145]. The ASP-mediated secretion of monocyte chemoattractant protein (MCP)-1 and keratinocyte chemoattractant (KC) in adipocytes is dependent on PI3K and NFκB phosphorylation [146]. Other than ASP, C5L2 also binds C3a with moderate affinity, but has a higher affinity for C5a and especially for $C5a^{desArg}$ [147].

1.4.3 C5a and its receptors

Human C5a is a 15 kDa glycosylated protein and consists of 74 amino acids [148], which is quickly desarginated by carboxypeptidase, forming $C5a^{desArg}$, with attenuated biological activities [149]. C5a is considered to be one of the most potent inflammatory factors, and functions via its receptor C5aR, but also binds C5L2 [121,131–133]. Although, C5a binds C5L2 with the same affinity as to C5aR, the sequential signaling pathways and C5L2-mediated functions are yet controversial [132,147], leading to the initial designation of C5L2 as a decoy receptor for C5a [132]. C5aR has similar tissue and cellular expression patterns to C5L2 [133,150,151], however C5aR mRNA levels are generally higher than C5L2. Both are abundantly expressed on innate immune cells including neutrophils, dendritic cells, macrophages, and lymphocytes, and at lower levels on non-myeloid cells such as adipocytes, hepatocytes, epithelial and endothelial cells [133,150,151]. Circulating C5a and $C5a^{desArg}$ are quickly cleared from the circulation [149], binding to receptors initiated

phosphorylation, β-arrestin protein association, and clathrin-mediated internalization [121,150,152].

The C5a immune activities, including chemotaxis, enzyme/cytokine release, and the respiratory burst, are mostly attributed to its binding to C5aR [132], with subsequent stimulation of MAPK, extracellular signal-regulated kinase (ERK), diacylglycerol and Akt signaling pathways [153]. The C5a-C5aR protective functions are tightly regulated and mostly tissue-localized, although the markedly increased plasma concentrations of C5a, for example in sepsis, are an indicator of an excessive imbalance in inflammatory response [101]. C5a induces a wide variety of responses depending on the cell and tissue type. It is not only a potent component of the innate immune system, [132], but also, as recently proposed, influences adipocyte metabolism with insulin-like effects such as lipogenesis, glucose uptake, and lipolysis [130]. C5a and its receptors, C5aR and C5L2, are also expressed in the central nervous system [154], and central administration of C5a induces food intake [155].

C5aR homodimerization as well as heterodimerization with the CCR5 chemokine receptor have been reported, and this is associated with C5a-induced co-internalization of the receptors [156,157]. These data contributed to my hypothesis that C5aR and C5L2 might be capable of heterodimerization, and thus might answer some unresolved questions regarding C5L2 function.

1.5 Animal models

The complement system provides a potential safeguard against pathogens. This conclusion has developed from many studies involving gene-knockout in mice that lack key complement components such as C2, C3, C4, and C5. These animal models result in serious susceptibility to infection and have clarified the pivotal role of complement factors in host defense [158,159]. Unlike immune-related roles, the potential for a metabolic role of the complement system is a newer perspective, and investigations using *in vitro* and *in vivo* methodologies are providing information.

1.5.1 ASP knockout (C3KO) mice

C3KO mice are obligatorily ASP deficient, as C3 is the direct precursor of ASP. These mice have altered energy metabolism and fat storage [128,160]. C3KO mice, on either a low fat or high fat diet, were leaner with reduced adipose tissue mass, delayed lipid clearance, and decreased leptin levels in comparison with WT animals [161]. In spite of being obesity resistant, ASP-deficient mice have an enhanced food consumption, which is over-compensated by augmented energy expenditure [162]. The imbalance between energy intake and energy expenditure is unrelated to BAT thermogenic function or physical activity, but is linked to increased basal metabolism, localized, at least in part, to elevated muscle fatty acid metabolism [160,162]. Of note, exogenous ASP injection in C3KO mice restores a normal TG-nonesterified fatty acid (NEFA) clearance pattern, as well as fat partitioning, demonstrating that this phenotype is due to absence of ASP (and not C3) [162]. Absence of the ASP pathway in mice highlights its importance on fatty acid metabolism, and consistently show a metabolic phenotype that is similar to C5L2 (ASP receptor) deficient mice [127].

1.5.2 C5L2KO mice

ASP deficient and C5L2KO mice exhibit similar phenotypes, defined by decreased fat storage despite increased energy intake, along with enhanced energy expenditure and delayed postprandial TG and NEFA clearance [127,128]. However the phenotypes are not identical; whole body weight and adipose tissue mass in C5L2KO were not different from WT models [127,160,163]. Based on earlier evidence, the disruption of the ASP-C5L2 signaling pathway was proposed to be metabolically beneficial, inducing energy usage over storage, and leading to a leaner phenotype [96]. However, some of the characteristics including delayed postprandial fat clearance, increased food consumption, and lipid redistribution may potentially lead to a proinflammatory state, and potential for ectopic fat deposition. This has been recently confirmed with mice treated with a diabetogenic diet, demonstrating that C5L2 absence exacerbates the development of insulin resistance and inflammation [164]. Further, additional studies on C5L2KO emphasized a pathophysiological role for C5L2 in onset of inflammation, inducing an insulin resistant state, imbalanced lipid metabolism, and also sepsis [165–167].

1.5.3 C5aRKO mice

Although C5aR appears to have a well-defined immune-related role, its potential immunometabolic role in energy expenditure and fat storage is only beginning to be considered [130,168]. This novel facet of C5aR is based on *in vivo* studies using C5aRKO mice [168] and a C5aR-selective antagonist [130]. C5aRKO mice are characterized, regardless of the diet, with decreased fat mass, lower plasma lipids, higher energy expenditure, and reduced fat storage [168]. However, the glucose clearance and insulin sensitivity of C5aRKO mice, has shown to be almost comparable to the WT model [168]. Further, the well-known C5aR-selective antagonist was shown to interfere with C5a-mediated fatty acid uptake, a novel function for C5a [130]. Although these whole-body metabolic features of C5aRKO have only been described recently, the related inflammatory and metabolic signaling pathways in adipocytes remained to be evaluated.

1.6 Clinical aspects

Normal circulating ASP concentration averages 20-30 nmol/L [169]. Plasma levels of ASP are altered in response to various pathophysiological conditions, including obesity, T2DM, dyslipidemia and cardiovascular disease, with reduction with exercise or weight loss [96,170]. It has been suggested that adiposity-associated complications, such as increased FFA and hyperinsulinemia, in addition to an imbalance in adipose tissue lipogenesis/lipolysis and adipokine production, could contribute to the augmentation of plasma ASP [96,171,172]. C5L2 levels have been recently reported to contribute to various inflammatory pathologies including insulin resistance, asthma, and coronary artery disease [173,174]. Despite these findings, many aspects on the role of C5L2 in acute and chronic inflammation are highly debated topics, including whether it attenuates or propagates inflammation, varies between disease states, and even between cell types in the same disease.

Although normal activation of the C5a-C5aR pathway is often beneficial against pathogenic threats, its excessive activation can lead to negative consequences [101,153]. C5a levels are increased in various pathologies, including sepsis [175], rheumatoid arthritis [176],

asthma and allergy [177,178], inflammatory bowel disease [179], and atherosclerosis [180]. Notwithstanding, most of these studies have only focused on C5a-C5aR interaction without particular investigation of the possible role for the other C5a receptor (C5L2).

1.7 Rationale

Despite many years of intensive investigations on complement, especially related to immune function, there are other aspects that have remained unexplored. It is now accepted that anaphylatoxins and their receptors have a broader range of functions than previously appreciated, both inside and outside of the immune system. This is particularly true of the potential metabolic aspects of ASP, C5a, and their receptors C5L2 and C5aR, which are controversial or unexplored:

(i) Evidence of ASP-C5L2 binding has been shown with various methods in endogenously expressing as well as stably transfected cells [125,147], yet this association is contested and controversial [166,181]. (ii) The physiological role of C5L2 is still debatable with two conflicting proposals: the first hypothesis describes an anti-inflammatory role for C5L2 in the immune system [139,150], whereas a second theory provides evidence for its proinflammatory role, triggering release of inflammatory cytokines, such as the damage-associated high mobility group box protein 1 (HMGB1) [182]. (iii) C5aR and C5L2 are endogenously expressed in similar cells and tissues, share common ligands, and recently have been proposed to have closely linked interactions [150]. However, a specific physical interaction, possibly via heterodimerization, and its functional importance, has not been studied yet. (iv) The proposed interaction of C5aR with C5L2, where C5L2 functionality depends on the presence of C5aR and *vice-versa,* contrasts with the decoy receptor hypothesis of C5L2 function. (v) The novel role of C5a–C5aR axis in WAT inflammation and the corresponding signaling pathways has not been addressed so far. (vi) Finally, the distinctive importance of C5aR and C5L2 in the context of obesity is presently clouded by controversy.

Accordingly, the aims of this thesis were to evaluate C5aR and C5L2 interaction, addressing different aspects of physical, functional and pathophysiological cooperation.

This has been addressed using transfected cells, 3T3-L1 adipocytes and J774 macrophages cells, primary cells from mice adipose tissue, as well as, human adipose tissue explants, as presented in three different studies in CHAPTERS 2-4.

CHAPTER 2: This chapter is a comparative study between 3T3-L1 adipocytes and J774 macrophages, intended to address the direct ASP/C5L2 interaction, C5L2 and C5aR interaction, in addition to the cell or ligand specificity of C5L2/C5aR interaction and subsequent signaling.

CHAPTER 3: This second paper was designed to investigate the C5a-C5aR functions and associated inflammatory signaling pathways and their possible regulatory effects on ASP-mediated activities and signaling pathways in primary adipocytes using C5aR deficient mice and *in vitro* studies.

CHAPTER 4: This third study is a large clinical study performed in adult women over a wide range of body size (non-obese to severely obese), to evaluate the expression of C5aR and C5L2 in both subcutaneous and omental adipose tissues and their association with obesity metabolic factors.

CHAPTER 2

C5L2 and C5aR Interaction in Adipocytes and Macrophages: Insights into Adipoimmunology

Poursharifi, P. *et al.* C5L2 and C5aR Interaction in Adipocytes and Macrophages: Insights into Adipoimmunology. *Cell. Signal.* **25,** 910–8 (2013).

Résumé

Depuis quelques années, on reconnait que l'obésité est accompagnée d'un état proinflammatoire et qu'il y a migration de macrophages vers le tissu adipeux. Les récepteurs membranaires C5L2 et C5aR, présentant une forte homologie, sont tous deux présents sur les adipocytes et macrophages et lient respectivement l'ASP (protéine stimulant l'acylation, dérivée du facteur C3 du complément et ayant un effet sur la synthèse des triglycérides) ainsi que le C5a (protéine du complément impliquée dans l'immunité innée). Notre objectif est donc d'évaluer les interactions entre ces deux récepteurs suite à la liaison avec leurs ligands respectifs chez les adipocytes et les macrophages en culture.

Les niveaux d'expression des récepteurs à la surface cellulaire furent analysés par cytométrie de flux (FACS). La localisation cellulaire des récepteurs (en surface ou intracellulaire) fut déterminée par immunofluorescence. La technique BRET (bioluminescence par transfert d'énergie de résonance) a permis l'évaluation de la dimérisation des récepteurs.

Lors de la différenciation des pré-adipocytes en adipocytes matures, il y a augmentation des niveaux d'expression de C5L2 et C5aR en surface (de 9 et 25 fois, respectivement). Les deux récepteurs sont aussi fortement exprimés chez les macrophages J774. En microscopie confocale, nous avons pu observer chez les adipocytes non-stimulés une distribution cellulaire diffuse (cytosol et membrane plasmique) des 2 types de récepteurs. Suite à une stimulation par l'ASP, il y a internalisation du C5L2 (30 minutes) et une augmentation de la colocalisation avec C5aR, avec un retour à la distribution de base après 90 minutes. Chez les macrophages non-stimulés, on observe plutôt une colocalisation des récepteurs de façon diffuse dans les cellules. Suite à une stimulation par C5a ou par l'ASP, une partie des récepteurs C5aR se déplacent vers la membrane plasmique (sans colocalisation avec C5L2). Les résultats du BRET démontrent une homodimérisation des récepteurs C5L2 et C5aR ainsi qu'une hétérodimérisation C5L2/C5aR.

L'ASP et le C5a stimulent tous les deux la colocalisation des récepteurs C5L2 et C5aR. Ce profil est cependant différent dépendamment du type de cellule (adipocytes ou macrophages) et pourrait être lié aux fonctions métaboliques divergentes de ces deux

lignées cellulaires. La suite de ces études permettra de mieux définir les rôles de la voie de signalisation ASP-C5L2 dans le tissu adipeux ainsi que de valider le choix du récepteur C5L2 en tant que cible thérapeutique pour le traitement de l'obésité et ses co-morbidités.

2.1 Abstract

Obesity is associated with inflammation characterized by increased infiltration of macrophages into adipose tissue. C5aR-like receptor 2 (C5L2) has been identified as a receptor for acylation-stimulating protein (ASP) and the inflammatory factor C5a, which also binds C5aR. The present study examines the effects of ligands ASP and C5a on interactions between the receptors C5L2 and C5aR in 3T3-L1 adipocytes and J774 macrophages.

BRET experiments indicate that C5L2 and C5aR form homo- and heterodimers in transfected HEK 293 cells, which were stable in the presence of ligand. Cell surface receptor levels of C5L2 and C5aR increased during 3T3-L1 adipocyte differentiation; both receptors are also highly expressed in J774 macrophages. Using confocal microscopy to evaluate endogenous receptors in adipocytes following stimulation with ASP or C5a, C5L2 is internalized with increasing perinuclear colocalization with C5aR. There is little C5a-dependent colocalization in macrophages. While adipocyte-conditioned medium (ACM) increased C5L2-C5aR colocalization in macrophages, this was blocked by C5a. ASP stimulation increased Akt (Ser473) phosphorylation in both cell types; C5a induced slight Akt phosphorylation in adipocytes with less effect in macrophages. ASP, but not C5a, increased fatty acid uptake/esterification in adipocytes.

C5L2-C5aR homodimerization versus heterodimerization may thus contribute to differential responses obtained following ASP vs C5a stimulation of adipocytes and macrophages, providing new insights into the complex interaction between these two cell types within adipose tissue. Studying the mechanisms involved in the differential responses of C5L2-C5aR activation based on cell type will further our understanding of inflammatory processes in obesity.

2.2 Introduction

The interaction between the immune system and adipose tissue is now well recognized in obesity, diabetes and cardiovascular disease, where adipocytes, macrophages and other immune cells maintain a biochemical interaction mediated by cytokines and adipokines [1-3]. Anaphylatoxins and their receptors play a crucial role in inflammation. While chronic low-grade inflammation is associated with obesity and metabolic dysfunction [1,3,4], the specific role of anaphylatoxins and their cognate receptors remains relatively less explored. C5a is a potent chemoattractant and pro-inflammatory anaphylatoxin that interacts with two receptors, C5aR-like receptor 2 (C5L2) and C5aR [5-9]. C5aR is a member of the rhodopsin family of G protein-coupled receptors (GPCR) which is expressed at varying levels in different immune and non-immune cells [10,11]. C5L2 is also a seven-transmembrane protein that resembles C5aR (58 % homology) [12] and has been shown to be expressed on various cell types concomitantly with C5aR [11,13,14]. Functional cell-dependent responses associated with C5a-C5aR binding include chemotaxis, oxidative burst and β-hexosaminidase release [15]. However, these responses are not observed in C5a-C5L2 interactions [6,8]. While it has been suggested that C5L2 acts as a decoy receptor for C5a [8,9,16,17], functional C5a-C5L2 interactions have also been proposed [13,18-20]. Studies on C5L2$^{-/-}$ knockout mice suggested an anti-inflammatory role for C5L2 [19,21] and, in a sepsis model, antibody blockade of C5L2 resulted in a dramatic increase in interleukin-6 levels [20].

To date, C5L2 has been shown to be the only known receptor for acylation-stimulating protein (ASP; C3adesArg), an adipose tissue-derived hormone involved in lipid storage and energy metabolism [14,22]. However, ASP-C5L2 binding is controversial, with other studies not reporting specific interactions [16,23]. By contrast, we have shown that ASP stimulates fatty acid uptake, glucose transport and triglyceride synthesis (TGS) in adipocytes as well as inhibiting lipolysis, effects that are additive to those of insulin [24-26]. These effects are dependent on the presence of C5L2 [14,27,28].

Ligand stimulation of C5L2 and C5aR which initiates receptor activation also leads to β-arrestin recruitment and internalization in neutrophils and transfected cells [7,11,29]. C5aR couples to Giα2, Giα3 or Gα16 and initiates several signaling pathways such as Akt,

phospholipase D and protein kinase C (PKC), which further result in increased intracellular calcium release or stimulation of transcriptional activators such as NF-κB [30,31]. Unlike C5aR, C5L2 signalling/trafficking and G protein coupling have not previously been detected in transfected cells [8,9,32], hence the proposal that C5L2 is a "decoy", "scavenger" or "non-signalling" receptor [6,8,9,16]. Specifically, no evidence for C5L2 activation of calcium mobilization or ERK phosphorylation has been found following C5a stimulation [21]. Our previous data on the ASP signalling pathway in cells endogenously expressing C5L2 showed sequential activation of PI3-kinase, phospholipase C, PKC, Akt, MAPK/ERK1/2 and cPLA2, which all lead to increased TGS in 3T3-L1 cells [33].

Bioluminescence and fluorescence resonance energy transfer (BRET and FRET, respectively) have provided evidence of dimerization of many GPCRs [34,35]. With co-internalization, cross phosphorylation can occur and un-liganded receptor can be activated as a result of dimerization [36]. Dimerization may affect receptor stability, and heterodimerization may, although it does not necessarily, modulate ligand binding, G protein signalling phenotypes, β-arrestin recruitment/internalization, and down regulation [37,38]. C5aR has been shown to homodimerize and also heterodimerize with the CCR5 chemokine receptor [36,39]. Moreover, co-internalization of homodimers has been observed with C5aR [37]. Rittirsch et al. reported a pro-inflammatory role for C5L2 in synergy with C5aR (in a mouse model of sepsis) and proposed C5L2 as a functional receptor [18]. Chen et al. demonstrated C5L2 as a positive regulator of C5aR in inflammatory responses [13]. Bamberg et al. reported interesting data regarding C5L2 and C5aR interactions in human neutrophils [11]. Based on their results, C5L2 was present primarily as an intracellular receptor, and colocalized with C5aR upon C5a stimulation, potentially negatively modulating its signalling [11].

The infiltration of immune cells into adipose tissue raises another aspect of potential C5aR and C5L2 interactions, which has not been explored. We hypothesized that the role of C5L2 as an inflammatory or metabolic mediator may be related to its diverse localization on different cell types (adipocytes, macrophages) present within adipose tissue and its physical interactions with different ligands (ASP or C5a), all of which may be influenced by possible homo- and heterodimerization with C5aR. Accordingly, the aim of this study

was to evaluate C5L2-C5aR interactions in 3T3-L1 differentiated adipocytes and J774 macrophages, with respect to functionality and signalling of C5L2 and C5aR following ASP or C5a stimulation.

2.3 Experimental Procedures

2.3.1 Recombinant ASP, C5a and C3a

Recombinant ASP (rASP) was prepared as previously described in detail [26], using a His-Tag labelled rASP, with purification using Ni^{+} affinity chromatography (GE Healthcare, Piscataway, NJ) followed by His-Tag removal and then further HPLC purification. Preparations are endotoxin free and are assessed for purity by mass spectrometry [26,40]. Recombinant C5a (rC5a) (purity ≥ 95% by SDS-PAGE) was purchased from EMD Biosciences (Gibbstown, NJ). Purified C3a was purchased from BD Pharmingen (Franklin Lakes, NJ). The experimental concentrations of ASP, C3a and C5a used reflect their physiological plasma concentrations, where ASP is normally more than 10-fold the concentration of C5a.

2.3.2 Culture conditions, transfection and differentiation

HEK 293 cells, 3T3-L1 preadipocytes and J774 macrophages were obtained from the American Type Culture Collection (Manassas, VA). All cells were routinely cultured in DMEM/F12 plus 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO2. Throughout all experiments, cells were transferred to serum free (SF) medium 2 h prior to the treatments.

HEK 293 cells were grown in DMEM supplemented with 5% (v/v) FBS and stably transfected with either mouse C5L2 cDNA (mC5L2) or human C3aR cDNA (HA-tagged) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions [26,28]. Following stable transfection, HEK-mC5L2 cells were sorted twice using Fluos-ASP binding as described previously [26]. Accordingly, this sorting procedure will bias the population towards cells expressing ASP-binding-competent surface C5L2. The culture medium was supplemented with 250 μg/ml of G418 for the stably transfected cells.

3T3-L1 preadipocytes were differentiated by plating them in DMEM/F12 containing 10% (v/v) bovine calf serum (BCS) and grown until confluent. Two days post-confluency, cell differentiation medium containing 10 μg/ml insulin, 1.0 μM dexamethasone, and 500 μM isobutylmethylxanthine (IBMX) was added. Two days later the differentiation cocktail was replaced with insulin supplementation for 2 more days, then changed to 10% FBS in DMEM/F12 only. The medium was changed every 2 days thereafter and adipocytes were used on day 8-10. The cells used for experimentation were >80% differentiated (as determined by microscopic evaluation of lipid droplet formation). Direct co-culture was achieved by adding J774 macrophages (≈1×105 cells/well) into 6-well culture plates containing differentiated 3T3-L1 adipocytes (≈1×105 cells/well). Adipocyte-conditioned medium (ACM), macrophage-conditioned medium (MCM), and co-culture conditioned medium (CoCM) were collected after overnight incubation in SF medium and centrifuged at 10,000xg for 5 min prior to the experiments.

2.3.3 Antibodies

The following antibodies were purchased commercially: anti-mouse C5aR (Biolegend, San Diego, CA), anti-rabbit IgG conjugated to FITC (Sigma, St Louis, MO), anti-rat IgG conjugated to FITC (Lifespan Biosciences, Seattle, WA), anti-mouse IgG conjugated to FITC (Sigma, St Louise, MO), anti-rabbit TRITC (Sigma, St Louis, MO), and anti-HA (Bethyl, Montgomery, TX). Polyclonal rabbit antibody to the N-terminal 23 amino acids of mouse C5L2 and monoclonal antibody against ASP were prepared locally as previously described [14,27,41]. Note this antibody recognizes both ASP and C3a equally.

2.3.4 Immunofluorescence, confocal microscopy and image analysis

HEK 293, HEK-mC5L2, HEK-hC3aR, 3T3-L1 adipocytes, and J774 macrophages adherent to glass coverslips were preincubated in SF medium for 2 h, followed by ligand stimulation with 200 nM ASP, 100 nM C3a or 20 nM C5a at 37°C as indicated. Cells were fixed, treated with blocking solution (1% BSA-PBS and 0.2% Triton X-100) for 15 min, and immunostained for C5L2, C5aR, HA-C3aR or ASP/C3a using primary antibodies:

rabbit anti-C5L2, rat anti-C5aR, rabbit anti-HA (for HA-C3aR) or mouse anti-ASP/C3a. After washing, cells were incubated with secondary fluorescent antibodies (goat anti-rabbit conjugated with TRITC, donkey anti-rat conjugated with FITC or sheep anti-mouse conjugated with FITC) and nuclei were visualized by using a mounting medium containing DAPI (Vector Labs, Burlingame, CA). For negative controls, cells were incubated with secondary antibodies only, omitting the primary antibodies, and there was no significant background staining. Data were collected by spinning-disk confocal microscopy (Olympus, IX81-DSU). Subsequently, images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD) and colocalization of C5L2 and C5aR was quantified with ImageJ software (National Institutes of Health, Bethesda, MD). The Colocalization Colormap Plugin was used to calculate the Correlation Index (Icorr) [42]. Icorr indicates the fraction of positively correlated pixels in the image. For each cell to be evaluated, a region of interest (ROI) was selected to enclose the selected cell and eliminate background for colocalization measurements (5-10 cells were analyzed for each construct).

2.3.5 Bioluminescence resonance energy transfer (BRET)

To analyze the interactions between C5L2 and C5aR, BRET was used as previously described [43-45]. Coelenterazine H was obtained from Biotium (Hayward, CA, USA). Briefly, HEK 293 cells were transiently transfected with a fixed amount of donor molecules tagged with Renilla luciferase (C5aR-Rluc or C5L2-Rluc), and co-transfected with increasing amounts of Venus-tagged acceptors (C5aR-Venus, C5L2-Venus, and Alk5-Venus). Alk5 is the type I receptor for TGF-β and would not be expected to interact with either C5L2 or C5aR, but is localized to the plasma membrane. HEK 293 cells were seeded into 6-well plates (1.5-2x105 cell/well) and transfected 2 days later with polyethylenimine (Sigma; 1 mg/ml stock) at 1:3 ratio with DNA. Total DNA amount was balanced in all conditions by adding pcDNA 3.1 as required. After the addition of the substrate coelenterazine H, the emission filter set was composed of 460/40 and 528/20 filters and the BRET ratio was calculated as described. In addition, transiently transfected HEK 293 cells were treated with ASP (200 nM and 1 μM) and/or C5a (20 nM and 100 nM) at 37°C, and agonist-induced BRET was measured at different time points following stimulation.

2.3.6 Flow cytometry

After 2 h preincubation in SF media, cells were detached with a non-enzymatic cell dissociation solution (Sigma, St Louis, MO), pelleted by centrifugation (600×g, 4 min), and resuspended in 0.5% BSA in PBS containing rabbit anti-C5L2 or rat anti-C5aR at the appropriate dilutions and incubated at 4°C for 30 min. Post pelleting and washing, cells were incubated again for 30 min at 4°C with goat anti-rabbit antibody conjugated with TRITC or donkey anti-rat antibody conjugated with FITC at 1:1000 dilution in 0.5% BSA in PBS, pelleted and washed. All samples were fixed in 4% paraformaldehyde, then transferred to 2% paraformaldehyde, and analyzed by FACScan flow cytometry (BD Biosciences, FACSCanto II, San Diego, CA). 10,000 events were considered for each analysis.

2.3.7 Akt phosphorylation

Akt activation was measured by Fast Activated Cell-based ELISA kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. This methodology measures total Akt and phospho-Akt directly in lysed cells using ELISA assays, without the need for separate extraction, homogenization, polyacrylamide gel separation and transfer to membranes, thereby reducing technical manipulation and the associated increased variability. Briefly, 3T3-L1 adipocytes and J774 macrophages seeded in 96-well plates were stimulated with ASP (200 nM) and C5a (20 nM) for the indicated incubation times, and rapidly fixed with 4% formaldehyde in PBS at the indicated times. After washing and blocking, cells were incubated overnight with rabbit anti-phospho-Ser473 or rabbit anti-total Akt. Following incubation with anti-HRP conjugated IgG for 1 h at room temperature, a colorimetric assay was performed and absorbance at 450 nm was determined. The values were subsequently corrected for cell number using the absorbance values at 595 nm determined by crystal violet cell staining.

2.3.8 Fluorescent fatty acid uptake into 3T3-L1 adipocytes

Uptake and incorporation into lipids of fluorescently-labeled fatty acid was measured over time at 37^{o}C in 3T3-L1 differentiated adipocytes, using a QBT TM fatty acid uptake assay kit (Molecular Devices, Sunnyvale, CA) as described by the manufacturer, with slight modifications as published [26].

2.3.9 Statistical Analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test or by Student's t test. All values are mean ± standard error of the mean (SEM) and statistical significance was as follows: **P*<0.05, ***P*<0.01, ****P*<0.001.

2.4 Results

2.4.1 C5L2 colocalized and internalized with ASP in HEK cells stably transfected with mC5L2

To support our previous results regarding ASP-C5L2 binding [26,28], visualization of ASP-C5L2 trafficking using fluorescently conjugated antibodies and confocal microscopy was assessed in HEK-mC5L2 cells. These cells were stably transfected and sorted based on Fluos-ASP binding (see Experimental Procedures). Both receptor (C5L2) and ligand (ASP) were immunostained to follow their localization by confocal microscopy. As shown in FIGURE 2.1A, prior to stimulation, C5L2 was localized on the cell surface (time=0). After 15 min of ASP stimulation, C5L2 was internalized and colocalized with ASP. This colocalization was maintained over the 90 minute time period as assessed by correlation index (Icorr; FIGURE 2.1B). Conversely in a control experiment with HEK-hC3aR, there was a complete absence of colocalization of C3aR and ASP (as indicated by separate signals in the merged panel, FIGURE 2.1C). However, when the cells are stimulated with C3a, there is ligand-mediated internalization of C3aR. As shown in FIGURE 2.1C, C3aR is associated with vesicles that accumulate in the perinuclear region.

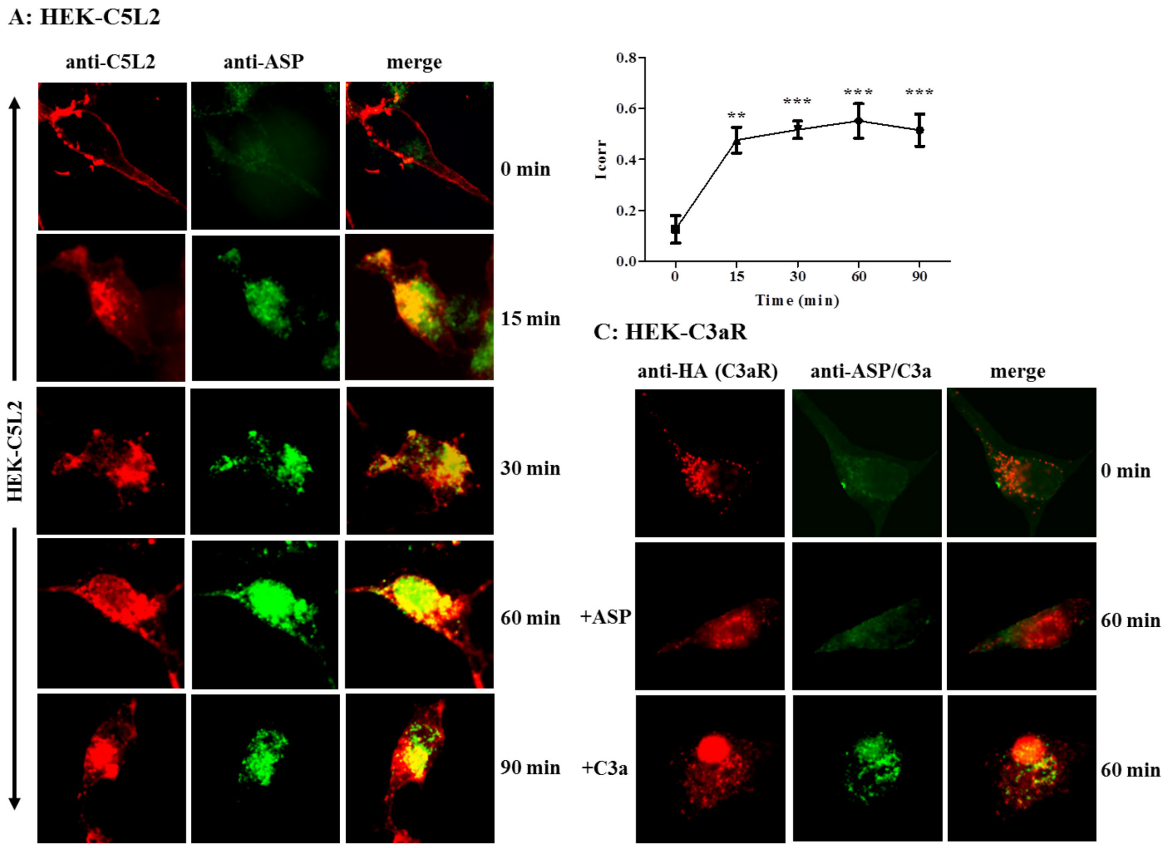


FIGURE 2.1 – Confocal microscopy of C5L2 and ASP colocalization in HEK-C5L2.
A, HEK-mC5L2 were treated with ASP (200 nM, T=0, 15, 30, 60, and 90 min) at 37 °C, then fixed and stained with mouse anti-human ASP (which also recognizes C3a, the arginated form of ASP) and rabbit anti-mouse C5L2 followed by FITC sheep anti-mouse (green) and TRITC goat anti-rabbit (red) antibodies. Cells were then mounted onto slides using a mounting medium and imaged with an Olympus spinning disk scanning confocal microscope (×100 oil immersion). *B,* Colocalization of C5L2 and ASP in HEK-mC5L2 cells was quantified in 5–10 cells using the colocalization colormap script, ImageJ plugin. *C,* As a control, HEK-hC3aR (containing an HA tag) were treated with ASP (200 nM) and C3a (100 nM) at 37 °C for the indicated times, then fixed and stained with mouse anti-human ASP/C3a and rabbit anti-HA followed by FITC sheep anti-mouse (green) and TRITC goat anti-rabbit (red) antibodies. Cells were then mounted onto slides using a mounting medium and imaged with an Olympus spinning disk scanning confocal microscope (×100 oil immersion). Results were analyzed by one-way ANOVA followed by Bonferroni post-hoc test (**P<0.01, ***P<0.001, versus T=0).

2.4.2 C5L2 and C5aR form homo- and heterodimers

To examine potential physical interactions between C5L2 and C5aR, homo- and heterodimerization of C5L2 and C5aR was assessed in transfected HEK 293 cells with BRET using receptor Venus-Luc pairs. First, to determine whether C5L2 self-association could be detected, BRET was evaluated in HEK 293 cells co-transfected with C5L2-Rluc and C5L2-Venus. As shown in FIGURE 2.2A, titration curves were performed in HEK 293 cells which were transfected with a fixed concentration of the energy donor C5L2-Rluc and increasing concentrations of the energy acceptor C5L2-Venus. As the ratio of energy acceptor to donor was increased, the BRET ratio increased and saturated, demonstrating homodimerization of C5L2. As a negative control, cells were co-transfected with Alk5 fused to Venus. Little or no BRET signal was detected with the negative control, which in any case was linear suggesting that the interactions detected for the C5L2 homodimer were specific (FIGURE 2.2A). HEK 293 cells were then co-transfected with C5aR-Rluc and C5aR-Venus. BRET saturation curves (FIGURE 2.2B) also indicate the potential of C5aR to homodimerize, while the negative control again showed no saturable BRET signal. HEK 293 cells were also transfected with a fixed amount of C5aR or C5L2 fused to Rluc and increasing concentration of C5aR-Venus or C5L2-Venus. Regardless as to which was donor or acceptor in the pair, these experiments revealed saturable heterodimerization between C5L2 and C5aR receptors (FIGURE 2.2C). Ligand effect on C5aR and C5L2 homo- and heterodimerization was also assessed over time, at two different concentrations of ASP and C5a. Agonist-induced BRET was measured at a point around the BRET50 values for both the heterodimer and the C5L2 homodimer. Results for agonist-induced effects on the C5L2/C5aR heterodimer with 200 nM ASP and 20 nM C5a are shown in FIGURE 2.2D. No changes in response to agonist were noted for the heterodimer. Moreover, addition of higher ligand concentration or a combination of ASP+C5a also had no affect on BRET between C5L2 and C5aR receptors (data not shown). Further, no change was detected in the C5L2 homodimer in response to agonist (data not shown).

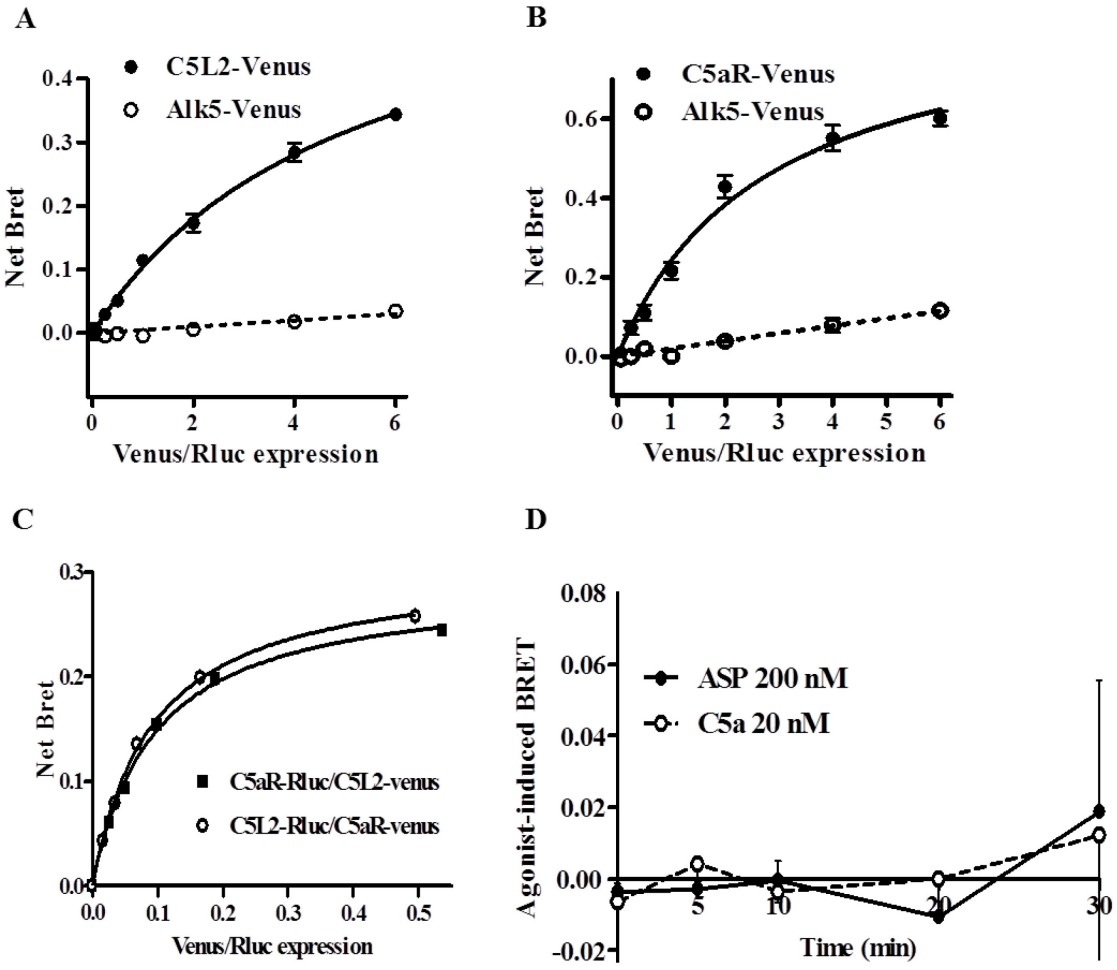


FIGURE 2.2 – BRET analysis of C5L2 and C5aR homo-/heterodimerization.

A, HEK 293 cells were transfected with a fixed amount of C5L2–Rluc and increasing concentrations of C5L2–Venus or Alk5–Venus (as a negative control). Data shown are the net BRET as a function of Venus/Rluc expression. *B,* Cells were transfected with a fixed amount of C5aR–Rluc and increasing concentration of C5aR–Venus or Alk5–Venus. Data shown are the net BRET as a function of Venus/Rluc expression. *C,* HEK 293 cells were co-transfected with C5aR–RLuc/C5L2–Venus or C5L2–Rluc/C5aR–Venus pairs to evaluate heterodimerization of the receptors. Data in all cases are representative of three independent experiments where multiple measures of BRET were taken and averaged. *D,* Agonist-induced BRET between C5L2–RLuc and C5aR–Venus determined by incubation of cells with ASP (200 nM) or C5a (20 nM) for 5, 10, 20, and 30 min, compared to unstimulated (T=0). Data are expressed as mean±SEM BRET, stimulated minus un-stimulated values (n=3).

2.4.3 3T3-L1 differentiation is associated with increased expression of C5L2 and C5aR

As transfected cells depend on receptor overexpression, interactions between C5aR and C5L2 were also evaluated in cells endogenously expressing both receptors. J774 macrophages as well as 3T3-L1 adipocytes expressed high endogenous levels of C5L2 and C5aR as detected by flow cytometry (FIGURE 2.3A and B). It has been reported that ASP production significantly increases during preadipocyte differentiation to mature adipocytes [46]. In parallel, the expression of ASP receptor (C5L2) and C5aR surface expression were clearly increased during different stages of adipocyte differentiation up until day 9 (FIGURE 2.3C).

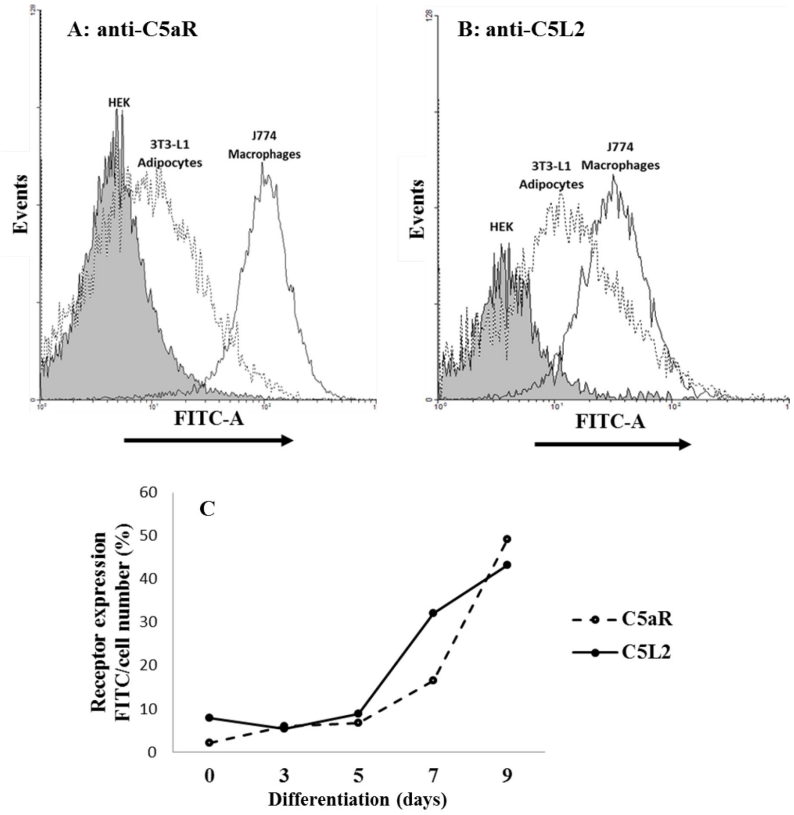


FIGURE 2.3 – Expression analysis of C5L2 and C5aR.
A, Flow cytometry analysis of HEK 293 cells (negative control), 3T3-L1 adipocytes and J774 macrophages using anti-mouse C5aR. *B,* Flow cytometric analysis of HEK 293 cells (negative control), 3T3-L1 adipocytes and J774 macrophages using anti-mouse C5L2. *C,* Flow cytometry staining of C5L2 and C5aR expression on 3T3-L1 cells over the course of 9 days of differentiation into adipocytes.

2.4.4 C5L2 and C5aR colocalized upon ASP and C5a stimulation in adipocytes and macrophages

Immunostaining and confocal microscopy of Triton-permeabilized cells to visualize C5L2 and C5aR localization without/with ligand stimulation at five time points (0, 15, 30, 60, 90 minutes) was evaluated in differentiated 3T3-L1 adipocytes and J774 macrophages. As shown in FIGURE 2.4A, before stimulation (t=0) both receptors displayed plasma membrane staining as well as a diffuse cytoplasmic localization. After stimulation with ASP or C5a (FIGURE 2.4A, selected time points shown), C5L2 internalization is markedly increased in 3T3-L1 adipocytes. A similar pattern, but with more diffuse intracellular vesicles, can be observed for C5aR upon ligand binding, with extensive perinuclear colocalization with C5L2 (yellow; FIGURE 2.4A). Similar visualizations were observed for J774 macrophages (FIGURE 2.4B). These data were quantified by ImageJ software to evaluate the degree of colocalization (Correlation Index=Icorr) over time. Noteworthy, in non-stimulated cells (t=0), there is greater basal colocalization (Icorr index) observed in macrophages than in adipocytes (FIGURE 2.4C). Quantification over the time course evaluated is shown for both adipocytes and macrophages with ASP (FIGURE 2.4D) and C5a (FIGURE 2.4E). In 3T3-L1 adipocytes, colocalization increased over time following both ASP and C5a stimulation, remained elevated up to 30-60 min (ASP and C5a, respectively), and then decreased to baseline by 90 min (FIGURE 2.4D and E). By contrast, the level of colocalization in macrophages was substantially lower in comparison to adipocytes for ASP, although still significant at specific time points (FIGURE 2.4D), while C5a had only a small transient effect at 15 min (FIGURE 2.4E).

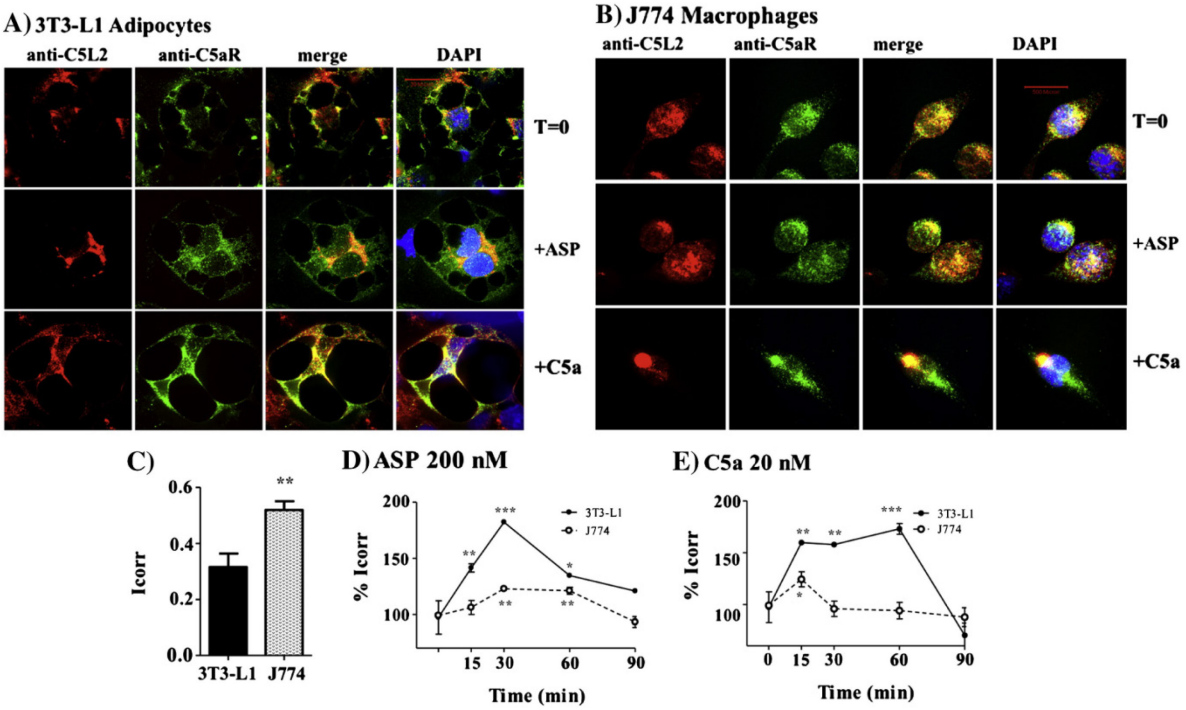


FIGURE 2.4 – Confocal microscopy and quantitative analysis of C5L2 and C5aR. *A,* 3T3-L1 adipocytes were treated with either C5a (20 nM, T=0, 15, 30, 60, 90 min) or ASP (200 nM, T=0, 15, 30, 60, 90 min) at 37 °C, then fixed and stained with rat anti-mouse C5aR and rabbit anti-mouse C5L2 followed by FITC anti-rat IgG (green), TRITC anti-rabbit (red) antibodies. Cells were then mounted onto slides using a mounting medium containing DAPI (nuclei staining, blue) and imaged with an Olympus spinning disk scanning confocal microscope (×100 oil immersion), stained for C5L2 (red) and C5aR (green) where colocalization is shown in the merged (yellow) panel. Only one time point for each treatment is presented (ASP 30 min; C5a 30 min). The results of a representative experiment of three conducted are shown. *B,* Following stimulation with either C5a (20 nM) or ASP (200 nM), the complete procedure (as indicated in panel A) was conducted on J774 macrophages. Time points presented here are ASP 60 min and C5a 15 min. *C,* Colocalization of C5L2 and C5aR in unstimulated cells; 3T3-L1 adipocytes (left) and J774 macrophages (right). Graphs represent the mean correlation index (Icorr)±SEM of FITC (green) and TRITC (red) *D* and *E,* Colocalization of C5L2 and C5aR in 3T3-L1 and J774 cells stimulated with ASP (200 nM, D) or C5a (20 nM, E) was quantified in 5–10 cells using the colocalization colormap script, ImageJ plugin. Results were analyzed by One-way ANOVA followed by Bonferroni post-hoc test (**P*<0.05, ***P*<0.01, ****P*<0.001, versus T=0).

2.4.5 Conditioned medium (CM) effects on C5L2 and C5aR colocalization are cell-specific

As adipocytes and macrophages are both detectable in adipose tissue in obesity, the effect of conditioned media from adipocytes (ACM) and macrophages (MCM) on C5L2

and C5aR colocalization in 3T3-L1 and J774, respectively, were evaluated. Three different conditioned medias were analyzed: i) ACM, i) MCM, and iii) CoCM (media collected from the direct co-culture of both adipocytes and macrophages together). All incubations were for 30 min, which is consistent with receptor activation and subsequent internalization based on data obtained by confocal microscopy above. Marked increases in colocalization (Icorr) were observed in adipocytes upon exposure to MCM and CoCM alone (FIGURE 2.5A). In adipocytes exposed to MCM, addition of C5a further increased C5L2-C5aR colocalization ($P<0.05$, FIGURE 2.5A), while addition of C5a or ASP to adipocytes treated with CoCM had no additional effect. In macrophages (FIGURE 2.5B), ACM and CoCM significantly increased C5aR/C5L2 colocalization, but ASP did not further enhance this. Interestingly, in macrophages, the effect of both ACM and CoCM was blocked with the addition of C5a (FIGURE 2.5B).

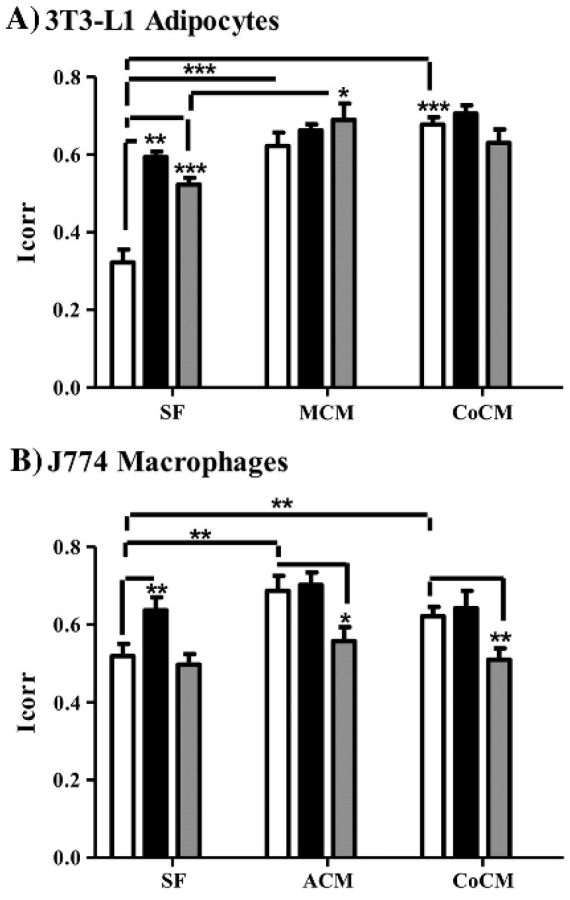


FIGURE 2.5 – Conditioned medium (CM) effects on C5L2 and C5aR colocalization.

A, Effect of PBS (Control, white bar), 200 nM ASP (black bar), and 20 nM C5a (gray bar) on C5L2 and C5aR colocalization in 3T3-L1 adipocytes supplemented with serum free (SF), macrophage-conditioned medium (MCM), and medium from co-culture of adipocytes and macrophages (CoCM). *B,* Effect of PBS (Control, white bar), 200 nM ASP (black bar), and 20 nM C5a (gray bar) on C5L2 and C5aR colocalization in J774 macrophages supplemented with serum-free (SF), adipocyte-conditioned media (ACM) and media from co-culture of adipocytes and macrophages (CoCM). All values are mean±SEM and results were analyzed by one-way ANOVA followed by Bonferroni post-hoc test (*P<0.05, **P<0.01, ***P<0.001).

2.4.6 Ligand-dependent Akt activation shows distinct profiles in adipocytes and macrophages

These cell-specific differences in colocalization suggested that there might also be cell-specific signalling differences. As shown in FIGURE 2.6A, ASP and C5a both induced rapid phosphorylation of Akt on Ser^{473} in 3T3-L1 adipocytes by 5 min (141.6±14.7% and 136.2±4.6%, respectively; P<0.05), followed by subsequent dephosphorylation. Furthermore, following ASP stimulation in J774 macrophages, phospho-Akt relative to total Akt was increased, reaching a peak at 15 min (211.7±14.7%; P<0.01) and remaining activated up to 30 min (FIGURE 2.6B). In contrast to adipocytes, C5a stimulation had no significant effect on Akt phosphorylation (Ser^{473}) in J774 macrophages (FIGURE 2.6B).

2.4.7 Only ASP (but not C5a) stimulates TGS in adipocytes

In order to evaluate the functional effects of ASP and C5a in adipocytes, we examined fatty acid uptake and incorporation into lipids, measured as fluorescently labeled real-time BODIPY-fatty acid uptake. As shown in FIGURE 2.6C, ASP stimulated fatty acid uptake at levels comparable to insulin, a known lipogenic hormone, as shown previously for both ASP and insulin [26]. By contrast, fatty acid uptake was not changed significantly at any concentration of C5a (1 nM - 60 nM) in comparison to the negative control (PBS) (FIGURE 2.6C).

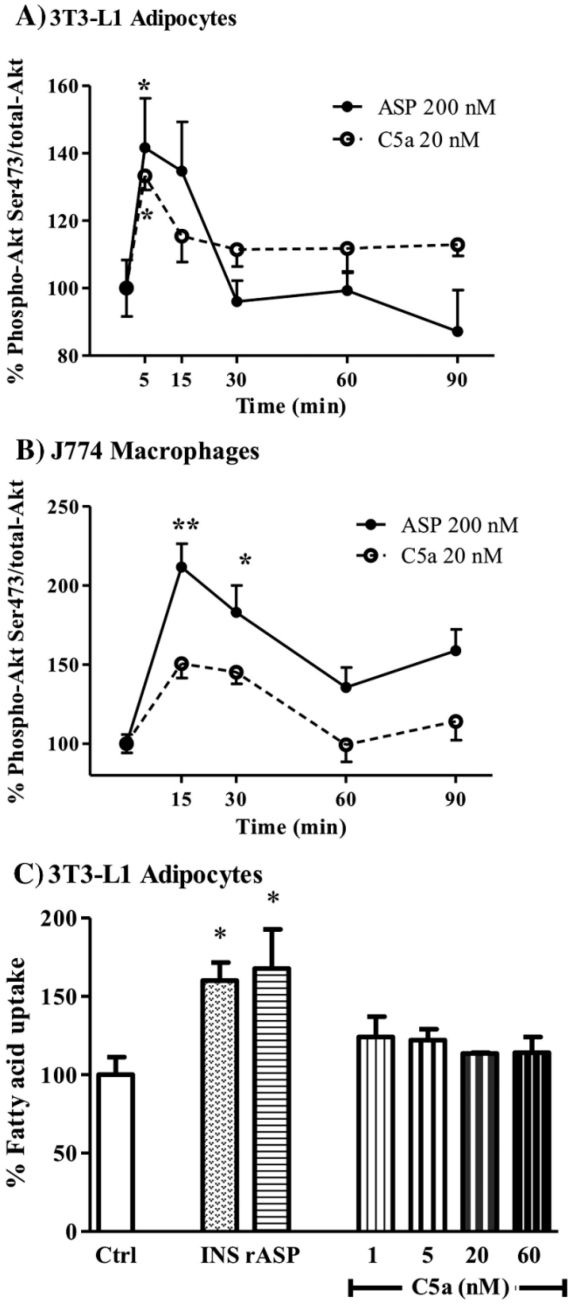


FIGURE 2.6 – Akt phosphorylation and fatty acid uptake into lipids.

A, ASP and C5a effects on Akt phosphorylation (normalized against total Akt) in 3T3-L1 adipocytes. *B,* ASP and C5a effect on Akt phosphorylation (normalized against total Akt) in J774 macrophages. All values are mean±SEM and results were analyzed by one-way ANOVA followed by Bonferroni post-hoc test (**P*<0.05, ***P*<0.01, ****P*<0.001, versus T=0). *C,* Bioactivity of ASP, insulin and C5a was tested in 3T3-L1 adipocytes. Treatments: PBS (Ctrl), insulin (INS; 100 nM), rASP (300 nM), and C5a (1 nM, 5 nM, 20 nM, and 60 nM). Results were analyzed by Student's t test (**P*<0.05, versus Ctrl).

2.5 Discussion

Here, a comparative study between 3T3-L1 adipocytes and J774 macrophages was performed to address several unresolved issues: (i) direct ASP/C5L2 interaction, (ii) C5L2/C5aR interaction and (iii) the cell or ligand specificity of C5L2/C5aR interaction and subsequent signalling. The results demonstrate that C5L2/C5aR colocalization is both cell- and ligand-specific and the functional outcomes are influenced by both.

Acknowledgment of ASP as a C5L2 ligand remains controversial. Johswish *et al.*, using transfected cells, found no evidence of ASP-C5L2 binding or functional responses [16]. Conversely, we have previously shown ASP-C5L2 binding with ^{125}I- and fluorescently labeled ASP in HEK 293 and CHO cells stably transfected with human hC5L2 and murine mC5L2 and sorted based on Fluos-ASP binding [26]. Consistent with these findings, we demonstrate in the present study that ASP and C5L2 colocalized in transfected HEK 293 cells, adipocytes and macrophages. These data complement our previous reports showing interaction between ASP and C5L2, functionality of ASP mediated through C5L2, and the related signalling mechanisms and metabolic outcomes [7,14,26-28,33].

Most cellular studies of C5L2 have been performed using transfected cells and few studies have focused on endogenously expressed receptors [11,47]. Bamberg *et al.* evaluated neutrophil expression of C5aR and C5L2 and suggested that C5L2 was primarily an intracellular receptor, with colocalization of C5L2 and C5aR following C5a stimulation [11]. Here, while adipocytes demonstrate lower basal colocalization (as compared to macrophages) in the absence of stimulation, there is greater response to both ASP and C5a. This is consistent with our observations regarding dimerization, showing the capability of both C5L2 and C5aR to homo- and heterodimerize. GPCR dimerization has been previously shown to be important for allosteric regulation and fine-tuning of signalling and receptor trafficking [36-38]. Based on our results, ASP and/or C5a stimulation revealed no significant changes in BRET under any conditions tested, suggesting that dimerization occurs constitutively in transfected HEK 293 cells and the complex remains stably associated during signal transduction. However, in endogenously expressing cells, ligand stimulation did result in rapid internalization of C5L2 and C5aR, which was associated with

increased intracellular colocalization in adipocytes and macrophages. Based on the confocal and BRET results presented here, coupled with the observed endogenous expression of C5L2 and C5aR in both adipocytes and macrophages, we propose that close physical interaction exists between C5L2 and C5aR, which may be tightly linked to signaling, including Akt-phosphorylation and functional responses (such as triglyceride synthesis).

Previously, we reported the involvement of Akt phosphorylation (Ser^{473}) in ASP signalling in 3T3-L1 preadipocytes [33]. The Akt pathway appears to play an important role in the regulation of cell metabolism, survival, migration, and inflammatory responses [48]. Furthermore, a study on peritoneal macrophages from C5aR knockout mice, reported involvement of Akt/PI3K pathway in C5a-C5L2 signalling [18]. Of note, macrophages and neutrophils from C5L2 knockout mice showed diminished Akt phosphorylation following C5a stimulation [13]. Akt can be phosphorylated independently at either Thr^{308} or Ser^{473} by different protein kinases [49]. Bosmann *et al.* showed that Akt phosphorylation in macrophages following C5a stimulation was mostly targeted to the Thr^{308} residue, while no significant phosphorylation was observed at Ser^{473} residue [50]. Although both ASP and C5a resulted in phosphorylation of Akt at Ser^{473} in adipocytes, our results indicate the absence of Akt signalling crosstalk between ASP and C5a in macrophages, where only ASP stimulated Akt Ser^{473} phosphorylation, but not C5a. While the contribution of C5L2 or C5aR to Akt phosphorylation in adipocytes cannot be assessed in the present context, the downstream consequences differ and this may depend on cell-specific differences in heterodimer function and trafficking. While ASP increases TGS, C5a does not. Although Akt activation is necessary for ASP activation of TGS [33], it may not be sufficient, as C5a, which increases adipocyte C5L2/C5aR colocalization and increases Akt phosphorylation, does not stimulate TGS. Additional signalling pathways such as PLC, PKC, MAPK/ERK1/2 and $cPLA_2$, which are all required for ASP induction of increased TGS in 3T3-L1 cells may be necessary [33]. Further, in macrophages, while both ASP and C5a had little effect on colocalization, ASP still increased Ser^{473}-Akt phosphorylation, suggesting that ASP may lead to alternate functions in macrophages different from those of C5a. Other reports suggest that in macrophages, C5a effects are mostly mediated through C5aR rather than C5L2 [50-53]. In accordance with these results, an *in vitro* study using macrophages from $C5aR^{-/-}$KO, and $C5L2^{-/-}$KO mice demonstrated the anti-inflammatory role of C5a (under the

specific circumstances of very high concentrations of C5a) through C5aR and not C5L2 [53]. Further, previous studies on macrophages and adipocytes in co-culture have demonstrated the generation of inflammatory responses and insulin resistance [54,55]. Our cell-conditioned media data suggest that C5a modulates the effects of adipocyte-conditioned medium on macrophages, and this is particularly relevant in the context of macrophage infiltration into adipose tissue [56]. Zhang et al, proposed C5L2 as a dual function receptor in an experimental allergic asthma setting, where C5L2 demonstrated both pro-inflammatory and anti-inflammatory effects [57]. Other reports have suggested a pro-inflammatory role of C5L2 in a mouse model of cecal ligation/puncture-induced sepsis, where C5a-C5L2 interaction contributed to the release of high-mobility-group-box 1 protein (HMGB1) [18]. In another study, inhibition of C5a by AcPepA in LPS-treated monkeys, effectively increased survival time, presumably via suppression of HMGB1 induction [58]. Furthermore, recent studies also proposed a pro-inflammatory cooperation between C5L2 and C5aR in the pathophysiology of sepsis [58,59]. Further, Atefi *et al.* demonstrated that the production of pro-inflammatory mediators in response to C5a is dependent on both C5L2 and C5aR in cardiomyocytes [59].

2.6 Conclusion

Taken together, our data support the idea of C5L2-C5aR homo- and heterodimerization, an association which is both cell- and ligand-specific. We propose that C5L2 is a functional receptor for ASP, especially relevant in adipocytes, but may also act as a decoy receptor for C5a in pathophysiological conditions where excessive pro-inflammatory activities of C5aR need to be down-regulated or in particular cells that respond actively to C5a. This may be particularly relevant in an obese state, where adipocytes and macrophages interact in adipose tissue. The present study demonstrates that the now well-accepted adipose tissue inflammation interconnectedness may well extend to the complement factors C5a and ASP and their receptors C5L2 and C5aR.

2.7 Bibliography

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CHAPTER 3

C5aR and C5L2 Act in Concert to Balance Immunometabolism in Adipose Tissue

Poursharifi, P. *et al.* C5aR and C5L2 Act in Concert to Balance Immunometabolism in Adipose Tissue. *Mol. Cel. Endocrinol.* **382,** 325–33 (2014).

Résumé

Le lien entre le système immunitaire et le métabolisme du tissu adipeux s'étend aussi au système du complément. De récentes études démontrent une hétérodimérisation du récepteur du facteur du complément C5a (le C5aR) avec le récepteur C5L2 de l'ASP, une hormone lipogène. Cette étude a pour objectif d'évaluer la contribution du C5aR à la régulation du métabolisme adipocytaire.

Des adipocytes matures ont été isolés à partir de tissu adipeux gonadal de souris de type sauvage et C5aR (-/-). La concentration de certaines cytokines et la phosphorylation de Akt/ERK/NFkB a été mesurée par ELISA. L'absorption et l'incorporation d'acides gras marqués avec un fluorochrome dans les triglycérides a été mesuré à l'aide d'un dosage d'incorporation d'acide gras via QBT™.

L'ASP ainsi que le C5a ont tous les deux des effets directs sur la sécrétion de MCP1 et KC, sur la synthèse des triglycérides ainsi que sur la phosphorylation d'AKT/NFkB dans les adipocytes. En contrepartie, dans les adipocytes C5aR (-/-), les effets de C5a ne sont plus mesurables alors que ceux de l'ASP sont maintenus. L'ajout de C5a, tant dans les adipocytes C5aR (-/-) que ceux de type sauvage, bloque complètement la signalisation et l'activité de l'ASP. Cet antagonisme peut aussi être observé dans les adipocytes 3T3-L1 différenciés. De plus, les adipocytes C5aR (-/-) démontrent aussi une détérioration de la capacité de l'insuline à stimuler la sécrétion de cytokines, une diminution de la signalisation intracellulaire et de la stimulation de la synthèse de triglycérides, des effets étant en continuité avec la diminution de l'expression génique d'IRS1 et de PGC1α dans le tissu adipeux.

Ces observations suggèrent que le C5aR possède un double rôle tant dans l'immunité que dans le métabolisme énergétique qui pourrait être modulé à travers son hétérodimérisation avec le récepteur C5L2.

3.1 Abstract

Recent studies suggested that the immunometabolic receptors; C5aR and C5L2, constitutively self-associate into homo-/heterodimers and that acylation stimulating protein (ASP/C3adesArg) or C5a treatment of adipocytes increased their colocalization. The present study evaluates the C5aR contribution in adipocytes to the metabolic and immune responses elicited by ligand stimulation.

The effects of C5a, ASP, and insulin on cytokine production, triglyceride synthesis (TGS), and key signaling pathways were evaluated in isolated primary adipocytes and cultured 3T3-L1 differentiated adipocytes. In addition, mRNA expression of *IRS1* and *PGC1α* was compared in adipose tissue samples from WT versus C5aRKO mice.

Both C5a and ASP directly increased MCP-1 (238±4%; $P<0.001$, and 377±2% vs basal 100%; $P<0.001$, respectively) and KC (413±11%; $P<0.001$, and 529±16%; $P<0.001$ vs basal 100%, respectively) secretion, TGS (131±1%; $P<0.001$, and 152±6%; $P<0.001$, vs basal 100% respectively), and Akt/NFκB phosphorylation pathways in adipocytes. However, in C5aRKO adipocytes, C5a effects were disrupted, while stimulatory effects of ASP were mostly maintained. Addition of C5a completely blocked ASP signaling and activity in both C5aRKO and WT adipocytes as well as 3T3-L1 adipocytes. Furthermore, C5aRKO adipocytes revealed impaired insulin stimulation of cytokine production, with partial impairment of signaling and TGS stimulation, consistent with decreased *IRS1* and *PGC1α* mRNA expression in adipose tissue.

These observations indicate the importance of C5aR in adipose tissue metabolism and immunity, which may be regulated through heterodimerization with C5L2.

3.2 Introduction

It is now well accepted that adipocytes play a dynamic role in metabolic regulation, with active synthesis and secretion of cytokines, adipokines, complement proteins, and molecules associated with inflammation [1]. Investigations have revealed major contributions of adipocytes to immunity, with adipocytes interacting and signaling with immune cells, including both resident and infiltrating macrophages, within the microenvironment of adipose tissue [2-4]. Recently, complement protein/receptor interactions with adipokines have attracted attention, with "immunometabolism" being the subject of a number of reviews [1,5-7].

Many inflammatory responses in the immune system are related to the complement cleavage fragments, C3a and C5a, also known as anaphylatoxins [2]. These proteins mediate their signaling activities through several serpentine 7-transmembrane G protein-coupled receptors (GPCRs), which are expressed by various immune as well as non-immune cells [2]. C5a is regarded as one of the most potent inflammatory factors known, and functions via its receptor C5aR, but also binds C5aR-like receptor 2 (C5L2) [8-11]. Both C5L2 and C5aR are expressed on myeloid and non-myeloid cells such as adipocytes and preadipocytes, although *C5aR* mRNA levels are typically higher than *C5L2* [10,12,13]. As previously shown, phosphorylation of C5aR and C5L2 leads to association with β-arrestin proteins and internalization into clathrin-coated vesicles [8,12,14]. Notably, the complex array of C5a functional activities, including chemotaxis, enzyme/cytokine release, and the respiratory burst, are mostly attributed to its binding to C5aR [11], with stimulation of MAPK, ERK, diacylglycerol and Akt signaling pathways [15,16]. While C5a binds C5L2 with the same high affinity as C5aR, the potential functions and signaling pathways mediated through C5L2 are still controversial [11,17].

C3a and acylation stimulating protein (ASP/C3adesArg) both stimulate triglyceride synthesis (TGS) and glucose transport in adipocytes [18,19]. Adipocytes produce C3, factor B, and adipsin, leading to production of ASP within the adipocyte microenvironment upon activation of the alternative complement pathway [20]. ASP induces Akt, MAPK, ERK1, and NFκb signaling pathways in 3T3-L1 adipocytes and/or preadipocytes [3,4,21]. Tom *et al.* demonstrated ASP stimulation of monocyte chemoattractant protein (MCP)-1 and

keratinocyte chemoattractant (KC) production in adipocytes, an effect blocked with PI3-kinase and NFκB inhibitors [4]. Further, complement C3 knockout mice, which are deficient in C3, the precursor of ASP, and therefore obligately deficient in ASP, demonstrated altered energy metabolism and fat storage [22,23].

The two receptors, C5aR and C5L2, have been proposed to have closely linked physical and functional interactions [3,12]. Likewise, it has been demonstrated that C5aR and C5L2 are both capable of forming homo- and heterodimers [3]. Interestingly, both ASP and C5a have been found to stimulate internalization/colocalization of C5aR and C5L2 in J774 macrophages and 3T3-L1 adipocytes [3]. The consequences of homo- or heterodimerization are not yet clear; however, this could be linked to alternative signaling or regulatory cell- and ligand-dependent responses to severe inflammatory conditions or metabolic modulations. Further, the distinctive roles of C5aR and C5L2 in immunity and adipocyte metabolism are presently clouded by controversy.

The aim of the current study was to investigate (i) C5a-C5aR functions and signaling pathways and (ii) their possible regulatory effects on ASP/C5a signalling pathways in adipocytes using C5aR knockout (C5aRKO) mice and *in vitro* studies.

3.3 Methods

3.3.1 Materials

All tissue culture reagents, including Dulbeccos's modified Eagle's medium/F-12 (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin and tissue culture supplies were from Gibco (Burlington, ON). Triglyceride (TG) mass was measured using an enzymatic colorimetric assay (Roche Diagnostic, Indianapolis, IN). Recombinant ASP (rASP) was prepared as previously described in detail [24], and assessed for purity by mass spectrometry [24,25], and was verified to be endotoxin-free. Recombinant C5a (rC5a) (purity≥95% by SDS-PAGE) was purchased from EMD Biosciences (Gibbstown, NJ). Physiological concentrations of C5a (20 nM) and ASP (100 nM) were used in all of the experiments.

3.3.2 Cell Culture of 3T3-L1 Preadipocytes and Differentiation into Mature Adipocytes

3T3-L1 preadipocytes, obtained from the American Type Culture Collection (Manassas, VA), were maintained in DMEM/F12 supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in 5% CO_2. Differentiation was induced two days post confluence in medium containing 10 μg/ml insulin, 1.0 μM dexamethasone, and 500 μM isobutylmethylxanthine (IBMX). After three days, the differentiation cocktail was replaced with insulin supplementation for 2 more days, then changed to 10% FBS in DMEM/F12 only. Media was changed on fully differentiated 3T3-L1 adipocytes (≥80% differentiated as determined by microscopic evaluation of multiple lipid droplets) every two days and were used for functional and signaling assays on days 9-10 after differentiation was initiated. Throughout all experiments, cells were transferred to serum free (SF) medium 2 h prior to the treatments.

3.3.3 Mice

BALB/c mice and C5aRKO mice on a BALB/c background were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a sterile barrier facility with a 12 h light: 12 h dark cycle. All protocols were pre-approved by the Laval University Animal Care Committee and were conducted in accordance with the Canadian Council of Animal Care (CACC) guidelines. Animals were fed a standard chow diet *ad libitum*.

3.3.4 Isolation and Culture of Primary Mouse Adipocytes

Mice were euthanized and gonadal fat pads from wild type (WT) and C5aRKO (n=9-11 in each group) were collected in Krebs-Ringer buffer (KRB), pH 7.4. The tissue was minced and incubated in KRB buffer containing collagenase (collagenase type II, 0.1% (w/v)) at 37 °C for 45 min. The resulting suspension was filtered through a nylon mesh (250 μm) and separated into two parts (floating mature adipocytes and pelleting stromal cells) by low-speed centrifugation. The mature adipocyte fraction was rinsed three times with KRB buffer, and adipocytes were counted and aliquoted for the various experiments of the study.

3.3.5 Fluorescent Fatty Acid Uptake into Mature Adipocytes

Uptake and incorporation into lipids of fluorescently-labeled fatty acid was measured using the QBT™ fatty acid uptake assay kit (Molecular Devices, Sunnyvale, CA) in 3T3-L1 adipocytes and primary adipocytes, according to the manufacturer's instructions. C5aR antagonist (3D53), which binds specifically to C5aR [15,26,27], was used to pre-treat 3T3-L1 adipocytes for 30 min prior to addition of the treatments where indicated.

3.3.6 Akt/NFκB/ERK Phosphorylation

Total and phosphorylated Akt (Ser^{473}), ERK ($Thr^{202/204-185/187}$), and NFκB (Ser^{536}) were quantified directly in lysed cells using ELISA-based assays, as previously published [3,4]. This methodology allows direct evaluation of both phosphorylated and total forms of the proteins simultaneously, reducing the technical manipulations of protein extraction, homogenization, and gel separation used in traditional western blot analysis.

Akt and NFκB activation were measured in 3T3-L1 adipocytes by Fast Activated Cell-based ELISA kit (Active Motif, Carlsbad, CA) as described by the manufacturer. Briefly, cells seeded in 96-well plates were stimulated with ASP (200 nM) and/or C5a (20 nM) for the indicated incubation times, and fixed with 4% formaldehyde. Following washing and blocking steps, cells were incubated overnight with anti-phospho or anti-total Akt and NFκB. Following incubation with anti-HRP conjugated IgG for 1 h at room temperature, a colorimetric assay was performed and absorbance at 450 nm was determined. The values were subsequently corrected for cell number using the absorbance values at 595 nm determined by crystal violet cell staining.

Akt, ERK, and NFκB phosphorylation in primary adipocytes were analyzed by InstantOne ELISA kits purchased from eBioscience (San Diego, CA), following the manufacturer's protocol for non-adherent cells. In summary, freshly isolated primary adipocytes were seeded in 96 well plates (20,000 cells/well), treated for stimulation, then lysed and incubated with antibodies against phospho- and total-Akt/ERK/NFκB overnight

and following colorimetric processing, absorbance readings were obtained at 450 nm/650 nm.

3.3.7 Hormone Measurements

MCP-1 and KC concentrations were measured using mouse ELISA kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Differentiated 3T3-L1 adipocytes and freshly isolated primary adipocytes (100,000 cells/1 mL) were incubated overnight in SF media with the indicated treatment concentrations, and the media was then collected. All samples were assayed in triplicate. Following colorimetric processing (according to the instructions), optical density was determined by a microplate reader set to 450 nm with the wavelength correction set to 540 nm.

3.3.8 RNA Extraction and qPCR Analysis

RNA was extracted from the whole gonadal fat pad of C5aRKO and WT mice (RNeasy Plus Universal Mini Kit) and cDNA was synthesized using RT^2 First Strand kit (Qiagen Inc., Mississauga, ON, Canada). mRNA expression level of each gene was quantified by CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada). Primers were designed using the Mouse qPrimerDepot resource site (mouseprimerdepot.nci.nih.gov) and were purchased from Alpha-DNA (Montreal, Canada). Results were analyzed by the ΔΔCt relative quantification method and normalized to *Eef2* As housekeeping gene. Primer sequences were as follows: mouse *IRS1*, forward 5'-CTATGCCAGCATCAGCTTCC-3' and reverse 5'-GGAGGATTTGCTGAGGTCAT-3'; mouse *PGC1α*, forward 5'-TGTAGCGACCAATCGGAAAT-3' and reverse 5'-TGAGGACCGCTAGCAAGTTT-3'; mouse *Eef2*, forward 5'-GCTTCCCTGTTCACCTCTGA-3' and reverse 5'- CGGATGTTGGCTTTCTTGTC-3'.

3.3.9 Statistical Analysis

All values are presented as mean ± standard error of the mean (SEM), with 5-9 mice per group, or multiple wells from cell experiments (as indicated). Groups were compared

using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA) by two-way analysis of variance (ANOVA) followed by Bonferroni post-test, or by Student's t test, as indicated. Statistical significance was indicated as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.4 Results

3.4.1 C5aR is Required for C5a Metabolic, Immune and Signaling in Adipocytes

As C5a has been shown to interact with both C5aR and C5L2, receptors involved in immune and lipid storage functions, the effects of C5a on MCP-1 and KC production, triglyceride synthesis (TGS), as well as Akt, ERK and NFκB signaling pathways were evaluated. MCP-1 and KC secretion increased significantly in WT adipocytes treated with 20 nM C5a ($P < 0.001$) (FIGURE 3.1A and B) where values are presented as percentage of basal levels for WT and C5aRKO individually. In adipocytes from C5aRKO mice, while basal MCP-1 and KC secretion from unstimulated C5aRKO adipocytes were significantly higher than WT (data not shown), the majority of C5a stimulation (expressed as % basal) was lost ($P < 0.001$, WT+C5a vs C5aRKO+C5a), although there still remained a small significant C5a effect as compared to basal levels (FIGURE 3.1A and B). C5a also increased TG storage, an important adipocyte function, in WT adipocytes ($P < 0.001$) (FIGURE 3.1C). By contrast, there was no stimulatory effect of C5a on TGS in C5aRKO adipocytes (FIGURE 3.1C). As shown in FIGURE 3.1D, in adipocytes from WT, C5a induced rapid phosphorylation of Akt on Ser^{473} by 5 min, reaching a peak at 30 min. In the absence of C5aR, C5a failed to induce Akt phosphorylation (two-way ANOVA, WT vs C5aRKO, P=0.005). There was no C5a activation of NFκB phosphorylation (Ser^{536}) in adipocytes from either WT or C5aRKO (FIGURE 3.1E). While there was a small stimulation of C5a on ERK phosphorylation ($Thr^{202/204\text{-}185/187}$) in WT adipocytes (FIGURE 3.1F), interestingly, the C5a impact on ERK phosphorylation increased in C5aRKO adipocytes (two-way ANOVA, WT vs C5aRKO, $P<0.0001$).

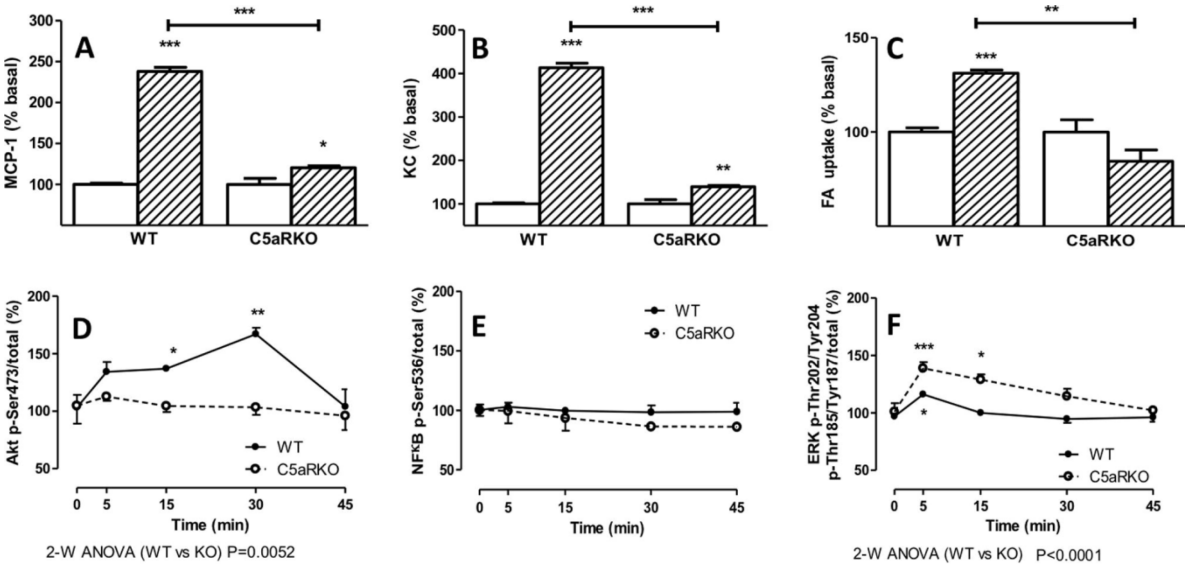

FIGURE 3.1 – C5aR is required for C5a signaling, metabolic, and immune functions. Mouse adipose tissue from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. *(A* and *B)* Mature adipocytes were treated with C5a for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. *(C)* Primary adipocytes were treated with C5a for 1 h, then uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured. *(D–F)* Freshly isolated adipocytes were seeded in 96-well plates (20,000 cells/well) and were treated with C5a (T = 0, 5, 15, 30, 45 min) at 37 °C, then Akt *(D)*, NFκB *(E)*, and ERK *(F)* phosphorylation and total protein were analyzed. Values are means ± SEM; white bars represent baseline with only PBS and striped bars represent C5a (20 nM). Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. BSL.

3.4.2 C5a Impaired ASP Activity in WT and C5aRKO Primary Adipocytes

ASP is known (i) to stimulate TGS through C5L2 interaction [19,28], (ii) to increase MCP-1 and KC secretion in adipocytes [4], and also (iii) to enhance colocalization of C5aR with C5L2 in adipocytes and macrophages [3]. Based on this, the effects of ASP and C5a combined were evaluated. Consistent with previous results, ASP alone strongly induced MCP-1 and KC production, as well as TGS in WT adipocytes (FIGURE 3.2A-C). In WT adipocytes, although addition of C5a had no effect on ASP stimulation of TGS (FIGURE 3.2C), C5a reduced the ASP effect on MCP-1 and KC (although still remaining significant). By contrast, in C5aRKO adipocytes, ASP effects on MCP-1 secretion and TGS were comparable, but were reduced for KC secretion although still remaining significant

(P<0.0001). However addition of C5a completely blocked all functional effects of ASP in C5aRKO adipocytes, with responses decreased to basal levels (FIGURE 3.2A-C).

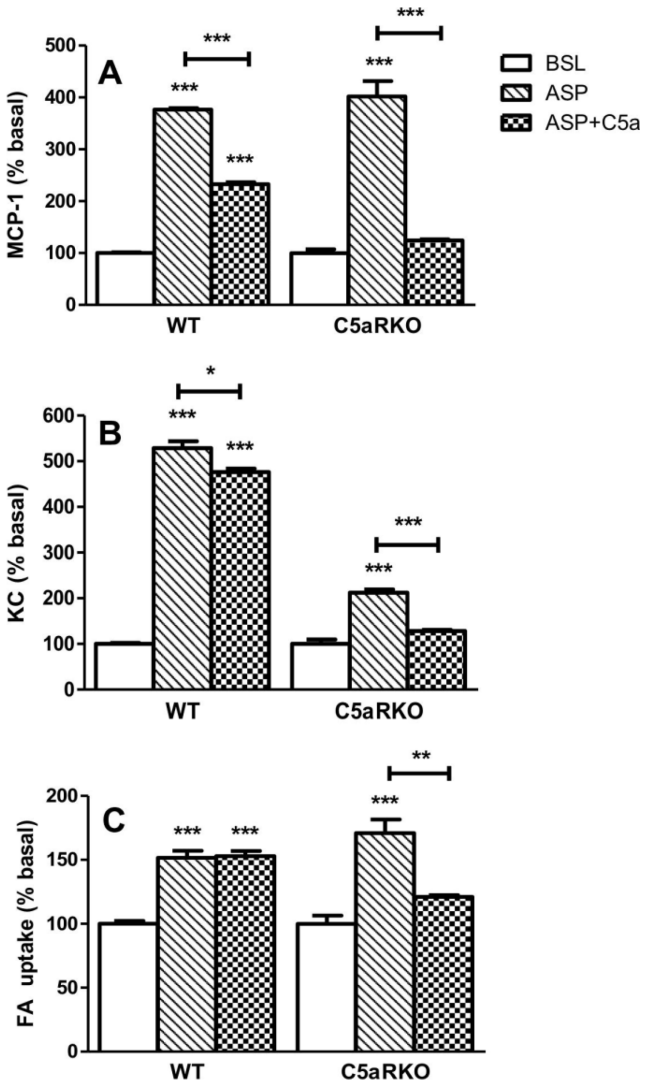

FIGURE 3.2 – C5a impaired ASP activity in WT and C5aRKO primary adipocytes.
(A and *B)* Mouse gonadal adipose tissue obtained from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. Mature adipocytes were treated with ASP and ASP + C5a for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. *(C)* Primary adipocytes were treated with ASP and ASP + C5a for 1 h then uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured. Values are means ± SEM, where white bars represent baseline with only PBS (BSL), striped bars represent ASP (200 nM), and checkered bars represent ASP (200 nM) + C5a (20 nM). Significant differences were analyzed by t-test: $^{**}P <$ 0.01, $^{***}P < 0.001$ vs. BSL.

3.4.3 C5a Blocked ASP Signaling Pathways in C5aRKO and WT Primary Adipocytes

Based on the C5a interference with ASP functionality, signaling pathways were further assessed to determine the mechanism of negative C5a feedback. As shown in FIGURE 3.3A, while ASP stimulated time-dependent Akt (Ser^{473}) phosphorylation in WT adipocytes, addition of C5a partially blocked the ASP effect. Similarly, ASP stimulation of NFκB (Ser^{536}) phosphorylation in WT adipocytes was blocked by addition of C5a (FIGURE 3.3D). By contrast, in C5aRKO adipocytes, there was little effect of either ASP or ASP+C5a (FIGURE 3.3B and E), as with the lack of C5a response (FIGURE 3.3C and F). ERK activation followed a different profile: while there was no significant change in phospho-ERK relative to total ERK between ASP treated versus ASP+C5a stimulated adipocytes (FIGURE 3.3G and H), adipocytes from C5aRKO mice were more responsive than WT, regardless of the ligand stimulation (ASP, ASP+C5a or C5a, FIGURE 3.3I).

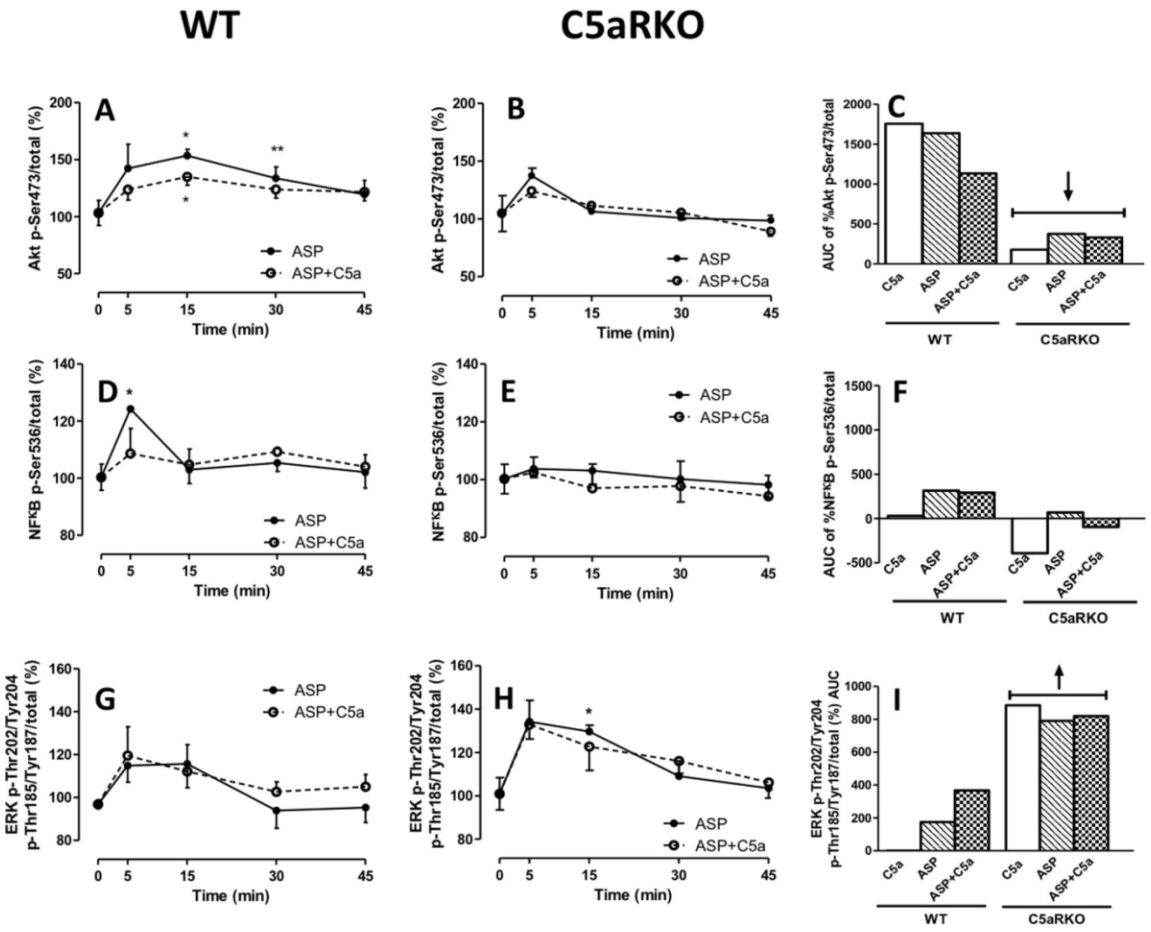

FIGURE 3.3 – C5a blocked ASP signaling pathways in C5aRKO and WT primary adipocytes.

Freshly isolated primary adipocytes from WT and C5aRKO mice were seeded in 96-well plates (20,000 cells/well) and were treated with ASP (200 nM) or the combination of ASP (200 nM) and C5a (20 nM) for 5, 15, 30, and 45 min at 37 °C, then Akt *(A* and *B)*, NFκB *(D* and *E)*, and ERK *(G* and *H)* phosphorylation and total protein were analyzed. The ratio of phosphorylation/total (%) area under the curve (AUC) for Akt *(C)*, NFκB *(F)*, and ERK *(I)* in WT and C5aRKO adipocytes are presented. Treatments: C5a (white bars), ASP (striped bars), and ASP + C5a (checkered bars). Values are presented as means ± SEM. Significant differences were analyzed by t-test or two-way ANOVA: $*P < 0.05$, $**P < 0.01$ vs. BSL.

3.4.4 C5a Interferes with ASP Function and Signaling in 3T3-L1 Adipocytes

Similar experiments were also conducted in 3T3-L1 adipocytes to confirm the results obtained from primary adipocytes. As shown in FIGURE 3.4A-D, C5a and ASP both independently increased MCP-1 and KC secretion as well as Akt and NFκB phosphorylation. However, treatment of 3T3-L1 adipocytes simultaneously with ASP and C5a significantly diminished the ASP effects on MCP-1 and KC secretion as well as on Akt and NFκB phosphorylation. Interestingly, treating the cells with the combination of ASP and 3D53, a specific C5aR antagonist that does not bind either C5L2 or C3aR [29], blocked the ASP effect, decreasing TGS to basal levels (FIGURE 3.4E and F).

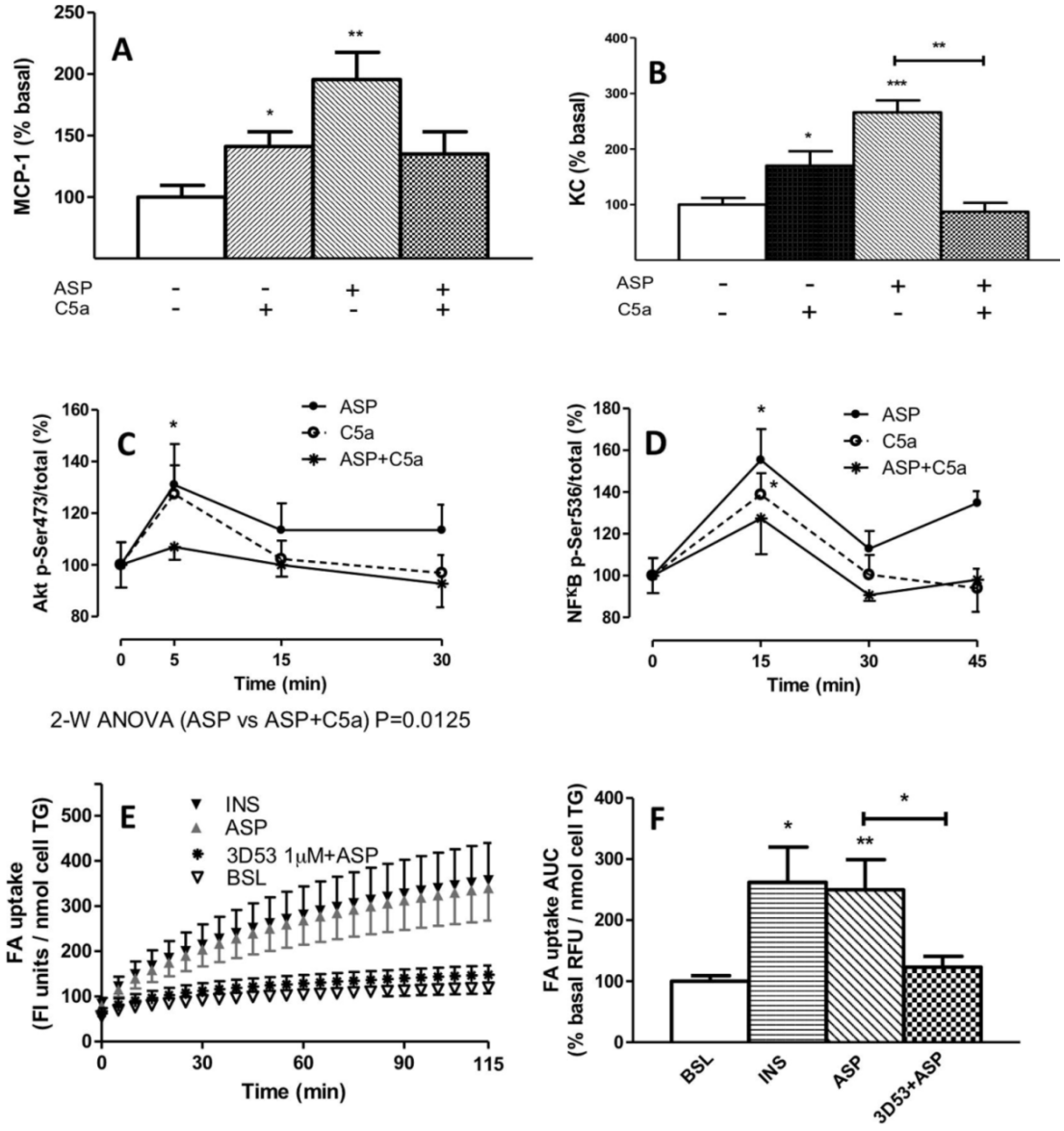

FIGURE 3.4 – C5a interferes with ASP function and signaling in 3T3-L1 adipocytes.
3T3-L1 adipocytes were differentiated and then incubated in serum-free media 2 h prior to treatments. Treatments: PBS (BSL), insulin (INS; 100 nM), ASP (200 nM), and C5a (20 nM). *(A* and *B)* 3T3-L1 adipocytes were treated with C5a and/or ASP for 24 h, then MCP-1 and KC secretion were evaluated in cell culture medium. 3T3-L1 adipocytes were treated with either ASP (T = 0, 5, 15, 30, 45 min) and/or C5a (T = 0, 5, 15, 30, 60, 90) at 37 °C, then Akt *(C)* and NFκB *(D)* phosphorylation and total protein were measured. *(E)* 3T3-L1 adipocytes were pre-treated with C5aR antagonist (3D53; 1 μM) for 30 min prior to addition of the treatments. Cells were incubated with the treatments for 1 h at 37 °C. Uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured over 115 min. *(F)* Results are presented for fatty acid uptake area-under-the-curve (AUC). Values are means ± SEM. Significant differences were analyzed by t-test or two-way ANOVA: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. BSL.

3.4.5 C5aR Disruption Induced Partial Insulin Resistance in Primary Adipocytes

As demonstrated above the presence of C5aR influenced most adipocyte immune and metabolic responses; this suggested a potential involvement of C5aR receptors in insulin resistance. To address this possibility, adipocytes from WT and C5aRKO mice were assessed for insulin effects on cytokine production, TGS, signaling pathways, and gene expression. As shown in FIGURE 3.5, insulin stimulated MCP-1 and KC secretion (FIGURE 3.5A and B), TGS (FIGURE 3.5C), and phosphorylation of Akt (FIGURE 3.5D), NFκB, and ERK (FIGURE 3.5G and H) in WT adipocytes. Further, the potential direct effects of C5a on insulin functionality in WT primary adipocytes were tested. As presented in FIGURE 3.5E and F, simultaneous addition of C5a to insulin treatment did not alter the robust insulin effects on MCP-1 and TGS. Interestingly, C5aRKO adipocytes revealed impaired insulin stimulation of MCP-1 and KC production (FIGURE 3.5A, B) (although basal MCP-1 and KC levels were greater in C5aRKO) and NFκB phosphorylation on Ser^{536} (FIGURE 3.5G) with partial impairment of TGS (FIGURE 3.5C) and Akt phosphorylation (Ser^{473}) (FIGURE 3.5D). Related to this, mRNA expression of *IRS1* and *PGC1α* was lower in adipose tissue from C5aRKO mice (FIGURE 3.5I). By contrast, as shown in FIGURE 3.5H, there was an increase of insulin-stimulated ERK phosphorylation ($Thr^{202/204-185/187}$) in C5aRKO mice in comparison to WT (two-way ANOVA, P=0.04).

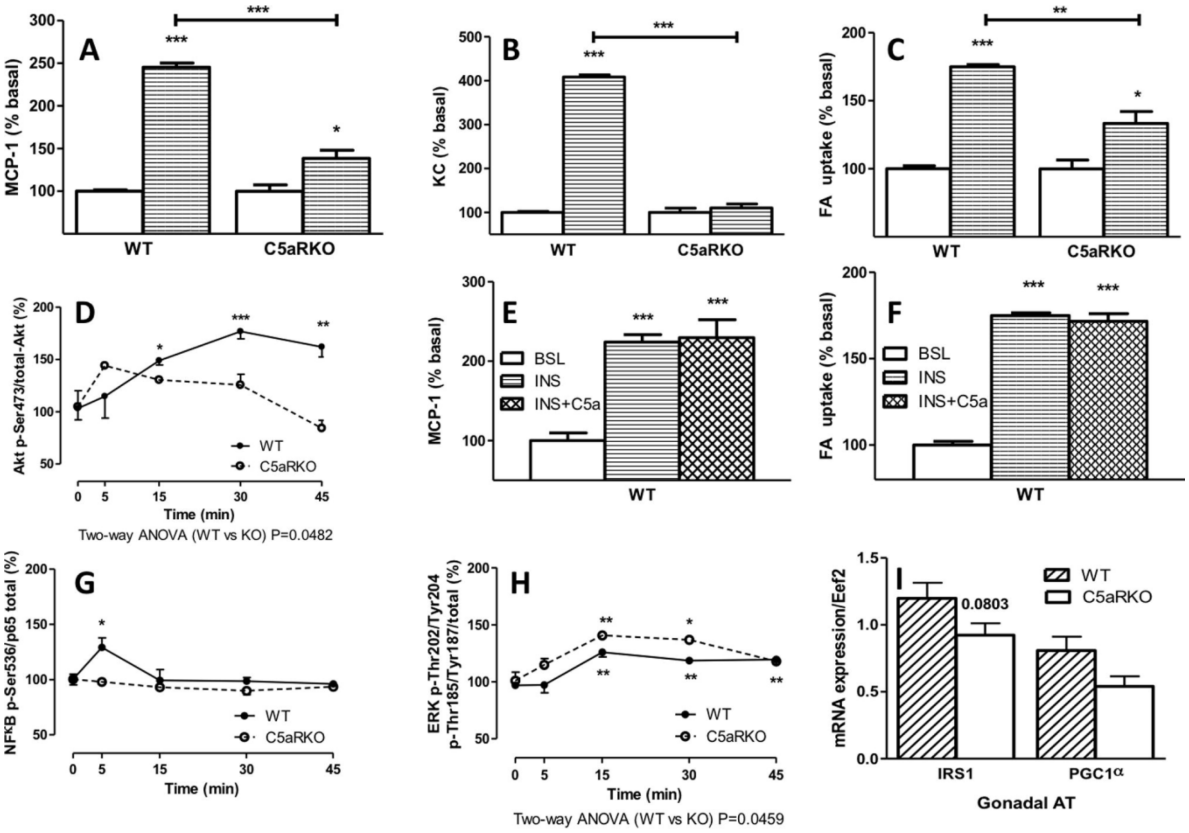

FIGURE 3.5 – C5aR disruption induced partial insulin resistance in primary adipocytes. *(A, B* and *E)* Mouse gonadal adipose tissue from WT and C5aRKO mice was dissected, and primary adipocytes were prepared by collagenase digestion. Mature adipocytes were treated with insulin or insulin + C5a for 24 h, and then MCP-1 and KC secretion were evaluated in cell culture medium. *(C* and *F)* Primary adipocytes were treated with insulin or insulin + C5a for 1 h then uptake and incorporation of fluorescently-labeled fatty acid into lipids was measured. White bars represent baseline with only PBS treatment, striped bars represent insulin (100 nM) treatment, and cross-hatched bars represent insulin (100 nM) + C5a (20 nM). Freshly isolated primary adipocytes were seeded in 96-well plates (20,000 cells/well) and were treated with insulin for 5, 15, 30, and 45 min at 37 °C, then analyzed for Akt *(D),* NFκB *(G),* and ERK *(H)* phosphorylation and total protein. *(I) IRS1* (insulin receptor substrate 1) and *PGC1α* (peroxisome proliferator- activated receptor-γ co-activator 1-α) gene expression was evaluated in adipose tissue obtained from WT (striped bars) and C5aRKO (white bars) mice by RT-quantitative PCR, expressed relative to the housekeeping gene Eef2 (eukaryotic elongation factor 2). Values are presented as means ± SEM; n = 5–9 per group. Significant differences were analyzed by t-test or two-way ANOVA: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. BSL.

3.5 Discussion

The present study extends previous findings for C5aR and C5L2 interaction, by proposing new insights into the C5a-C5aR role in adipocyte metabolism and immunity, and demonstrating regulatory impact on ASP and insulin-mediated functions. The close physical interaction of C5aR and C5L2 has been recently demonstrated in several studies, with heterodimerization of C5aR and C5L2 in transfected cells [3], colocalization of internalized C5aR with both C5L2 and β-arrestin in neutrophils [12], and internalization/colocalization of C5aR and C5L2 in C5a- or ASP-stimulated macrophages and adipocytes [3]. Although there are only a few studies using C5aR and/or C5L2 antibodies/antagonists or knockout models, the results are consistent with coupling of immune and metabolic aspects in adipocytes [28-30]. However, the individual contributions of C5aR and C5L2 to adipose tissue functions and signaling are not yet clear. Roy *et al.* suggested an immune-metabolic role for C5aR in energy expenditure and fat storage in *in vivo* studies using C5aRKO mice [30], while Lim *et al.* demonstrated C5a effects on lipid and glucose metabolism in adipocytes by using a C5aR-selective antagonist which interfered with C5a-mediated fatty acid uptake [29]. In the present study, where the potential cross-talk between C5aR and C5L2 in adipocytes was evaluated, the use of C5aRKO models allowed us to investigate (i) C5a-C5L2 function and signaling, (ii) C5a effects on ASP function and signaling (through C5L2), and (iii) the potential role of C5aR on insulin sensitivity in adipocytes.

ASP is well-known for its metabolic mediator roles in obesity, atherosclerosis, and type 2 diabetes [31-32]. C5a, on the other hand, is a well-appreciated immune mediator, possessing regulatory roles in sepsis and other immunoinflammatory disorders [33-37]. Interestingly, a C5a metabolic facet in adipocytes has recently been suggested based on its effects on lipogenesis, glucose uptake, and lipolysis [29]. These insulin-like effects in adipocytes have previously been well-established for ASP [18,19]. Moreover, Akt and ERK have been shown to be involved in insulin- and ASP-mediated TGS lipid storage pathways in 3T3-L1 preadipocytes [21]. Additionally, *in vitro* studies by Tom *et al.* suggested that ASP-mediated stimulation of the inflammatory factors MCP-1 and KC involved both NFκB and Akt mediated pathways [4]. In accordance with our findings, both C5a and ASP

independently enhanced adipocyte function and signaling, however the disruption of C5aR reduced the C5a effects on inflammatory cytokine secretion, TGS, and Akt phosphorylation, whereas ASP effects remained mostly intact. Collectively, we speculate that the signalling/functional responses to C5a in adipocytes are primarily mediated through C5aR, while C5a may interfere with ASP function via C5L2 interaction. On the other hand, ASP effects, in certain cases, were manifested only in the presence of both C5aR and C5L2 receptors, possibly as heterodimers. Accordingly, *in vitro* assays with 3T3-L1 adipocytes pre-treated with a C5aR antagonist showed significantly reduced ASP effects on TGS. Of note, C5aR does not seem to play a role in C5a or ASP activation of ERK, since ERK phosphorylation remained comparable or greater in C5aRKO adipocytes, possibly pointing to a role for C5L2 in this process. Of relevance, neutrophils and macrophages obtained from C5L2KO mice showed absence of ERK phosphorylation following C3a stimulation, in contrast to WT mice [13].

This study has shown that C5a interfered with ASP functions and signaling in primary adipocytes and that the effects were more pronounced in C5aRKO adipocytes. In like manner, combination of C5a and ASP stimulation on 3T3-L1 adipocytes, negatively influenced ASP impact on cytokine secretion and signaling. Notably, ligand concentrations, cell surface GPCR expression levels, dimerization-induced conformational modulation, and desensitization processes have all been shown to influence ligand competitive effects [38,39]. Ligand binding could also potentially alter receptor conformation and subsequent interaction with a second ligand [40,41]. Further, we have recently demonstrated that C5L2 and C5aR are internalized upon stimulation by both C5a and ASP individually [3], which might suggest C5L2 desensitization and internalization post-interaction with either ASP or C5a. Since ASP and C5a do not appear to occupy the same binding site on C5L2 [42], then the C5a negative influence on ASP action could support the hypothesis that ASP does indeed bind to C5L2. This contrasts with the interpretation of other studies [43,44] reporting no such binding, but those studies were based solely on lack of competitive radioligand displacement with labelled C5a. If so, this would be consistent with C5a-C5aR/C5L2 exerting regulatory effects via the ASP-C5L2 pathway in adipocytes. This type of ligand-receptor blocking has been demonstrated elsewhere, and could be another model explaining the negative C5a regulatory effects. In an analogous fashion, a study by Pello *et al.*

indicated that the simultaneous ligand stimulation of CXCR4- and δ-opioid receptor (DOR)-expressing cells blocked the individual responses of the receptors. It was proposed that formation of CXCR4/DOR heterodimers, as a silent signaling complex, resulted following exposure to the combination of ligands [38].

Studies with C5L2KO mice have demonstrated a pathophysiological role for C5L2 in insulin resistance, lipid metabolism and sepsis [45-48]. Further, it is suggested that disruption of an ASP-C5L2 dependent signaling pathway may contribute to altered energy metabolism [31] and induce some beneficial effects [23,48]. By contrast, in the presence of a high-fat-high-sucrose diet, the absence of C5L2 has been shown to accentuate insulin resistance [45]. Further, recent *in vivo* studies with C5aR and C3aR selective inhibitors revealed an improved inflammatory profile, suggesting specific roles for C5aR and C3aR in metabolic dysfunction such as obesity [29]. This is in accordance with the recently published study showing that C5aRKO mice have decreased adipose tissue weight, lower plasma lipids, higher energy expenditure, and reduced fat storage [30]. Based on our current results, co-treatment with C5a and insulin maintained elevated insulin sensitivity of WT adipocytes. However, in the absence of C5aR, adipocytes appear to lose the pro-inflammatory effects of insulin on MCP-1 and KC production, and NFκB phosphorylation, while maintaining ERK activation along with a trend towards lower expression of genes responsible for metabolic disorders, including insulin resistance (*IRS1* and *PGC1α*). Interestingly, survival in high-grade (100% lethality) sepsis required simultaneous blockade of C5aR and C5L2 receptors [37]. Thus, synergic pro-inflammatory contributions of C5aR and C5L2 in sepsis might reflect an adaptive C5L2 mechanism in response to chronically increased levels of C5a.

3.6 Conclusion

In conclusion, these novel findings support a model depicted schematically in FIGURE 3.6, in which C5aR is associated with C5a-dependent signaling which could be regulated by heterodimerization with C5L2. The negative influence of C5a and ASP co-stimulation on key downstream signaling pathways and adipocyte activity in both hetero- and homodimerization conditions has also been summarized (FIGURE 3.6: with conclusions

drawn from FIGURES 3.2-3.4). Preclinical/clinical trials have been undertaken to target C5a-C5aR interaction, following development of compounds acting on either C5 (to prevent its cleavage), or on C5aR (to prevent activation by C5a). The chronic antagonism of C5aR may adversely affect host defense and other beneficial complement functions, however this has recently been addressed by developing alternative tissue specific antibody therapies [49]. Given the potential therapeutic value of inhibiting C5a-C5aR function to prevent or slow progression of inflammation [15], further studies are essential to better understand how C5a-C5aR/C5L2 alternative pathways adjust to different pathophysiological environments, and might have unexpected influences on adipose tissue and metabolism.

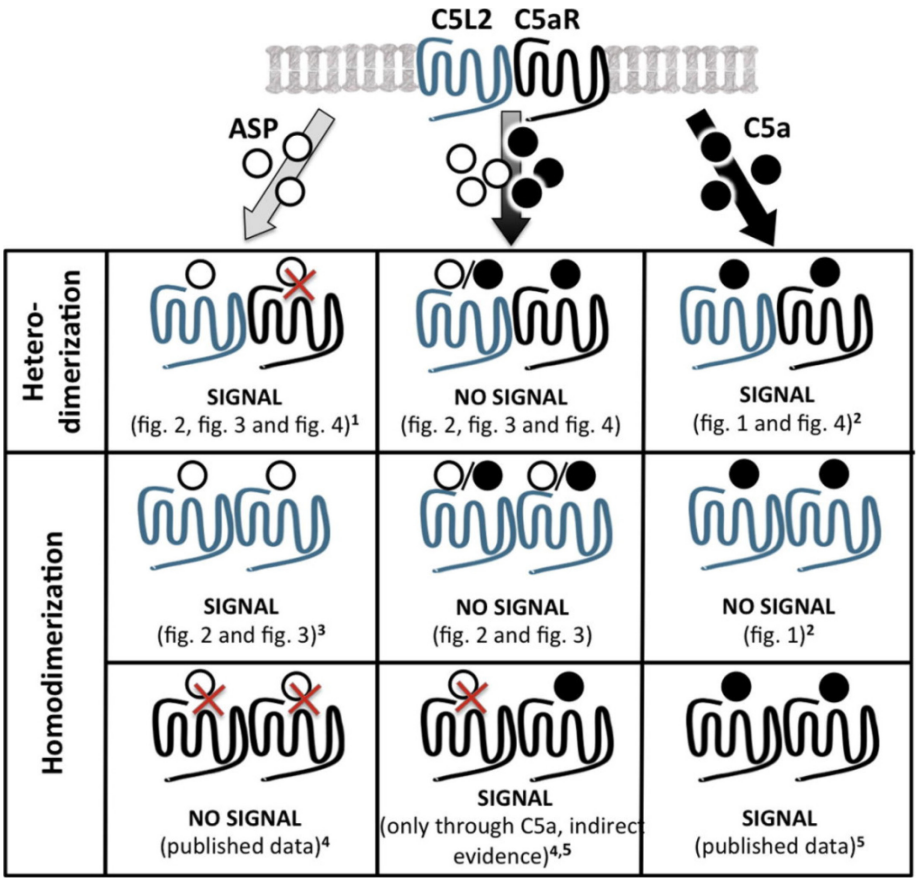

FIGURE 3.6 – Schematic representation of proposed interactions between C5aR and C5L2. Summary of results from FIGURES 3.2, 3.3 and 3.4 (C5a, ASP, and ASP + C5a effects) in addition to published data on ASP and C5a effects based on *in vitro* and *ex vivo* experiments on 3T3-L1 and primary adipocytes from WT, C5aRKO and C5L2KO models as well as transfected cells and

treatment using C5aR-specific antagonists. Data supported by: 1([3,4,21]); 2([11,29]); 3([19]); 4([28,48]); 5([11,15,47]).

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CHAPTER 4

Association of Receptors C5aR and C5L2 with Adiposity in Women

Poursharifi, P. *et al.* Association of Receptors C5aR and C5L2 with Adiposity in Women. *Mediators Inflamm.* **2014**, 413921 (2014).

Résumé

Des études ont déjà évalué la fonction des récepteurs C5L2 et C5aR ainsi que leur hétérodimérisation au niveau du tissu adipeux suite à une interaction avec leurs ligands (C5a et la protéine stimulant l'acylation (ASP)) chez des modèles cellulaires et animaux. Malgré cela, leur contribution chez l'humain à l'obésité et aux facteurs qui la compose, demeure nébuleuse. Nous offrons l'hypothèse que ces récepteurs, déjà associés à la défense immunitaire, seraient aussi associés à l'adiposité.

Nous avons donc récolté des données anthropométriques ainsi que mesuré différents paramètres sanguins chez 136 femmes que nous avons séparé en quatre groupes selon leur indice de masse corporelle (IMC): normal/surpoids (≤30kg/m2; n=34), obèse I (≤45 kg/m2; n=33), obèse II (≤51 kg/m2; n=33), et obèse III (≤80 kg/m2; n=36). L'expression des récepteurs C5L2 et C5aR dans les tissus adipeux omental et sous-cutané a été analysée.

L'expression de C5L2 s'est démontrée comparable entre les tissus adipeux sous-cutané et omental et aucune difference n'a été observée entre les groupes d'IMC. Par contre, les niveaux plasmatiques d'ASP et le ratio ASP/C5L2 (expression omentale) augmentait avec une hausse de l'IMC ($P<0.001$ et $P<0.01$, respectivement). Les niveaux de C5a en circulation restait similaires entre les groupes tandis que l'expression du récepteur C5aR diminuait dans les tissus adipeux omental et sous-cutané ($P<0.01$ and $P<0.05$, respectivement) lorsque l'IMC augmentait, et ce, toujours en présentant une quantité plus grande dans le tissu sous-cutané versus omental. Le ratio de C5L2/C5aR au niveau du tissu omental augmentait lui aussi avec l'IMC ($P<0.01$) et corrélait de façon significative avec le tour de taille et les niveaux sanguins de HDL-C et d'adiponectine des sujets.

Les différences observées au niveau de ces récepteurs et de leurs ligands, variant selon le tissu ou le degré d'obésité, particulièrement dans le tissu adipeux omental, suggèrent l'existence de rôles possibles de ceux-ci en lien avec des perturbations métaboliques reliées à l'obésité. De plus, ces différences mettent en valeur l'interaction qui existe entre le tissu adipeux et le système immunitaire.

4.1 Abstract

Adipose tissue receptors C5aR and C5L2, and their heterodimerization/functionality and interaction with ligands C5a and acylation stimulating protein (ASP) have been evaluated in cell and rodent studies. Their contribution to obesity factors in humans remains unclear. We hypothesized that these C5a receptors, classically required for host defense, are also associated with adiposity.

Anthropometry and fasting blood parameters were measured in 136 women divided by body mass index (BMI): normal/overweight (≤30kg/m^2; n=34), obese I (≤45 kg/m^2; n=33), obese II (≤51 kg/m^2; n=33), and obese III (≤80 kg/m^2; n=36). Subcutaneous and omental adipose tissue *C5aR* and *C5L2* expression were analysed.

C5L2 expression was comparable between subcutaneous and omental across all BMI groups. Plasma ASP and ASP/omental *C5L2* expression increased with BMI (P<0.001 and P<0.01, resp.). While plasma C5a was unchanged, *C5aR* expression decreased with increasing BMI in subcutaneous and omental tissues (P<0.01 and P<0.05, resp.), with subcutaneous>omental depots. Omental *C5L2/C5aR* ratio increased with BMI (P<0.01) with correlations between *C5L2/C5aR* and waist circumference, HDL-C, and adiponectin.

Tissue and BMI differences in receptors and ligands, particularly in omental, suggest relationship to metabolic disturbances and highlight adipose-immune interactions.

4.2 Introduction

The classical heat insulator and fat storage organ, adipose tissue, is now recognized as an active metabolic regulator, which synthesizes and/or secretes various cytokines and hormones [1]. White adipose tissue is found subcutaneously throughout the body, while internal organs are surrounded by omental or visceral adipose tissue. Increased omental fat mass (central obesity) strongly contributes to the pool of circulating inflammatory adipokines associated with metabolic complications such as dyslipidemia, insulin resistance, type 2 diabetes, and increased risk of metabolic syndrome [2,3]. The precise mechanisms linking omental depots and metabolic complications are yet unclear. However, recent emphasis on "immunometabolism" has become a major focus of both metabolic and immunologic research, with the demonstration of crosstalk between adipokines and the innate immune system (including complement components) [4,5].

One example of a protein bridging immunity and metabolism is acylation stimulating protein (ASP), an adipose tissue-derived hormone, which is the product of complement component C3 cleavage [6]. Circulating ASP levels are associated with atherosclerosis, type 2 diabetes, and are increased by several fold in obese versus normal weight controls [6,7]. ASP manifests its insulin-like effects on differentiated human adipocytes via the receptor C5L2 [8]. However, the most potent anaphylatoxin, complement C5a, also binds C5L2, as well as its own classical receptor C5aR [9,10]. C5a is a multifunctional protein, stimulating chemotaxis, enzyme/cytokine release, and the respiratory burst [10]. Pathological conditions such as sepsis and various immunoinflammatory disorders are accompanied by increases in circulating C5a [11–13].

C5L2, initially proposed as a nonfunctional receptor, has been shown to be actively involved in inflammatory conditions such as insulin resistance, asthma, and coronary artery disease [14,15]. Accumulating evidence demonstrates direct interactions between C5L2 and C5aR [16], and this has been implicated in inflammatory conditions such as sepsis [13,17]. Recently, the well-defined pro-inflammatory C5a-C5aR pathway has been targeted for pharmacological therapy via inhibition of C5 cleavage, C5a blocking antibodies or C5aR antagonists for treatment of sepsis, cardiovascular diseases, autoimmune disorders, asthma,

and psoriasis [18,19]. However, the consequences of interfering with the C5a-C5aR pathway could also have a metabolic impact on C5L2 signaling and this requires clear knowledge and consideration of C5L2 and its ligand- and tissue-specific effects.

Given the documented homo- and heterodimerization of C5L2 and C5aR, and the resulting potential alternative signaling pathways in adipocytes [16,20,21], the C5L2/C5aR ratio was evaluated in a human study. We hypothesized that proportional expression of *C5L2* relative to *C5aR* would vary between specific adipose tissue depots and would be influenced by obesity. As such, this was evaluated in both subcutaneous and omental adipose tissues and its association with metabolic factors in adult women over a wide range of BMI values was investigated.

4.3 Methods

4.3.1 Subjects

Samples were obtained from (i) severely obese women who had undergone weight-loss surgery (biliopancreatic diversion, BPD) at the CRIUCPQ (Centre de Recherche de l'Institut Universitaire de Cardiologie et Pneumologie de Québec) and (ii) healthy women who had undergone elective surgery at the Gynecology Unit, Laval University Medical Center. All patients met the following eligibility criteria for entry into the study: women aged between 21 and 69 years, nondiabetic, not taking medication for dyslipidemia, had not previously undergone ovarectomy, and with availability of matched blood and adipose tissue samples. The total group contained samples from 136 women, with body mass indexes (BMI; weight/height^2) ranging from 19.5 to 78.9 kg/m^2. Research protocols were approved by the CRIUCPQ and CHUL institutional review boards. Subjects with severe obesity were recruited through our institution-approved tissue bank for the study of the causes and consequences of obesity (http://www.criucpq.ulaval.ca/index.php/en/tissue-bank). All the participants provided written informed consent prior to the enrollment.

4.3.2 Study design

Individuals were classified into quartile groups based on their BMI. In addition to the normal/overweight category (n=34), defined as a BMI of less than or equal to 30 kg/m^2, additional obese groups were defined as follows: BMI>30 to BMI≤45 (obese group I; n=33), BMI>45 to BMI≤51 (obese group II; n=33), and BMI>51 to BMI≤80 (obese group III; n=36).

4.3.3 Physical measures

Anthropometric measurements including body weight, height, and waist circumference were measured the day before surgery. BMI was calculated by standard formula (weight in kilograms divided by height in meters squared). Blood pressure was measured in the right arm with the participant seated after at least 5 minutes of rest. The average of two sequential measures was used.

4.3.4 Blood lipids and hormones

Blood samples were collected in a fasted state and immediately centrifuged to obtain plasma. Biochemical parameters (fasting plasma glucose, triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), apolipoprotein B (ApoB), and apolipoprotein A1 (ApoA1)) were measured by the hospital biochemistry laboratory (IUCPQ and CHUL, QC) according to validated clinical procedures. The remaining plasma was transferred to the research laboratory for the following measurements: adiponectin by commercial radioimmunological assay according to the manufacturers' protocol (Millipore, MA) and C5a by commercially available ELISA kit (BD Biosciences, San Jose, CA). Plasma ASP concentration was measured using an in-house sandwich ELISA following previously published methodology [22].

4.3.5 Tissues

Adipose tissue samples were obtained from the subcutaneous and omental depots during surgery. The adipose tissue samples were rinsed with sterile Krebs-Ringer-HEPES buffer, placed in liquid nitrogen, and frozen at -80^{o}C until analysis.

4.3.6 RNA extraction and Real Time qPCR

Omental and subcutaneous adipose tissues (maximum 100 mg) were homogenized in Qiazole lysis reagent (Qiagen, Mississauga, ON). Following mRNA extraction using RNeasy Plus Universal Mini Kit (Qiagen, Mississauga, ON), a total amount of 0.1 µg RNA was reverse transcribed to cDNA (final volume of 20 µL) using QuantiTect® Reverse Transcription Kit (Qiagen, Mississauga, ON). Genomic DNA contamination was eliminated by DNase treatment included in QuantiTec Reverse Transcription Kit. All Real-Time PCR reactions were performed in a 25 µL mixture containing cDNA (1 µL), RT^2 SYBR Green qPCR Master Mix (Qiagen, Mississauga, ON) (12.5 µL), RNase-free water (10.5 µL), and 0.5 µL of each primer. Negative controls (without cDNA or reverse transcription) were also performed. Three-step PCR amplification was conducted using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON) with the following instrumental settings: a denaturation step at 95°C for 10 min, 39 cycles of 95°C for 15 s, 55°C for 40 s, 72°C for 30 s, and a final extension step of 95°C for 10 s. C5L2 primers were purchased from Qiagen (GPR77: QT00243971, QuantiTect Primer Assay, Qiagen, Mississauga, ON). C5aR and GAPDH primers were ordered from Alpha-DNA (Montreal, QC) with the following sequences: C5R1 forward: 5'-GCCCAGGAGACCAGAACAT-3' reverse: 5'-TATCCACAGGGGTGTTGAGG-3', GAPDH forward: 5´-AAGGTGAAGGTCGGAGTCAA-3´ reverse: 5´- AATGAAGGGGTCATTGATGG-3´. Results were analysed by the ΔΔCt relative quantification method using Bio-Rad CFX manager software (version 1.5) (Bio-Rad Laboratories, Mississauga, ON) and normalized to *GAPDH* (housekeeping gene). All procedures followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines including specificity, appropriate controls, and assay performance [23].

4.3.7 Statistical analysis

All anthropometric measurements, plasma parameters, and adipose tissue gene expression data are expressed as mean ± SEM for normally distributed data and median interquartile range for nonnormally distributed data. Groups were compared by two-way analysis of variance (ANOVA) followed by Bonferroni posttest, one-way ANOVA, or Student's t-test, as indicated, using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Relationships between variables in each group were assessed by linear regression analysis using Pearson correlation. Statistical significance was indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, where P NS indicates no significant difference.

4.4 Results

4.4.1 Anthropometric and blood characteristics of normal/overweight and obese groups

TABLE 4.1 shows the anthropometric, lipid, and hormone characteristics of the normal/overweight and obese groups I, II, and III. There was no significant difference in the average age of the women in these four groups. In addition to the expected differences in BMI (assigned groups), there were marked differences in waist circumference and systolic and diastolic blood pressure between normal/overweight group and obese groups ($P<0.001$). While there was no significant difference between normal/overweight and obese groups (I, II, and III) for fasting glucose, total cholesterol, LDL-C, and ApoB, the obese groups had, however, significantly lower HDL-C, ApoA1, and adiponectin than normal/overweight women.

TABLE 4.1 – Anthropometric and blood characteristics of normal/overweight and obese groups.

Characteristic N = 136	N/O (*n* = 34)	Obese I (*n* = 33)	Obese II (*n* = 33)	Obese III (*n* = 36)
Age (yrs)	46.7±0.7	42.5±1.6	38.8±1.2	41.4±1.9
BMI (kg/m^2)	25.1 (22.5-27.7)	41.5 (35.5-43.2)***	48.6 (46.4-49.9)***	56.4 (53.4-63.2)***
Waist circumference (cm)	87.2±1.4	126.1±2.7***	141.5±1.5***	156.8±2.7***
BP systolic (mmHg)	115 (102-126)	132 (120-140)***	138 (129-146)***	139.5 (129-145)***
BP diastolic (mmHg)	70 (60-80)	83 (73-90)***	84 (77-93)***	84 (78-93)***
Glucose (mmol/L)	5.7 (5.1-6.1)	5.4 (4.6-6.0)	5.3 (4.8-5.7)	5.7 (5.0-6.8)
Cholesterol (mmol/L)	4.9±0.1	4.8±0.2	4.9±0.2	4.9±0.1
HDL-C (mmol/L)	1.7 (1.6-2.1)	1.5 (1.3-2.3)*	1.4 (1.2-2.2)**	1.4 (1.3-2.2)*
LDL-C (mmol/L)	2.8±0.1	2.8±0.2	2.8±0.1	2.8±0.1
Triglyceride (mmol/L)	1.5 (1.0-2.5)	1.9 (1.4-3.7)	2.2 (1.5-4.4)*	2.0 (1.4-3.8)
ApoB (g/L)	1.1 (0.9-1.4)	1.1 (0.7-2.2)	1.1 (0.7-1.6)	1.1 (0.7-1.9)
ApoA1 (g/L)	1.6 (1.4-1.8)	1.4 (1.2-1.8)**	0.9 (0.7-1.9)***	1.0 (0.9-1.9)***
Adiponectin (μg/mL)	22.6 (19.1-47.0)	16.8 (7.1-32.2)**	10.5 (6.6-12.2)***	8.5 (5.4-10.5)***

136 patients were classified into four weight categories based on their BMI: normal/overweight (BMI ≤ 30; n=34), obese group I (BMI > 30 to BMI ≤ 45; n=33), obese group II (BMI > 45 to BMI ≤ 51; n=33), and obese group III (BMI > 51 to BMI ≤ 80; n=36). Values are expressed as mean ± SEM for normally distributed data and median interquartile range for nonnormally distributed data. Results were compared by one-way ANOVA versus normal/overweight group where **P*<0.05, ***P*<0.01, and ****P*<0.001. BMI: body mass index; BP: blood pressure; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; apoB: apolipoprotein B; apoA1: apolipoprotein A1.

4.4.2 Circulating ASP and ASP to C5L2 ratio are associated with adiposity

As shown in FIGURES 4.1A and 4.1B, no significant differences in *C5L2* expression were observed between normal/overweight and obese groups in either subcutaneous or omental adipose tissues, and there was no significant difference between subcutaneous versus omental (*P*=NS, 2wayANOVA). However, relative to body mass index, plasma ASP increased by up to twofold in the obese III group (FIGURE 4.1C, linear trend *P*<0.001). The ASP/*C5L2* ratio, representing the ligand/receptor ratio, was calculated individually for each subject, and, as shown in FIGURE 4.1D, this ratio increased proportionately to BMI in omental adipose tissue (up to 300% in group III versus normal/overweight, linear trend *P*<0.01). By contrast, in subcutaneous adipose tissue, the ASP/*C5L2* ratio remained comparable in all four groups with no significant differences between normal/overweight and obese groups (FIGURE 4.1D).

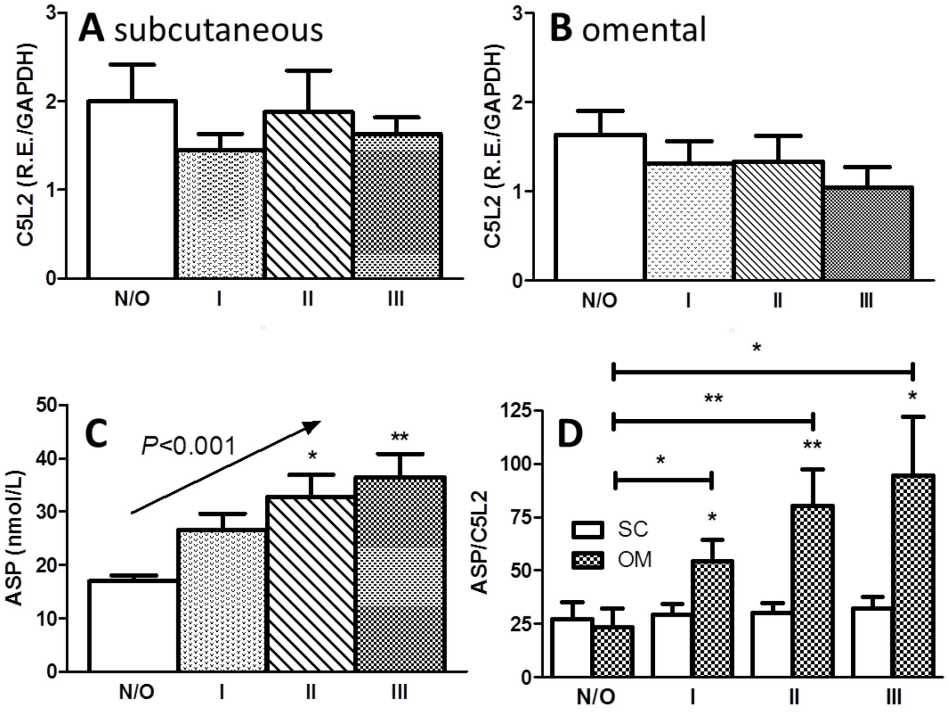

FIGURE 4.1 – Circulating ASP and ASP to *C5L2* ratio are associated with adiposity. Subcutaneous *(A)* and omental *(B) C5L2* mRNA expression for normal/overweight (open bar), obese group I (dotted bar), obese group II (striped bar), and obese group III (checkered bars), *(C)* plasma ASP for normal/overweight (open bar), obese group I (dotted bar), obese group II (striped bar), and obese group III (checkered bars), and *(D)* ASP/*C5L2* ratio in subcutaneous adipose tissue (open bars) and omental adipose tissue (checkered bars). Results are expressed as means ± SEM; n=33–36 per group. Statistical differences were determined by Student's t-test and one-way ANOVA, for normal/overweight versus obese groups and for SC versus OM groups, where **P*<0.05 and ***P*<0.001.

4.4.3 C5aR expression in both subcutaneous and omental adipose tissue is downregulated in obesity

As shown in FIGURE 4.2, *C5aR* expression decreased with increasing BMI in both subcutaneous and omental adipose tissues (FIGURES 4.2A and B, linear trend $P<0.01$ and $P<0.05$, resp.). In contrast to ASP, there was no significant difference in C5a concentration between normal/overweight and all levels of obesity (FIGURE 4.2C). Interestingly, *C5aR* expression in subcutaneous tissue was significantly greater than omental tissue at all levels of obesity ($P<0.05$, 2wayANOVA). Further, as demonstrated in FIGURE 4.2D, although

C5aR expression decreased in both tissues with increasing obesity levels, there was a proportionally greater decrease in omental tissue, such that the subcutaneous/omental ratio of *C5aR* expression tended to increase with increasing levels of obesity (linear trend $P<0.05$).

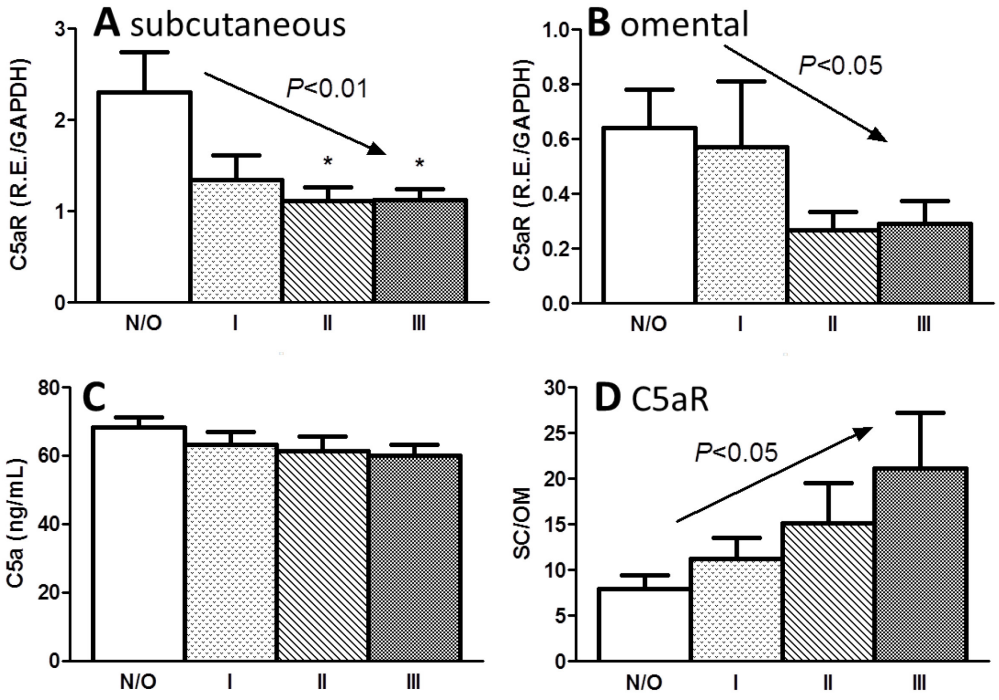

FIGURE 4.2 – *C5aR* expression in both depots is downregulated in obesity.
Subcutaneous *(A)* and omental *(B) C5aR* mRNA expression for normal/overweight (open bar), obese group I (dotted bar), obese group II (striped bar), and obese group III (checkered bars), *(C)* plasma C5a for normal/overweight (open bar), obese group I (dotted bar), obese group II (striped bar), and obese group III (checkered bars), and *(D)* subcutaneous/omental *C5aR* expression for normal/overweight (open bar), obese group I (dotted bar), obese group II (striped bar), and obese group III (checkered bars). Results are expressed as means ± SEM; n=33–36 per group. Statistical differences were determined by Student's -test and one-way ANOVA, for normal/overweight versus obese groups, where *$P<0.05$.

4.4.4 Omental C5L2/C5aR ratio as a potential marker of obesity

As C5L2 and C5aR heterodimerize [16], the ratio of C5L2/C5aR was evaluated. As shown in FIGURE 4.3A, omental *C5L2/C5aR* ratio differed significantly from subcutaneous *C5L2/C5aR* ratio (*P*=0.0012, 2wayANOVA). While the *C5L2/C5aR* ratio remained constant in subcutaneous tissue over the range in BMI, in omental tissue there was a significant increase in *C5L2/C5aR* ratio (linear trend *P*<0.01) (FIGURE 4.3A). There was also a significant increase in ASP/C5a ratio with increasing obesity (linear trend *P*<0.05; data not shown). Additionally, a positive correlation (r=0.259, *P*=0.003) between *C5L2/C5aR* ratios was observed between subcutaneous and omental adipose tissue (FIGURE 4.3B).

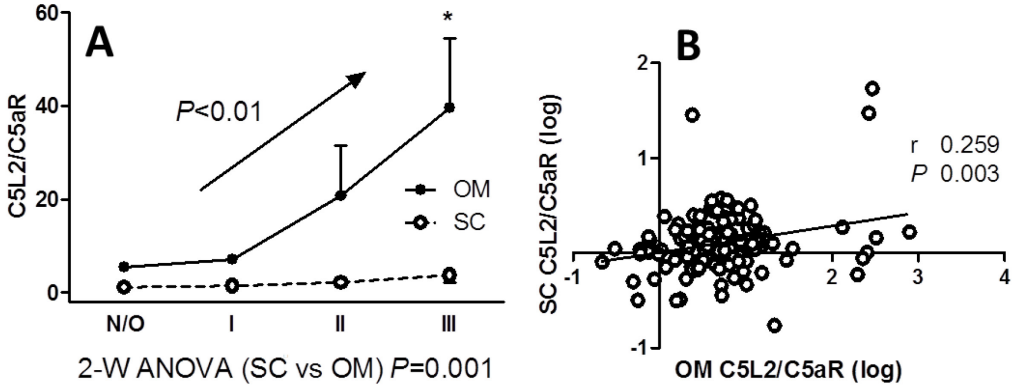

FIGURE 4.3 – Omental *C5L2/C5aR* ratio in relation to obesity.
(A) C5L2/C5aR ratio in omental adipose tissue (solid circles) versus subcutaneous adipose tissue (open circles), *(B)* linear regression analysis of subcutaneous versus omental *C5L2/C5aR* ratio (log-log values). Reported r and *P* values were calculated by Pearson correlation. Data are expressed as mean ± SEM and were compared by Student's t-test and one-way ANOVA (versus normal/overweight group), as well as two-way ANOVA (subcutaneous versus omental) where **P*<0.05.

4.4.5 C5L2/C5aR is associated with anthropometric indices, HDL and adiponectin

Omental *C5L2/C5aR* demonstrated positive associations with anthropometric parameters such as weight (r=0.262, *P*=0.002), BMI (r=0.223, *P*=0.009, FIGURE 4.4A), and

waist circumference (r=0.228, *P*=0.009). Comparable significant correlations were also found between subcutaneous *C5L2/C5aR* ratio and weight (r=0.318, *P*=0.0001), BMI (r=0.300, *P*=0.0004), and waist circumference (r=0.333, *P*=0.0001; FIGURE 4.4B). Furthermore, there were significant inverse relationships between omental *C5L2/C5aR* and plasma HDL-C (r=-0.172, *P*=0.048; FIGURE 4.4C), as well as subcutaneous *C5L2/C5aR* ratio and circulating adiponectin (r=-0.293, *P*=0.009; FIGURE 4.4D).

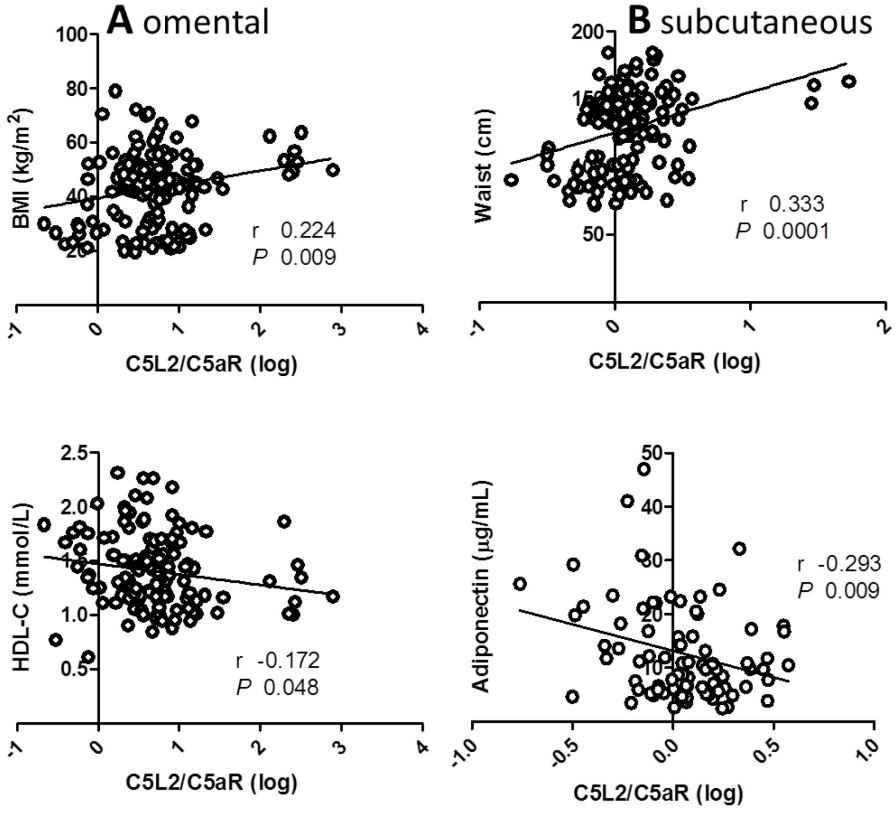

FIGURE 4.4 – *C5L2/C5aR* ratio is associated with anthropometric indices, HDL and adiponectin. Linear regression analysis of omental *C5L2/C5aR* versus BMI *(A),* subcutaneous *C5L2/C5aR* versus waist circumference *(B),* omental *C5L2/C5aR* versus HDL-C *(C),* and subcutaneous *C5L2/C5aR* versus adiponectin *(D)*. Reported r and *P* values were calculated by Pearson correlation.

4.5 Discussion

Despite the marked increase in bariatric surgery procedures in the last decade, the rate of severe obesity continues to increase and exceeds that of moderate obesity in the United States [24]. To add to that, recent clinical evidence indicates that complications stemming from obesity are not only related to the extent of fat accumulation but also to the pattern of fat distribution [25,26]. More recently, research detailing the contribution of the immune system to the observed obesity-induced inflammation has enhanced our understanding of this multifactorial disorder. This current study adds to this, with the emerging concept that C5a and its receptor C5aR, traditionally considered to be required only for host defense, are also associated with adipose tissue metabolic dysfunction, as discussed below. However, the limitations of the study should be noted: all data were obtained in women, and due to the cross-sectional nature of the analyses, cause-and-effect relationships cannot be determined. In addition, based on the limited availability of frozen adipose tissue in small quantities, the present study relied on *C5aR* and *C5L2* mRNA expression without addressing the possible posttranslational modifications and protein levels of the examined receptors. Further, as only frozen tissue was available, direct ligand functional assays in tissues could not be performed

It has been repeatedly shown that circulating ASP levels are altered in response to pathophysiological conditions in humans, including augmentation in obesity, cardiovascular disease, and type II diabetes (even in the absence of obesity), and their reduction with exercise or weight loss [6,7]. In the present study, not unexpectedly, plasma ASP increased markedly with increasing BMI. While the associated consequences of this increase in ASP in humans remain speculative, its causes can at least be partially explained by the observations that dietary fatty acids, chylomicrons, and insulin can increase ASP production [27,28]. Furthermore, obesity-associated adipose tissue metabolic complications such as an imbalance in lipogenesis/lipolysis, delayed triglyceride clearance, dysregulated adipokine, and C3 (ASP precursor) production, as well as fat accumulation, all exert profound impacts on ASP secretion [6,27]. Based on *in vitro* and *in vivo* experiments, ASP stimulates triglyceride synthesis and fat storage in adipose tissue, while disruption of the ASP-C5L2 pathway in mice resulted in delayed lipid clearance and redistribution of lipids

towards skeletal muscle for oxidation, a consequence which has been shown to be reversible with ASP administration [6,29].

Beyond the proposed role of C5L2 in lipid storage and adipose metabolism, Huber-Lang *et al.* demonstrated a reduction of C5L2 protein content in neutrophils during sepsis [30]. Likewise, *C5L2* expression was downregulated in neutrophils from patients with Familial Mediterranean Fever (an autoinflammatory syndrome) [31]. Furthermore, Raby *et al.* proposed a negative regulatory effect of TLR on *C5L2* expression following C5a stimulation [32]. Alteration of C5L2 expression in inflammatory-based disorders together with previous evidences that *C5L2* expression in adipocytes is regulated by differentiation, TNF-α, and rosiglitazone all indicate a potent pathophysiological role for the ASP–C5L2 pathway in adipose tissue inflammation [33,34]. Interestingly, Fisette *et al.* demonstrated that a combined high fat–high sucrose diabetogenic diet worsens the inflammatory state of *C5L2*(-/-) mice [35]. This phenotype, demonstrated experimentally in mice, is consistent with metabolic features of the obese women in the current study, exhibiting increased plasma ASP and a corresponding ASP/*C5L2* ratio increment in the omental fat depot. This coupling of increased ligand to decreased receptor is suggestive of a downregulated pathway, which could be consistent with an "ASP resistant" state in humans. In addition, as ASP is an important regulator of postprandial lipemia [6] an increase in basal plasma lipids in obesity could be the consequence of putative ASP resistance, analogous to the hyperglycemia in insulin-resistant states. A recent publication has provided a "proof-of-concept" of ASP resistance in diet-induced obesity [36]. Feeding wild-type mice a high fat-high sucrose diet led to a decrease in *C5L2* expression, increased plasma ASP, and reduced ASP functional activity as evidenced by decreased *in vivo* ASP-mediated postprandial fat clearance and decreased *in vitro* ASP-mediated Akt phosphorylation in gonadal fat depots following ASP injection [36]. Taken together with ASP proinflammatory effects on adipose tissue, such as stimulation of inflammatory cytokine production and macrophage infiltration/M1 polarization [37,38], the altered ASP/*C5L2* ratio in omental tissue may be both reflective of impaired ASP functionality as well as contributing to the dyslipidemia and metabolic disturbances of obesity.

Recent studies have demonstrated that *C5aR*(-/-) mice have decreased adipose tissue weight, lower plasma lipids, and reduced fat storage regardless of diet [39]. Further, administration of C5aR-selective antagonists in diet-induced obese rats resulted in weight loss and improvement in insulin resistance and adipose tissue inflammation [40]. Other studies indicate that C5a stimulates increased fatty acid [20,40] and glucose uptake in adipocytes, while inhibiting cAMP stimulation and lipid lipolysis [40]. These findings highlight the recently identified role of C5a-C5aR in metabolic disorders such as obesity, while supporting an anti-inflammatory role for C5aR antagonists in animal models of inflammatory diseases [41,42]. Blogowski *et al.* demonstrated a constant plasma level of C5a between lean, overweight and obese individuals [43], consistent with data presented here. However, the marked downregulation of *C5aR* in both subcutaneous and omental depots of obese women in this study raises an interesting question: could C5aR antagonists, which are currently being used in Phase I and II clinical trials for treatment of asthma, psoriasis, and rheumatoid arthritis [19], have additional metabolic-related effects? Further, the potential C5L2 regulatory impact on C5aR also needs to be taken into account.

We had hypothesized a potential physiological role for the *C5L2*/*C5aR* ratio, based on the following evidence: (i) *in vitro* studies on transfected cells have indicated the presence of constitutive C5aR-C5L2 heterodimers, in addition to evidence of cell-specific localization and the cointernalization/colocalization of C5aR and C5L2 following C5a or ASP treatment [16,21,44]; (ii) synergic contributions of both C5aR and C5L2 are required for the production of G-CSF during acute inflammation [17] and the harmful consequences and lethality observed during sepsis [45]; (iii) recent publications on C5aR or C5L2 knockout mice have emphasized that disruption of either receptor resulted in decreased expression of the complementary receptor in retroperirenal and gonadal adipose tissues but not in liver [39,46]. Moreover, elevated *C5L2* expression was shown to be accompanied by a likewise increase in *C5aR* gene expression in adipose tissue, muscle, and liver of wild-type mice on a diet-induced obesity (DIO) regimen [39]. Thus, while there appears to be a coupled relationship between C5L2 and C5aR, this relationship appears to be tissue-specific and can be regulated differentially based on disease conditions [31,47]. For example, in neutrophils from patients with Familial Mediterranean Fever, *C5L2* is decreased but not *C5aR* [31]. In kidney biopsies from patients with antineutrophil cytoplasmic antibody- (ANCA-)

associated glomerulonephritis, C5aR is downregulated but C5L2 is upregulated [47]. In the current study, there are adipose tissue depot-specific changes leading to increases in the *C5L2/C5aR* ratio, which are also associated with obesity indicators.

This issue of receptor dimerization has been suggested to regulate many aspects of receptor function including synthesis, ligand binding, and intracellular trafficking and downstream signaling [48]. However, the heterodimerization of C5L2-C5aR is a recently observed phenomenon and the functional consequences with respect to signaling pathways, particularly in obesity pathophysiology, are still unexplored. In adipocytes from C5aRKO mice, C5a induces a greater increase in ERK phosphorylation than in wild-type adipocytes [20]. In the present study the strong downregulation of *C5aR* along with ASP resistance may potentially increase C5a-C5L2 interaction which may further induce ERK phosphorylation. Interestingly, ERK/MAPK activation has been associated with the regulation of adipocyte differentiation, adiposity, high-fat diet induced obesity, as well as type 2 diabetes [49,50].

It is striking that the increased ASP/*C5L2* and *C5L2/C5aR* ratios occur specifically in omental adipose tissue. We speculate that the decreased C5aR relative to C5L2, in the face of maintained C5a levels, could divert C5a towards C5L2 interaction (promoting a proinflammatory response), simultaneously interfering with ASP action, increasing internalization/downregulation of C5L2, leading to compensatory increases in circulating ASP (as demonstrated in this study and others in obese subjects). In *vitro* studies in adipocytes demonstrated that the simultaneous treatment of C5aRKO adipocytes with ASP and C5a blocks the ASP-C5L2 pathway [20]. Accordingly, we hypothesize that this interference may consequently induce a state of "ASP resistance" in omental adipocytes from obese subjects, as evidenced by increased plasma ASP concentrations. Of note, ASP binding affinity and ASP functional stimulation are more pronounced in subcutaneous versus omental adipocytes [51], which supports the potential for ASP resistance in the omental depot. This could decrease the capacity of adipose tissue to efficiently uptake postprandial glucose and free fatty acid, which, coupled with inefficient anti-inflammatory C5L2 effects, could contribute to systemic inflammation associated with obesity and insulin resistance and is consistent with the associations with high levels of circulating lipids and lower adiponectin and HDL-C. Thus altogether, increased ASP/*C5L2* and *C5L2/C5aR*

ratios in omental adipose tissue are commensurate with the known pathophysiology of omental adiposity and its role in obesity-induced metabolic alterations [26].

4.6 Conclusion

Collectively, these findings shed light on the complexity of C5L2-C5aR interaction, providing further insight into the immunopathology of obesity while suggesting a potential role for the *C5L2/C5aR* ratio in omental adiposity. The balance between *C5aR* and *C5L2* expression can thus be observed in the ratio of *C5L2/C5aR* and is postulated to contribute to tissue-dependent ASP resistance and the adverse physiological effects that stem from it.

4.7 Bibliography

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CHAPTER 5

Discussion

5.1 Summary

Obesity is accompanied by the development of systemic chronic low-grade inflammation, differing from classic acute inflammation [11,183]. In addition to adipocyte hypertrophy, adipose tissue becomes an inflamed tissue characterized by immune cell (neutrophils, eosinophils and macrophages) infiltration and altered secretion of adipokines [69,184]. From the functional interface of cells of primarily immune (such as macrophages) and metabolic (such as adipocytes) natures, has emerged the new field of immunometabolism as a dynamic study of these two interwoven aspects of physiology [82–84,185]. Several components of the immune response including immune cells and cytokines contribute directly and significantly to the metabolic dysfunction seen in adipose tissue [82,84,185]. Over the last decade, increasing evidence points to multiple functions of the complement system, an ancient branch of innate immunity, beyond pathogen killing, such as role in metabolic disorders [98,99,186]. However this contemporary aspect of the complement cascade and its role in the complexity of obesity, especially adipose tissue metabolism, has yet to be clearly delineated. The aim of the present PhD thesis was to elaborate the importance of a particular aspect of the complement system in the course of adiposity, with a special focus on the complement receptors, C5aR and C5L2, and their ligands (C5a and ASP). To fulfill this objective, a series of studies were carried out utilizing cell culture, mice models, and human subjects.

5.2 New developments in C5aR and C5L2 interaction

5.2.1 Chapter 2: Innovations in C5aR and C5L2 physical interaction

Cell-based comparative experiments between 3T3-L1 adipocytes and J774 macrophages were performed to address the physical interaction between the two immunometabolic receptors, C5aR and C5L2. These *in vitro* assays permitted a level of simplification of the complex cellular and molecular networks, while providing the ability to control the testing environment. However, *in vitro* studies are not without limitations, as they are primarily confined to cell culture studies, outside of the natural environment, and the physiological implications can only be inferred. For instance, the co-culture and conditioned media approaches were used to mimic adipose tissue inflammation, where macrophage infiltration is present. Nonetheless, this still represents an imperfect scheme, and does not capture the adipose tissue inflammation observed in obese subjects in relation to heterogeneity in adipocyte size, diversity in macrophage/immune cell phenotypes and even gender and age.

CHAPTER 2 has answered many of our original questions regarding basic C5aR and C5L2 interaction in 3T3-L1 adipocytes and J774 macrophages. We reported, for the first time, that C5aR and C5L2 are capable of homo- and heterodimerization, based on BRET data obtained in HEK transfected cells. Just recently, another team has also demonstrated this phenomenon in both transfected and endogenously expressing cells [187], thus confirming our results. BRET is a newly developed energy transfer based technique that offers the possibility to study complex protein-protein interactions in living cells. It is a naturally occurring phenomenon without the need for an excitation light source. Therefore, the problems associated with FRET-based assays such as photobleaching and autofluorescence are ruled out [188]. In addition, immunofluorescence/microscopy approach in endogenously expressing cells was adopted to complement BRET data in transected cells. While others have reported the C5aR-C5L2 colocalization with C5a treatment in immune cells [150,187], CHAPTER 2 has presented this association in both adipocytes and macrophages upon ASP or C5a individual inductions.

Functionality of ASP through C5L2 and the related signaling mechanisms and metabolic consequences is now well-documented [121,125,126,129,138,145,189]. While C5L2 was initially postulated to act only as a recycling decoy receptor for C5a [132,147,181,190], more recent reports indicate regulation of both pro- and anti-inflammatory responses, depending on the disease state studied [151,165,166,173,182,191,192]. Interestingly, CHAPTER 2 has demonstrated that the signaling and functional outcome of C5aR and C5L2 association is both ligand- and cell-specific. For instance, only ASP, but not C5a, stimulates triglyceride synthesis in 3T3-L1 adipocytes. Further, ligand-dependent Akt phosphorylation has shown distinct profiles in 3T3-L1 adipocytes versus J774 macrophages. Since C5aR and C5L2 are capable of heterodimerization (CHAPTER 2) these signaling and functional effects cannot be specifically attributed to the individual receptors. Thus, it could be speculated that while ASP-C5L2 metabolic activities are well-documented, particularly in adipocytes, C5L2 may act as a less-functional receptor for C5a depending on the pathophysiological conditions. A recent paper emphasized the C5L2 dual inflammatory facet and hypothesized that at high concentrations of C5a, C5L2 may negatively modulate C5aR pro-inflammatory signaling, while at lower concentrations of C5a, it mediates pro-inflammatory responses [187]. It should be mentioned that the extremely high concentration of C5a that was used in their study (500 nM) [187], may not be an accurate implication of C5a physiological or even pathophysiological concentrations (which range from 1-10 nM in humans [193]). Despite the significance of their hypothesis, which could explain some of the controversial pro- *vs* anti-inflammatory roles of C5L2, they have not addressed ASP-C5L2 pathway and its regulatory effects on C5a activities.

Finally, by assessing conditioned media and direct co-culture experiments in adipocytes and macrophages, the extent of C5aR-C5L2 colocalization altered in a ligand-specific manner. These results have opened a wide window of possibilities regarding C5aR and C5L2 physical interaction and their associated functional/signaling consequences under inflammatory conditions such as obesity, where adipose tissue is inflamed by immune cell migration. Accordingly, previous findings suggested that ASP, especially at high physiologic doses, may stimulate secretion of specific cytokines in adipocytes through activation of pro-inflammatory pathways, thus further promoting macrophage infiltration and local inflammation in obese adipose tissue [146].

Taken together, the accepted adipose tissue-inflammation interconnectedness may well extent to the complement factors C5a and ASP and their receptors C5L2 and C5aR. The individual contributions of C5aR in metabolism and immunity of adipocytes has been further analyzed in CHAPTER 3.

5.2.2 Chapter 3: Proposing a new role for C5aR in adipocyte metabolism

CHAPTER 3 extended the previous findings for C5aR and C5L2 adjacent physical interaction [150,187,189], by proposing novel insights into the C5a-C5aR role in adipocyte metabolism and immunity, while demonstrating the important contribution of C5aR in insulin sensitivity of adipose tissue. Utilization of C5aRKO models allowed us to bridge the complex ASP and C5a pathways and further investigate the associated C5aR role in adipocyte metabolism and inflammation. It should be noted that although differentiated 3T3-L1 adipocytes are a reliable *in vitro* adipocyte model [10], they may not completely resemble freshly isolated primary adipocytes, which are subject to local environmental influences and circumvent some of the *in vitro* caveats. Utilization of mature adipocytes strengthened this study, as the signaling and functional interpretations are specific to adipocytes, not the mix of adipocytes, preadipocytes, endothelial, and immune cells present in adipose tissue. Amongst all adipose depots in mice, the gonadal depots are the largest and the most easily dissected. Notwithstanding the limitations on tissue amount, using isolated adipocytes only from gonadal tissue in this study, limits interpretations to this adipose tissue depot.

CHAPTER 3 has clarified C5a-C5aR signaling in adipocytes, suggesting that the majority of C5a functions and signaling pathways are transmitted through C5aR. In addition, we have proposed new insights into the C5aR role in adipocyte metabolism, beyond its classic role as host defense. A similar interpretation has also been reported earlier by Lim *et al.*, demonstrating metabolic role for C5aR in 3T3-L1 adipocytes, using alternate methodology (C5aR-selective antagonist), which interfered with C5a-mediated fatty acid uptake [130]. Roy *et al.,* in studies using C5aRKO mice, has pointed towards the importance of C5aR in energy expenditure and fat storage [168].

Based on the results obtained in C5aRKO adipocytes, it is suggested that the heterodimerization could positively influence the ASP-C5L2 pathway, as seen in C5aRKO adipocytes ASP functions remained significant but decreased. Very recently, a study on monocyte-derived cells reported that the blocking function of C5aR specific antagonist (3D53) not only affects C5aR function, but interestingly inhibits C5aR and C5L2 heterodimerization [187]. We used the same antagonist (3D53) on the same cell-type (3T3-L1) and observed diminished ASP-mediated fatty acid uptake. This is in accordance with our current hypothesis regarding ASP function, which is completely manifest only in the presence of both C5aR and C5L2, possibly as heteromers. As presented in CHAPTER 3, the C5aR antagonist completely disrupted the ASP-mediated fatty acid uptake in 3T3-L1 adipocytes, while the absence of C5aR in C5aRKO primary adipocytes resulted in a decreased but still significant triglyceride synthetic capacity in comparison to WT adipocytes. This cell-dependent difference could be due to (i) differences in 3T3-L1 *vs* primary adipocyte differentiation levels (3T3–L1 adipocytes have multilocular fat droplets while mature adipocytes are unilocular), (ii) maintained pre-existing environmental influences in the *ex vivo* adipocytes, or (iii) the fact that the C5aR antagonist only blocks the receptor activity, while C5aRKO adipocytes may have generated compensatory pathways that are different from the WT adipocytes.

Another original finding presented in CHAPTER 3 is that ASP and C5a co-treatment disrupt C5L2 activities, in 3T3-L1 adipocytes as well as C5aRKO and WT primary adipocytes. Although blocking effects of simultaneous addition of ligands on heteromer GPCR constructs has been reported previously [194], the analysis of ASP+C5a impact on adipocyte inflammatory signaling and hormone production is original. This negative regulatory effect of C5a on ASP-C5L2 pathway may partly be a result of ligand competition or receptor conformational modulation, and partly influenced by the heterodimerization phenomenon. It is speculated that since ASP and C5a do not appear to occupy the same binding site on C5L2 [126] and as both ligands are capable of C5aR-C5L2 internalization in adipocytes (CHAPTER 2), the ligand competition effect could also be associated with desensitization processes.

Through C5aR, C5a mediates inflammatory responses, and as such, is involved in various pathologies including sepsis, rheumatoid arthritis, asthma, ischemia-reperfusion injuries, and inflammatory bowel disease [153]. Further, *in vivo* studies with the C5aR selective inhibitor suggested a specific role for C5aR in metabolic dysfunction such as obesity [130]. Accordingly, Roy *et al.* reported decreased adipose tissue weight, lower plasma lipids, higher energy expenditure, and reduced fat storage in C5aRKO mice [168]. Based on data obtained from insulin-treated adipocytes, as well as metabolic gene expressions in adipose tissue (CHAPTER 3), absence of C5aR induced partial insulin resistance, mostly regarding the insulin-mediated pro-inflammatory function/signaling. This is relevant to the recently proposed role for C5aR in the physiology and metabolism of adipose tissue, as a major insulin target tissue. Beyond the proposed pathophysiological role for C5L2 in insulin resistance, lipid metabolism, allergies, asthma, and sepsis [127,164–166,173,195], an anti-inflammatory role has also been assigned to this multi-faceted GPCR, suggesting its balancing effects on the biological activity of C5aR [140,165,166]. In addition, recent studies proposed a pro-inflammatory cooperation between C5L2 and C5aR in the pathophysiology of sepsis [182,191,192]. Taken together, both C5aR and C5L2 are important role players in adipose tissue metabolic homeostasis and their regulatory interaction appears to be ligand-dependent, akin to observed negative influence of C5a on ASP-C5L2 pathway in adipocytes. Finally, a schematic figure representing possible homo- and heterodimer interactions between C5aR and C5L2 has been proposed in CHAPTER 3, which has shed more light on C5aR-C5L2 ligand-induced interactions, signaling pathways and the subsequent functionalities in adipocytes. The clinical aspect of C5aR and C5L2 interaction in adiposity is further discussed in CHAPTER 4.

5.2.3 Chapter 4: C5aR and C5L2 relevant importance in adiposity

Mutual interaction of C5L2 and C5aR may be particularly relevant in an obese state, where adipocytes and macrophages interact in adipose tissue. To our knowledge, this kind of *C5aR* and *C5L2* gene expression assessment in a large scale human study, with analysis of their circulating ligands, and their association with obesity parameters, has never been done before. This chapter reported that the adipose tissue-specific expression of *C5L2* and

C5aR is strongly associated with adiposity, which supports findings obtained from *in vitro* and animal studies. However the limitations of the study should be noted: all data were acquired in women, and due to the cross-sectional nature of the analyses, cause-and-effect relationships cannot be determined. In addition, based on the limited availability of frozen adipose tissue in small quantities, the present study relied on *C5aR* and *C5L2* mRNA expression without addressing the possible post-translational modifications of the examined receptors, which could also contribute to adiposity. It should be noted that the intact adipose tissue, represents a pool of adipocytes and infiltrated immune cells, which was specifically chosen for these analyses. Direct study of the tissue prevented the introduction of artefactual changes resulting from processing and separating the various cell populations. This can be viewed as a strength, specifically for this clinical study, as results represent the intact adipose tissue microenvironment in obesity, relevant to the study objectives of CHAPTER 4. Further, the large number of subjects with a wide range in BMI values particularly strengthened our study, as no samples were pooled and all PCR analyses have been conducted individually on all samples.

Insulin resistance is associated with hyperglycemia, as well as increased insulin levels in T2DM subjects [73]. Likewise, raised plasma ASP, elevated omental-specific ASP/*C5L2* ratio along with an increase in basal plasma lipids in morbidly obese individuals, provide evidences to support an ASP resistant state. The term "ASP resistance" was previously proposed based on human studies [196,197], but is supported by direct evidence in a study on diet-induced obesity (DIO) in mice [198], as well as *in vitro* studies using 3T3-L1 adipocytes [172,199]. Taken together with the ASP pro-inflammatory effects on adipose tissue cytokine production and macrophage infiltration [146], the increased ASP/*C5L2* ratio in the omental adipose tissue depot may be both reflective of impaired tissue-specific ASP functionality as well as contributing to the dyslipidemia and metabolic disturbances of obesity.

The coupling of increased plasma ASP, down-regulation of C5aR, and the elevated omental adipose tissue ASP/*C5L2* in obese subjects has highlighted the importance of C5aR and C5L2 interaction and their obesity-relevant expression in fat. In addition, the following evidences provide support for the potential physiological relevance of the *C5L2/C5aR* ratio:

(i) C5L2 and C5aR direct physical and functional interaction in adipocytes, transfected cells and immune cells (CHAPTER 2 and 3) [150,187,189,200], (ii) their synergic pro-inflammatory contribution in inflammatory conditions [182,201], and (iii) their tightly linked tissue-specific expression levels based on data from either C5aRKO or C5L2KO models [167,168]. Thus, based on our data, the C5aR-C5L2 interaction appears to be adipose tissue depot-specific and the ratio *(C5L2/C5aR)* correlates with obesity metabolic parameters. Further, as demonstrated elsewhere, C5L2 and C5aR expression are modulated in different disease conditions [202,203]: *C5L2*, but not *C5aR,* downregulated in neutrophils from patients with Familial Mediterranean Fever, while in kidney biopsies of anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, C5aR is downregulated but C5L2 is upregulated. Further mechanistic studies on primary adipocytes from obese subjects are essential to elucidate the consequence of obesity on C5aR-C5L2 interaction and the mediated signaling pathways.

We speculate that the decreased *C5aR* relative to *C5L2*, along with maintained C5a level, could divert C5a towards interaction with C5L2, simultaneously interfering with ASP function (CHAPTER 3), increasing internalization/downregulation of C5L2 (CHAPTER 2), and leading to compensatory increases in plasma ASP (CHAPTER 4). The C5a interference with the ASP-C5L2 pathway may consequently induce a state of "ASP resistance" in omental adipocytes from obese subjects. Previous studies reported that subcutaneous adipocytes are more efficient regarding ASP binding affinity and its functional stimulation in comparison with the omental adipocytes [204,205], which also supports the potential for ASP resistance in omental depot. Further, ASP dysfunction could disrupt adipose tissue glucose and FFA uptake, which would then contribute to systemic inflammation as well as high levels of circulating lipids, lower adiponectin and HDL-C. Thus, an increased *C5aR/C5L2* ratio in the omental depot is in accordance with the accepted pathophysiological role for omental tissue in metabolic disorders [5].

5.3 Strategies to control the undesired effects of C5a receptors

The complement system has been associated with several pathologies such as Alzheimers, ischemia-reperfusion injury, acute lung injury, asthma sepsis, rheumatoid arthritis, atherosclerosis, glomerulonephritis, multiple sclerosis [206], and, as presented here, obesity. Currently, there are a plethora of compounds that are either synthetic molecules or antibodies which target inhibition of C5/C5a and C5a receptors (C5aR and C5L2) [207].

5.3.1 Targeting C5/C5a and/or C5aR

Complement component C5 appears to be an optimal target to manipulate, as its activation result in the generation and release of the well-known pro-inflammatory agent C5a and consequently the assembly of the MAC complex, which contributes to tissue damage and inflammation [208]. Enhanced C5a production has been implicated in the pathophysiology of numerous immunoinflammatory diseases such of sepsis, leading to tissue damage and multiorgan failure [206]. Among the various reports regarding the novel compounds neutralizing the damaging effects of anaphylatoxins, very few have made it through to human clinical trials and only the monoclonal C5 antibody, Eculizumab (Soliris®), has received approval from the U.S. Food and Drug Administration in 2007 [209]. Eculizumab, prevents the formation of C5a and C5b and has been approved for the treatment of paroxysmal nocturnal hemoglobinuria, as well as preclinical trials for several diseases including autoimmune disorders, asthma, age-related macular degeneration, and myocardial infarction [207,210]. The clinical success of Eculizumab is a proof-of-concept for targeting C5a formation as a potent therapeutic direction, leading to attenuation of harmful systematic inflammation.

Modulation of the C5a-C5aR interaction is crucial for elimination of pathogens and equally important in maintaining homeostasis and preventing the pathophysiology of the disease process. The main effort has been concentrated on C5aR through the use of anti-C5aR antibodies, and also peptides or small molecule antagonists [153]. These include AcF-[OPdChaWR] (termed PMX53 or 3D53) [153,211]. However, despite the benefits of C5aR inhibition in certain inflammatory conditions, conversely, it can trigger inflammatory

reactions to pulmonary allergens [153]. Further, inhibition of C5aR may adversely affect host defense and other beneficial complement functions. Hence, this has recently been addressed by developing alternative tissue-specific therapies in preventing joint inflammation in a rat model of antigen-induced arthritis [212]. This new approach allows a selective accumulation of the drug at the specific target tissue level thus avoiding side effects that may derive from long-term systemic administration of therapeutic compounds.

However, the rationale of targeting C5a in obesity may be more complex, as plasma C5a concentrations in obese individuals remains unchanged (CHAPTER 4). On the other hand, as discussed in CHAPTER 4, increased plasma ASP together with omental-specific ASP resistance, could play a contributing role in obesity-associated inflammation. Thus, in contrast to other pro-inflammatory diseases (as mentioned above), low-grade inflammation in obesity might not be successfully manipulated solely through targeting C5a-C5aR inhibition. While C5a inflammatory and metabolic effects appear to be transmitted primarily through C5aR, in contrast, ASP effects appear to require heterodimer constructs to deliver maximal signals (CHAPTER 3). Collectively, given the potential therapeutic value of controlling C5a formation or C5a-C5aR function in excessive pro-inflammatory responses in humans, the issue of how this may alter the complex interaction with ASP and C5L2-C5aR heterodimerization needs to be considered.

5.3.2 Targeting C5L2

Contrary to the classical receptor for C5a, C5aR, which plays an accepted pro-inflammatory role, the controverisal nature of C5L2 has generated sometimes contradictory interpretations within different research groups. A clearer profile of the tissue-specific inflammatory aspects of C5L2 in pathological conditions needs to be known prior to its any clinical targeting. If C5L2 has an anti-inflammatory role, stimulating its expression/activity may be beneficial. By contrast, from its pro-inflammatory aspect, such as suggested in sepsis [182], then blocking may provide benefits. To add to this confusion of the specific inflammatory role for C5L2, there is the observation demonstrating that the severity of sepsis affects C5L2 functionality [182], perhaps in association with changing ligand (ASP and/or C5a) concentrations and C5aR regulation. Because of these multifarious roles for

C5L2, its potential for therapeutic targeting via activation or inhibition, is a more difficult endeavor and has not been successful [213].

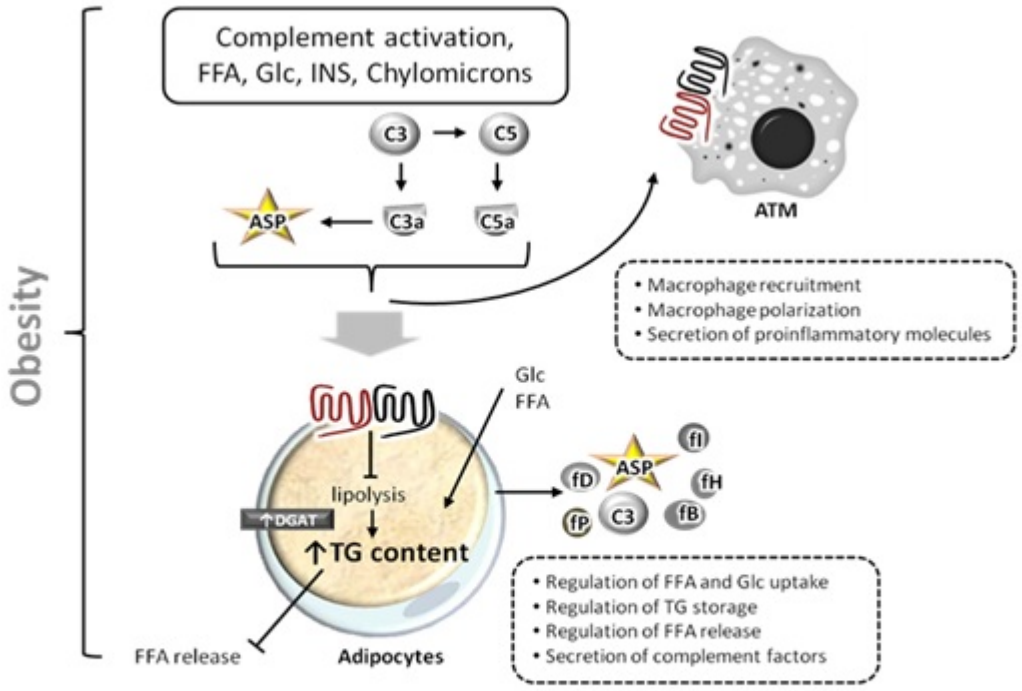
To date, there are few potential selective C5L2-activating or blocking peptides that have been identified and this area is still under further investigation [214]. Although, polyclonal [182] and monoclonal [150] antibodies have been developed to target mouse and human C5L2, these have mainly been used to understand the complex C5L2 signal transduction and functions, along with various mouse C5L2KO studies [127,164,215]. Further, the C5a mutant A8Δ71-73, originally reported as an inhibitor of C5aR, is also shown to inhibit C5L2 [216].

Taken together, diverse cell-dependent localization of C5L2 (intracellular versus plasma membrane) [150,187,189,213], as well as controversial evidences for C5L2 G-protein coupling [140,145], and its dual pro- *vs* anti-inflammatory potential [213], has made C5L2 a difficult receptor to target pharmacologically. C5L2 heterodimerization with C5aR (CHAPTER 2) and its functional dependency on this association (CHAPTER 3), means that therapeutic targets developed using transfected cell lines or KO mouse studies may not translate well into relevant clinical scenario. The place for C5L2 in complement pathophysiology is yet to be clearly unraveled, but it certainly appears to be a worthy and challenging target.

5.4 Conclusion

Evidence suggests that basal levels of complement activation occur in normal adipose tissue and have beneficial immune as well as metabolic effects, ranging from insulin-like actions, to adipocyte maturation and energy regulation (ASP, C5a, C5L2, and C5aR) [97–99]. Although "immunometabolism" has emerged as an area of research, the actions of immune cells in the pathophysiology of fat storing tissues, hence, in obesity, are still be elucidated. The recognition of adipose tissue complement components and their mediated pathways has raised the possibility that the complement system contributes to adiposity-related complications. However, it still remains unclear whether the complement activation

as a whole aggravates adiposity, or if obesity induces an exaggerated complement activation. Notwithstanding the "chicken-and-the-egg" conundrum, we speculate that the activation of the alternative complement pathway and its derivatives (ASP, C5a, and the receptors) are indeed one central player in the development of adiposity-related inflammation (FIGURE 5.1).



Adapted from:

Phieler J, *Semin Immunol* 25, 47-53

FIGURE 5.1 – The central role of complement in adiposity-related complications.

Overall, these three studies present fundamental relationships between innate immune responses in the regulation of adiposity and adipose tissue inflammation, demonstrating how excessive immunometabolic responses may exacerbate adiposity. This has altered our understanding of C5aR and C5L2 interaction in various concepts of physical (CHAPTER 2), functional (CHAPTER 3), and physiological (CHAPTER 4) aspects, regarding the specific ligand, cell, tissue, species, and also the pathological situations. C5aR and C5L2 activate signaling pathways that are important in defending against pathogens and

maintaining metabolic homeostasis of adipose tissue. In addition, these findings suggest that although inhibition of C5a-C5aR might appear to be effective in inflammation management, the effectiveness may stem, at least in part, from regulation of the C5aR-C5L2 heterodimer. Interestingly, this is not the only case in which GPCR heteromers have been implicated as therapeutic targets for the treatment of disease. For example, a previous study on dopamine D2 and adenosine A2A receptor interaction in Parkinson's disease has highlighted the potential therapeutic strategies that target their associated dimerization [217,218]. Taken together, despite the acquired knowledge regarding C5a receptors and their role in metabolism (mostly from transfected cells or animal studies), much more still needs to be established in terms of human studies, such as C5aR and C5L2 tissue distribution, their metabolic *vs* inflammatory function, as well as investigation of compounds specifically targeting heteromer constructs. Although, this would be a difficult challenge, it is supported by the general agreement that C5aR-C5L2 interaction and function may well have therapeutic potential.

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