TUMOR NECROSIS FACTOR-α AMPLIFIES ADIPOSE-DERIVED CHEMERIN PRODUCTION AND BIOACTIVATION

by

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Signature of Author

DEDICATION

TO MY FAMILY,
AS I STAND HERE ON THE SHOULDER OF GIANTS,
IT WAS YOU THAT FIRST TAUGHT ME TO STAND.

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ABSTRACT

Due to its escalating prevalence, obesity is becoming a leading cause of morbidity and mortality worldwide. Obesity is a complex health problem accompanied by metabolic abnormalities and low-grade inflammation that increases the risk for developing comorbidities including type 2 diabetes. Recent evidence supports a role for fat (adipose) tissue derived factors, called adipokines, in the development of obesity and obesity-related metabolic pathologies.

Chemerin is an adipokine that mediates immune and metabolic effects through the chemokine-like receptor 1 (CMKLR1). Chemerin is secreted as an inactive proform, prochemerin, which subsequently undergoes enzymatic cleavage into multiple chemerin products that differentially activate CMKLR1. Multiple studies have reported elevated total chemerin (a combination of prochemerin and various chemerin products) in obese humans suggesting chemerin involvement in obesity pathophysiology. However, the observational nature of these human studies have restricted them from identifying specific forms of chemerin that are elevated in obesity and the mechanisms that govern them.

Herein, I have reported that the levels of both serum total chemerin and chemerin products capable of activating CMKLR1 are elevated in obese mice and in wild type mice following treatment with an obesity-associated inflammatory mediator tumor necrosis factor-α (TNFα). Likewise, cultured adipocytes produced active chemerin under basal conditions and highly active chemerin following TNFα treatment as measured by CMKLR1 activation. The current belief is that prochemerin circulates through blood primed for activation by immune and fibrinolytic enzymes present within injured tissues. My results challenge this theory, identifying adipocytes as cells alone produce and proteolytically activate chemerin. Under basal conditions, a balance between activating serine proteases and deactivating aminopeptidases governed the amount of CMKLR1-activating chemerin formed by adipocytes. Treatment of adipocytes with TNFα elevated the levels of serine proteases elastase and tryptase, which cumulatively shifted the proteolytic balance toward the production of chemerin products that highly activate CMKLR1.

Taken together, my results are the first to identify that local TNFα triggers increased adipocyte production of chemerin providing an explanation for the elevated concentrations of chemerin in obese animals and humans. Furthermore, adipocyte processing represents a novel mechanism that likely governs the amount and type of circulating chemerin in obesity.

LIST OF ABBREVIATIONS AND SYMBOLS USED

1: TNFα- Tumor necrosis factor-α

2: T2DM- Type 2 diabetes, Non-insulin dependent diabetes mellitus

3: CVD- Cardiovascular disease

4: BMI- Quetelet's index, Body mass index

5: WC- Waist circumference

6: WHR- Waist-to-hip ratio

7: MRI- Magnetic resonance imaging

8: CT- Computerized tomography

9: DEXA- Dual x-ray absorptiometry

10: WHO- World Health Organization

11: EOSS- Edmonton obesity staging system

12: FFA- Free fatty acid

13: VLDL- Very low-density lipoprotein

14: ATGL- Adipose triglyceride lipase

15: HSL- Hormone-sensitive lipase

16: β-AR- β-adrenergic receptor

17: NPRA- Natriuretic peptides receptor A

18: bMSC- Bone mesenchymal stromal cells

19: CEBP- CCATT/enhancer-binding protein

20: PPAR-γ- Peroxisome proliferator activated receptor-γ

21: SREPB1c- Sterol regulatory element-binding protein 1c

22: IFγ- Interferon-gamma

23: IL- (Interleukin)

24: MCP1- Monocyte-chemotactic protein-1

25: M2- Alternatively activated macrophages

26: M1- Classical activated macrophages

27: Ob/Ob- Leptin-deficient mice

28: Db/Db- Leptin-receptor deficient mice

29: Ob-Rb- Leptin receptor-B

30: CCR2- Chemokine receptor-2

31: Fa/Fa- Leptin-deficient rats

32: TZD- Thiazolidinediones

33: Adip-Ob/Ob- Adiponectin overexpressing leptin-deficient mice

34: CMKLR1- Chemokine-like receptor-1

35: GPR1- G-protein coupled receptor-1

36: CCRL2- Chemokine-receptor-like 2

37: ERK1/2- p42 and p44 MAP kinases

38: LPS- Lipopolysaccharide

39: PolyI:C- Polyinosinic:polycytidylic acid

40: OLP- Oral lichen planus

41: RvE1- Resolvin E1

42: TNFRsf11a$^{-/-}$/1b$^{-/-}$- Tumor necrosis factor-α receptor 1a/1b deficient mice

43: PBS- Phosphate buffered saline

44: BSA- Bovine serum albumin

45: DMEM- Dulbecco's modified Eagle's medium

46: DMSO- Dimethyl sulfoxide

47: QPCR- Quantitative polymerase chain reaction

48: Fw- Forward

49: Rv- Reverse

50: I.P.- Intraperitoneal injection

51: PAI-1- Plasminogen activator inhibitor-1

52: RT- Room temperature

53: TEV- Tobacco etch virus

54: tTA- Transcriptional-transactivator

55: AUC- Area under the curve

56: CE- Recombinant bioactive mouse chemerin$_{156}$

57: tPA- Tissue plasminogen activator

58: uPA- Plasminogen activator urokinase

59: PIC- Protease inhibitor cocktail

60: PAR– Protease-activated receptor

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CHAPTER 1.00.00: INTRODUCTION

1.01.00: PREFACE

With the proportion of adult obesity reaching in excess of 20% globally, obesity is being recognized as a 21st century epidemic. Groundbreaking research over the past two decades has identified that adipose tissue is an endocrine organ secreting a multitude of factors that regulate diverse processes including adipocyte differentiation, adipose tissue metabolism and insulin resistance. These factors, called adipokines, are at the forefront of metabolic research as many of them have been implicated in the development of obesity and exacerbation of obesity-related inflammatory and metabolic comorbidities. The first chapter serves to introduce the epidemiological, clinical and pathological features of obesity and the mechanisms, including adipokines, which govern obesity and obesity-related diseases. The work presented in the second and third chapters will subsequently focus on original research concerning the regulation of a novel adipokine, chemerin, by an obesity-associated inflammatory mediator tumor necrosis factor-α (TNFα). The final chapter will discuss the implication of these results in the context of chemerin biology and obesity.

1.02.00: THE EPIDEMIOLOGY OF OBESITY

In 2010 the Surgeon General outlined that obesity was one of the fastest growing threats against public health in the United States of America with an estimated 112 000 preventable deaths a year [1]. The proportion of individuals diagnosed as obese continues to rise throughout industrialized and non-industrialized countries with ~23% (approximately 1.2 billion) of adults worldwide estimated as being overweight and obese (**Table 1**). If effective clinical interventions are not found and implemented, it is

estimated that the proportion of the population developing obesity will continue to rise by ~2% annually [2]. In parallel with these world trends, ~25% of Canadian adults are considered obese, having increased from 10% in the past two decades (**Table 2**) [3, 4]. The increased prevalence of obesity is not confined to the adult population. Childhood obesity has risen 3-fold over the past 20 years in Canada with ~28% of Canadian children now considered overweight or obese. This is concerning as obese children are more likely to remain obese as adults, and are more likely to suffer and die from an obesity-associated illness [5-8]. The prevalence of childhood obesity has risen so dramatically, it is suggested children of each successive generation will continue to become more obese and progressively more ill than their parents [9].

Characterized by excess adipose tissue, as well as low-grade systemic inflammation, obesity is an independent risk factor for numerous comorbidities including type 2 diabetes (T2DM), and cardiovascular disease (CVD) [10-13]. This clustering of metabolic abnormalities including elevated abdominal obesity and elevated blood triglyceride, blood pressure and fasting glucose constitute the metabolic syndrome and contributes to the major health problems of obese patients [14]. Obesity-associated morbidity and mortality is therefore a considerable burden on the Canadian health care system incurring ~2.1 billion dollars a year in direct health care costs including hospital and physician care. This equates to ~2.4% of the annual Canadian health care budget [15]. Obesity additionally costs the public ~2.7 billion dollars annually in indirect costs, including the cost of years of life lost and value of activity days lost [16]. Although obesity has a detrimental effect on the health of the individual and is of great cost to the public, it is a multi-factorial disease governed by genetic, social, behavioral and

biochemical processes which has made clinical interventions at the early developmental and late pathological stages of obesity relatively unsuccessful. Research investigating adipocyte and fat tissue functions is for that reason of utmost importance for understanding the basic pathologies of the disease as well as identifying much-needed therapeutic targets.

1.03.00: CLINICAL DIAGNOSIS

An individual's weight reflects a controlled balance of energy intake and energy output [17]. While energy input constitutes any food ingested and the relative ability of endogenous gut microbes to process this ingested food into a useable form, energy output is a combination of three metabolic processes: physical activity, basal metabolism and adaptive thermogenesis [18, 19]. White adipose tissue acts as a long-term energy storage organ that grows in times of caloric/energy excess and in turn is mobilized in times of caloric deprivation or need [20]. Obesity, in simplest terms, develops when there is a long-term imbalance in energy input exceeding energy output. Excess energy is then stored in white adipose tissue as triglycerides [21]. This is exemplified by individuals on a high caloric diet and/or with sedentary life styles, both of which are correlated with total body fat [22-26]. Diagnosis of obesity relies on correlative, anthropometric and direct measurements of total body fat and metabolic fitness including the body mass index (Quetelet's Index or BMI), waist circumference (WC), waist to hip ratio (WHR), magnetic resonance imaging (MRI), computerized tomography (CT) and dual X-ray absorptiometry (DEXA) [27]. MRI, CT and DEXA provide a direct quantification of body adiposity, although, high cost, limited supply, lengthy wait times, use of radiation and unsuitability for use in young children severely limit their use [28, 29]. In contrast,

anthropometric measurements, including BMI, WC and WHR, which measure body proportions (e.g. weight & height) are in contrast easily performed in clinics and therefore take precedence [30-32]. Unique in their approach, each clinical measurement divides obesity into distinctive classes of severity based on correlation to total body fat and obesity-associated comorbidities. Definitely the most commonly used diagnostic criterion is BMI. BMI is a measurement of weight (Kg) divided by height (m) squared. Identified by Adolph Quetelet (1796-1874) during a study of human growth, it was noted that outside of few growth spurts, an individual's weight increases in proportion to the square of their height [33]. As a diagnostic tool, BMI assumes that variability in weight at a given height is dependent on changes in fat mass [34]. A positive correlation between BMI and the likelihood of acquiring obesity-associated comorbidities is greatly supported [10, 35-37]. BMI therefore remains an easily calculated and clinically useful indicator of clinical obesity and propensity for obesity-related diseases. Accordingly, the expert committee at the World Health Organization (WHO) utilizes BMI as its primary method of obesity classification. The WHO classifies an individual as overweight and obese at 4 different BMI cut off points of 25, 30, 35 and 40 based on their increasing associations with mortality (**Table 3**) [38]. BMI is not used without contention. Assuming equal distribution of both lean and fat mass, BMI fails to take into consideration age, sex, bone structure, fat distribution or muscle mass [39]. This assumption can result in overestimation of fat mass in athletes whom have greater lean mass, while overestimating lean mass in certain cultural and age groups whom have greater fat mass [40]. The WHO therefore identifies important criteria when interpreting BMI for intervention. A combinatory approach of BMI and other determinants of morbidity and mortality (e.g.

insulin resistance) should always be used in consideration of clinical intervention. Second this categorization identifies only the extent of the patient's weight and does not provide endpoints for intervention. Finally, the large ranges within each BMI category should not suggest that fluctuations within a classification go without consequences. Even minor changes within a BMI category can result in significant effects on human health [41].

1.04.00: BROWN VERSUS WHITE ADIPOCYTES

Humans and rodents contain two separate adipocyte lineages; white and brown, both of which vary greatly in structure and function. White adipocytes are large spherical cells with a single lipid droplet, few mitochondria and play a prominent role in energy storage. In contrast, brown adipocytes contain numerous small lipid-filled vacuoles and large spherical mitochondria. Brown adipocytes play a contrasting role from white adipocytes in energy metabolism by dissipating energy via the uncoupling protein-1 in the form of non-shivering thermogenesis in small mammals and newborns. Developing very early during fetal development, brown adipocyte numbers are highest relative to body weight at birth, sharply reducing thereafter [42]. The relatively few brown adipocytes that remain in human adulthood localize and deposit to the neck, supraclavicular, para-aortic, paravertebral and suprarenal areas within white adipose tissue depots and muscle tissue [43, 44]. Brown adipocyte development is thought to occur through two processes. A study by Timmon et al. identified brown and white adipocytes are derived from two different preadipocyte lineages. Preadipocytes destined to become brown adipocytes are found to express a number of myocyte-associated genes including *Myogenin*, *MyoD* and *Myf5* [45]. These genes, which were not expressed in

preadipocytes destined to become white adipocytes, are thought to contribute to brown adipocytes heightened oxidization of triglycerides and dissipation of energy. Evidence also supports that white and brown adipocytes are derived from a single precursor and have the ability to transdifferentiate under certain conditions. This is supported by the increase in the number of brown adipocytes with a simultaneous decrease in white adipocytes that occurs in rodents exposed to cold temperatures [20]. Although the extent brown adipocytes contribute to adult human energy metabolism remains controversial, brown adipocytes are being explored as possible therapy for treating obesity. By increasing brown adipocyte numbers through differentiation from progenitor cells or through transdifferentation from white adipocytes it is believed that an individual would be protected from obesity by increasing the amount of basal energy output. Despite the promising nature of this research, brown adipocytes are not the focus of the current dissertation; therefore, any further reference to adipocytes or adipose tissue will refer to white adipocytes.

Table 1: Percentage of individuals considered overweight and obese grouped by country			
Country	**Year**	**% Overweight (BMI ≥ 25.00)**	**% Obese (BMI ≥ 30.00)**
United States	2008	68	34
Mexico	2006	70	30
New Zealand	2007	63	27
Australia	2007	61	25
United Kingdom	2008	61	25
Canada	**2008**	**60**	**24**
Ireland	2007	61	23
Chile	2005	60	22
Iceland	2007	60	20
Spain	2009	55	17
Germany	2009	52	16
Finland	2008	49	16
Portugal	2006	52	15
Belgium	2008	47	14
Austria	2006	47	14
Netherlands	2009	48	14
Denmark	2005	45	11
France	2008	38	11
Sweden	2007	44	10
Norway	2008	46	10
*Adapted from [3]			

Table 2: Obesity rates by age group in Canada in 1978 and 2004		
Age	**Year**	**Percentage**
18-24	1978/9	6
	2004	11
25-34	1978/9	9
	2004	21
35-44	1978/9	13
	2004	20
45-54	1978/9	17
	2004	30
55-64	1978/9	20
	2004	30
65-74	1978/9	20
	2004	25
75+	1978/9	11
	2004	24
* Adapted from [4]		
Obese defined as a BMI≥30		

Table 3: The international classification of adult overweight and obesity according to BMI		
BMI (Kg m^{-2}) Cut-off Points	**WHO classification**	**Popular description**
<18.5	Underweight	Thin
18.5-24.99	Normal Range	"Healthy"
25.0-29.9	Pre-Obese	Overweight
30.0-34.99	Obese Class I	Obesity
35.00-39.99	Obese Class II	Morbid Obesity
≥ 40.00	Obese Class III	Super Obese
*adapted from [38]		

1.05.00: ANATOMICAL LOCALIZATION OF ADIPOSE TISSUE.

Adipose depots of humans and rodents are similar both in anatomical deposition and cellular morphology, composed of subcutaneous or visceral depots. Subcutaneous adipose depots, representing ~80% of adipose tissue in humans, are found spread out beneath the dermis of the skin. In contrast, visceral adipose depots are located within the thorax (mediastinic) and abdomen (omental, mesenteric, perirenal, retroperitoneal and parametrial). Adipose tissue is the most modifiable source of overall body weight in adulthood, varying between ~3% of total body weight in elite athletes to ~ 70% in the severely obese [46]. Although fat mass is an excellent indicator of morbidity and mortality, obesity is a heterogeneous condition with variable quantities and deposition patterns of adipose tissue and development of comorbidities. These differences in adipose tissue distribution may account for the ~20% of individuals that are diagnosed as "obese" in BMI terms yet remain metabolically healthy [47]. This paradox suggests total body fat does not necessarily determine metabolic fitness. Rather, recent studies indicated that fat distribution and adipocyte dysfunction play an equally important role in contributing to obesity-associated diseases. In particular intra-abdominal or visceral adipose tissue is closely associated with T2DM [48]. To investigate the relative contribution of the visceral versus subcutaneous adipose depots on insulin resistance, an elegant human study separated obese patients into two groups; those considered insulin-resistant and those considered insulin-sensitive ("normal"). Insulin resistance is a state of reduced responsiveness to normal circulating levels of insulin representing the basic pathology of T2DM [49, 50]. Independent of BMI, body fat mass, age and sex, patients found to be insulin-resistant had higher quantities of visceral fat and a larger waist circumference,

compared with insulin sensitive patients. No difference in the quantities of lean mass was found [51]. Obese post-menopausal women classified as metabolically abnormal were equally identified to have ~50% higher visceral adipose tissue weight then those classified as metabolically normal. CT analysis identified no other difference in subcutaneous adipose tissue accumulations [52]. This link between visceral adipose depots and increased risk for obesity associated morbidity and mortality have been made in several other patient populations [53, 54]. Collectively these results indicate that in addition to total body fat, deposition of abdominal adipose tissue is a contributor to the development of obesity-comorbidities.

Given that BMI alone is inherently unable to distinguish between metabolically normal and abnormal obesity, new diagnostic criteria are being devised to complement anthropometric indices and provide more clinically relevant prognostic information. The Edmonton obesity staging system (EOSS) is a system based on anthropometric and simple clinical assessments, which provides a measurement for the presence and severity of risk factors, comorbidities and functional limitations serving as a guide for clinical management (**Table 4**) [55]. EOSS thereby defines obesity in four stages based on the presence of deleterious health problems and not simply excess weight. Statistical analysis has validated the ability of EOSS to better identify increased risk of morbidity and mortality then BMI alone [56, 57]. Although BMI remains the predominant method of diagnosing obesity, adoption of more stringent and relevant diagnostic criteria like EOSS in the future may help exclude healthy obese patients from getting unsuitable clinical therapies while better identifying those are in desperate need of intervention.

Table 4: Edmonton obesity staging system		
Stage	**Description**	**Management**
0	No apparent obesity-related risk factors (e.g., blood pressure, serum lipids, fasting glucose, etc. within normal range), no physical symptoms, no psychopathology, no functional limitations and/or impairment of well being	Identification of factors contributing to increased body weight. Counseling to prevent further weight gain through lifestyle measures including healthy eating and increased physical activity.
1	Presence of obesity-related subclinical risk factors (e.g., borderline hypertension, impaired fasting glucose, elevated liver enzymes, etc.), mild physical symptoms (e.g., dyspnea on moderate exertion, occasional aches and pains, fatigue, etc.), mild psychopathology, mild functional limitations and/or mild impairment of well being	Investigation for other (non-weight related) contributors to risk factors. More intense lifestyle interventions, including diet and exercise to prevent further weight gain. Monitoring of risk factors and health status.
2	Presence of established obesity-related chronic disease (e.g., hypertension, type 2 diabetes, sleep apnea, osteoarthritis, reflux disease, polycystic ovary syndrome, anxiety disorder, etc.), moderate limitations in activities of daily living and/or well being	Initiation of obesity treatments including considerations of all behavioral, pharmacological and surgical treatment options. Close monitoring and management of comorbidities as indicated.
3	Established end-organ damage such as myocardial infarction, heart failure, diabetic complications, incapacitating osteoarthritis, significant psychopathology, significant functional limitations and/or impairment of well being	More intensive obesity treatment including consideration of all behavioral, pharmacological and surgical treatment options. Aggressive management of comorbidities as indicated.
4	Severe (potentially end-stage) disabilities from obesity-related chronic diseases, severe disabling psychopathology, severe functional limitations and/or severe impairment of well being	Aggressive obesity management as deemed feasible. Palliative measures including pain management, occupational therapy and psychosocial support.
Adapted from [55]		

1.06.00: ADIPOCYTE DYSFUNCTION: ADIPOCYTE HYPERTROPHY

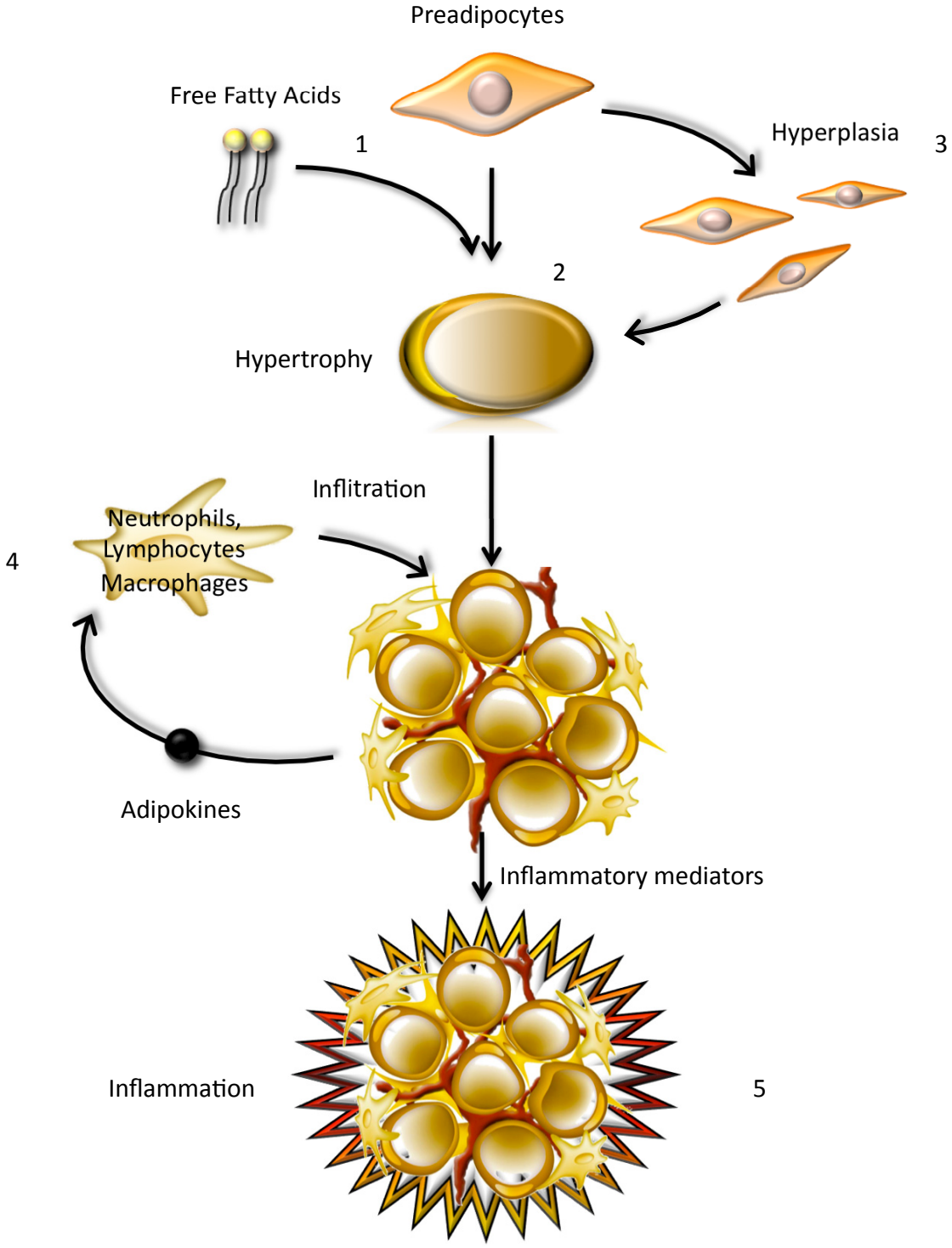
Adipose tissue is a heterogeneous tissue, made up of adipocytes, preadipocytes, macrophages, endothelial cells and fibroblasts although adipocytes make up the single largest component of the adipose tissue [58, 59]. In obesity, tissue histology, morphology and function of adipocytes and adipose depots become altered. This process is referred to as adipose tissue remodeling (**Figure 1**). Adipose remodeling therefore occurs in coincidence with an increased energy imbalance and is implicated in the development of obesity-associated co-morbidities.

Adipose tissue remodeling begins when continued overconsumption of energy combined with reduced energy output results in increased accumulation and deposition of triglycerides within adipocytes. Adipocyte triglycerides are maintained through a balance of free fatty acid (FFA) deposition and release by enzymatic lipolysis [60]. The majority of stored triglycerides result from ingested or re-esterified fatty acids brought to adipocytes by circulating chylomicrons and very low-density lipoproteins (VLDL) [61-65]. After reaching capillary endothelial cells of the adipose tissue, triglycerides from chylomicrons and VLDLs are broken down by lipoprotein lipase releasing FFA and glycerol. FFA and glycerol move into the adipocyte where they are re-esterified and stored as triacylglycerol. In times of energy need, triacylglycerol can be broken down through three sequential enzymatic steps by adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase resulting in glycerol and 3 FFA [60]. Although FFA storage and release are normally in equilibrium, in times of energy restriction or exercise, triacylglycerides can be mobilized by hormonal induction of lipolysis. Hormones, including natriuretic peptides and catecholamines, increase the

basal rate of lipolysis, releasing FFA. Stimulation of β-adrenergic receptors (β_1AR, β_2AR and β_3AR) and natriuretic peptides receptor A (NPRA) have been readily implicated in this process by activating HSL [60, 66, 67]. Activated HSL subsequently releases the FFA and glycerol from internal adipose lipid stores [65]. Adipocyte hypertrophy and subsequent obesity occurs because of increased triacylglycerol deposition and decreased hormonal-induced lipolysis. Visceral depots in particular have higher rates of lipid absorption and decreased *HSL* and *ATGL* expression in addition to lowered catecholamine-induced lipolysis [66, 68-71]. Together, the increase in triglyceride deposition and inhibition of triglyceride breakdown increases adipocyte expansion by up to 20-fold in diameter and 1000-fold in volume [72]. This engorgement of the adipocyte is thought to contribute to metabolic dysfunction by simultaneously reducing adipocyte membrane cholesterol content through activation of sterol regulatory element-binding protein 2 as well as inducing endoplasmic reticular stress. These changes to the adipocyte result in a reduced response to insulin and increase pro-inflammatory cytokine production (i.e. TNFα, IL-6) both underlying pathologies associated obesity [73, 74]. As long as energy continues to be consumed, adipocytes will continue to absorb lipids until they reach a "critical cell size" ~1 μg lipid per cell, at which point preadipocytes undergo hyperplasia [61, 64, 72]. Accordingly, "moderate" obesity is characterized by hypertrophic adipocytes while "severe" obesity is thought to be more tightly associated with an increase in hypertrophic and hyperplasic adipocytes [72, 75].

Figure 1: Adipose tissue remodeling. Adipose tissue remodeling begins when ingested dietary free fatty acids are stored by adipocytes (1). Increased free fatty acid storage and decreased lipolysis results in adipocyte hypertrophy (2). Once adipocytes reach their hypertrophic cap and are unable to absorb any further lipids, preadipocytes undergo cell division or hyperplasia as well as differentiation into new adipocytes. The total number of adipocytes thereby increases (3). The expanded adipose tissue subsequently secretes adipokines, which recruit neutrophils, T-lymphocytes and macrophages to the adipose tissue (4). A low-grade inflammation results from a combination of macrophage- and adipocyte-secreted inflammatory mediators (5).

Figure 1


Preadipocytes
Free Fatty Acids
1
Hyperplasia
3
2
Hypertrophy
Inflitration
Neutrophils,
Lymphocytes
Macrophages
4
Adipokines
Inflammatory mediators
Inflammation
5

1.07.00: ADIPOCYTE DYSFUNCTION: ADIPOCYTE HYPERPLASIA

Adipocyte hyperplasia, or increased preadipocyte (lipid-free undifferentiated mesenchymal cells) mitosis, is a characteristic phenotype of tissue remodeling. Its exact onset in humans however remains controversial. Studies using tritiated thymidine incorporated into genomic DNA to monitor cell division have identified that preadipocytes are highly proliferative, reaching a state of quiescence prior to differentiating and accumulating lipid [76]. In postnatal development, rodents undergo a high succession of preadipocyte division up until ~3-5 weeks after birth. From the 4^{th} until the 14^{th} week of life, both hyperplasia and hypertrophy contribute to the growth of the adipose tissue mass. After the ~14^{th} week, hypertrophic growth is shown to predominate [59]. The store of preadipocytes remaining within adult rodents can differentiate into mature adipocytes, individually growing in size, but the sum total number of preadipocyte cells remains constant after early rodent development [77]. Given the parallel function and distribution of adipocytes in rodents and humans, studies suggested that humans have a similar defined number of preadipocytes at birth. This number can increase with changes in nutrition during childhood, after which the number remains stable in adulthood [78]. Accordingly the number of adipocytes is significantly higher amongst those individuals who developed obesity as children versus those who developed obesity in later life [72, 79]. Adipocyte DNA incorporation of ^{14}C through increased environmental exposure to radioactive material released during nuclear testing in the 1950s, identified that although a basal rate of loss of adipocytes in lean and obese individuals exists, this rate is in equilibrium with preadipocyte division. This finding

argues that the total number of adipocytes although larger in obesity, had to have been established in childhood and/or early adolescence [78].

Animals or humans that continuously consume energy will gain weight and adipose tissue as long as this energy imbalance remains. If placed on a calorie restricted diet, the overall weight and adipose depot size will decrease, yet the number of adipose cells will be more numerous than prior to weight gain [59, 80]. Accordingly these studies argue that adult adipocyte hyperplasia must occur [81-83]. To identify the proliferative potential of the preadipocytes in the adipose tissue, Maumus *et al*. isolated preadipocytes from adipose tissue of obese and lean women and subsequently measured the number of Ki-67 positive cells by flow cytometry. Ki-67 is expressed only during the active phases of cell cycle (G_1, S, G_2 and mitosis) but not at rest (G_0). The number of positively labeled Ki-67 progenitor cells was significantly higher in obesity and positively correlated with BMI suggesting the presence of adult preadipocyte hyperplasia [84]. Likewise, lean subjects placed on an acute high-fat high-caloric diet had an increase in body fat which resulted from the creation of ~2.6 billion new adipocytes [80]. Hypertrophy and hyperplasia therefore contribute to tissue remodeling in obesity.

1.08.00: ADIPOGENESIS

Adipogenesis, or adipocyte differentiation describes the process of adipocyte formation from preadipocyte precursors [85]. Cell culture models are preferentially used to study adipocyte differentiation versus *in vivo* developmental models due to the difficulties in tracking preadipocyte developmental programs *in vivo* [82, 86]. Use of primary preadipocyte cell cultures are similarly limited due to contaminating fibroblast-

like cells, the large amount of fat tissue required for preadipocyte isolation and the limited life span of preadipocytes in culture. Most of the work investigating the molecular determinants of adipocyte differentiation has been studied in 3T3-L1 and 3T3-F442A cells derived from non-clonal Swiss 3T3 cells. These cell lines recapitulate both the phenotypic and genotypic responses of adipocytes *in vivo* [87]. Recently, multipotent bone mesenchymal stromal cells (bMSC), a non-hematopoietic subpopulation of cells from bone marrow capable of differentiating into adipocytes, have become a novel model for studying adipocyte function. bMSCs are particularly useful given that they: are easily isolated, expanded in culture without loss of differentiation potential, can be manipulated genetically and, phenotypically model adipocytes *in vivo* [88, 89].

Adipogenesis is divided into two distinct phases, which are tightly and temporally regulated by transcriptional factors. These phases are the early determination phase and the late differentiation phase. The early determination phase pertains to the commitment of pluripotent stem cells into their preadipocyte lineage. Terminal differentiation involves the conversion of preadipocytes into adipocytes and is more widely studied (**Figure 2**) [90]. 3T3-L1 and bMSC adipocyte models differentiate spontaneously and to a limited degree when exposed only to fetal bovine serum. Treating with isobutylmethylxanthine, dexamethasone and insulin can enhance this adipogenic process [86, 91, 92]. During differentiation, preadipocytes must first undergo growth-arrest at the G_0/G_1 cell cycle boundary. In cell culture this is reached through proliferation and contact inhibition. This process in human adipocytes is less clear. Adipogenesis is initiated by signaling convergence on early transcription factors resulting in re-entry of preadipocytes into the cell cycle [81, 93]. 3T3-L1 adipocytes undergo two rounds of mitotic clonal expansion

terminating between 24-36 hr and 48-72 hr following hormonal induction [94]. Clonal expansion provides the needed DNA replication and chromatin structural changes allowing transcriptional regulators like CCATT/enhancer-binding protein (CEBP) β and δ access to specific promoters. These transcriptional regulators control genes required for differentiation [95, 96]. The importance of clonal expansion is supported by the loss of adipogenic potential in preadipocytes treated with rapamycin (inhibitor of mitogen-activated p70 S6 Kinase) or aphidicolin (DNA polymerase inhibitor), inhibitors of cell division [97, 98]. Mitotic clonal expansion has not, however been fully elucidated in humans. Work by Hauner et al. showed preadipocytes derived from human adipose tissue do not require post-confluent cell division to undergo differentiation. Rather, they hypothesize that preadipocytes in adipose tissue have already undergone clonal expansion and therefore exist in a later stage of differentiation than the established rodent cell culture models [99]. This hypothesis remains to be validated. Regardless, work in *in vitro* rodent preadipocyte models have identified the early onset of cell division and induction of transcription regulation results in up-regulation of C/EBP β and C/EBPδ between days 2 and 4 following induction by IBMX, dexamethasone and insulin. This forces preadipocytes from the cell cycle into terminal differentiation [100]. The fundamental role for C/EBPβ and δ in adipogenesis is exemplified by the reduction in adipose mass in mice lacking either protein [101]. Temporal expression of C/EBPβ and δ drives terminal differentiation by increasing the transcription regulators C/EBPα and peroxisome proliferator activated receptor γ (PPARγ) (**Figure 2**). C/EBPα and PPARγ induce expression of adipose factors involved in insulin sensitivity, lipid accumulation and metabolism [100]. The simultaneous increase in sterol regulatory element-binding protein

(SREBP1c) promotes adipogenesis by increasing the expression of genes linked to fatty acid metabolism and a reciprocal increase in PPARγ expression. C/EBPα and PPARγ, like C/EBPβ and δ are essential in adipogenesis. Mice with absent or lowered C/EBPα and PPARγ signaling have resulting abnormalities in adipocyte development [102-104]. Given that no other factor has yet to be identified that can induce adipogenesis in its absence, PPARγ in particular is considered the "Master Regulator" of adipogenesis [105, 106]. This is exemplified by the sufficiency in which PPARγ expression rapidly induces adipogenesis in dividing fibroblasts [107]. Two PPARγ receptors have been currently identified (PPARγ 1 and 2). While PPARγ1 is more ubiquitously expressed, PPARγ2 is localized to the adipose tissue and has been proposed as the major contributor to adipogenesis [107-109].

1.09.00: IMMUNOCYTE INFILTRATION AND ADIPOSE INFLAMMATION

The complicated nature of adipose tissue remodeling and obesity is now understood to include an immunological and inflammatory component. The importance of this contribution is highlighted by the categorization of obesity as an inflammatory disease. Adipose tissue remodeling is characterized by sequential neutrophil, T-lymphocyte and macrophage recruitment and infiltration into the adipose tissue with subsequent pro-inflammatory cytokine production [110-112]. This infiltration, or diapedesis, refers to passage of the immunocytes from the blood through intact capillary walls into the adipose tissue [113]. Diapedesis begins shortly after the consumption of a high-fat diet, and is characterized by acute and long–term components. Neutrophil diapedesis reflects the most acute stage of immunocyte infiltration into adipose tissue.

Neutrophils invade adipose tissue of mice in as little as three days after the introduction of a high-fat diet in response to adipose tissue interleukin (IL)-8, complement factor-3, and fatty acid metabolite production. Neutrophil infiltration is transient, as few neutrophils remain in the adipose tissue of mice after 7 days on a high-fat diet [110]. The exact role of this initial neutrophil infiltration is unknown. Given the role neutrophils play in initiating other long-term inflammatory reactions, infiltrated and activated neutrophils are thought to produce pro-inflammatory factors including reactive oxygen species, thromboxane, and matrix metalloproteinase-9 which initiate both an inflammatory response as well infiltration of adipose tissue by other immunocytes including macrophages. Accumulation of T-lymphocytes in obese mouse and human adipose tissue may also constitute a proportion of this early infiltrate [114-116]. Kintscher *et al*. identified that T-lymphocytes were already present in visceral adipose tissue of C57/BL6 mice fed a high-fat diet in as little as 5 weeks after initiation of high-fat feeding. Macrophages were not detectable until the 10-week mark. Kintscher *et al*. theorized that like neutrophils, infiltrated and activated T-lymphocytes might contribute to late stage macrophage diapedesis via pro-inflammatory cytokine production. Accordingly, adipocytes treated with t-lymphocyte interferon-γ (IFγ) increased the expression of *monocyte-chemotactic protein-1* (MCP1) a chemoattractant characteristically involved in macrophage recruitment.

Macrophage infiltration in adipose tissue occurs much later after ~8 week consumption of a high-fat diet in mice [117]. Macrophage content of adipose tissue is consequently positively correlated with body mass, degree of adiposity and the cross sectional area of adipocytes and represents the long-term infiltration phase of adipose

tissue remodeling [117, 118]. Macrophages are essential components of the innate immune system and are normally activated by microbe-derived factors [119]. They function as the primary defense against foreign cells by phagocytosing them, presenting antigens to lymphocytes in addition to secreting inflammatory mediators (e.g. TNFα) which influence resident cells (e.g. endothelial cells) and attract additional immunocytes (e.g. lymphocytes) to sites of injury and infection [120]. These are conventionally acute affects, which resolve quickly. On the other hand, chronic inflammation such as occurs in obesity is characterized by continued pathological interaction between the macrophages and tissues resulting in abnormal tissue responses and remodeling [121]. Although adipose tissues from lean mice contain macrophages, they are small, isolated and scattered throughout the adipose tissue. Lean adipose tissue macrophages express genes associated with an alternatively "anti-inflammatory" activated macrophage (M2) phenotype. M2 macrophages express cytokines such as IL-4 and IL-13 that down-regulate pro-inflammatory cytokines [122]. In contrast, macrophages found within the obese adipose tissue remain activated, fusing into multinucleated giant cells, surrounding necrotic adipocytes, and expressing genes more closely associated with a classical "pro-inflammatory" activated macrophage phenotype (M1) [122]. These active multinucleated macrophages will subsequently continue to produce pro-inflammatory cytokines (e.g. TNFα) until the "insult" is cleared [20, 112, 117]. Although the role the obese adipose tissue macrophages play has yet to be fully elucidated, studies suggest they are involved with removal of the necrotic adipocytes they surround as well as removing their leftover lipid droplets [123]. Nonetheless, macrophage infiltration into adipose tissue precedes both the development of adipose tissue associated inflammation and severe insulin

resistance in mice and humans suggesting an important link for adipose tissue macrophages in governing obesity-associated inflammation and altered glucose homeostasis [124-126].

Collectively the current data supports a sequential infiltration of adipose tissue and pro-inflammatory factor production by neutrophils, lymphocytes and macrophages, respectfully. The early infiltration of neutrophils and lymphocytes may initiate both early inflammation and later macrophage infiltration. Once macrophages have infiltrated the tissue, they surround hypertrophic adipocytes increasing secretion of inflammatory mediators like TNFα, which propagates inflammation and insulin resistance.

1.10.00: ADIPOKINES: ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

Adipose tissue has traditionally been understood to function as a storage vessel for energy in the form of triglycerides or fats. Our understanding of adipose tissue has, however, undergone a considerable paradigm shift with the discovery that adipose tissue is an important endocrine organ secreting biologically active molecules called adipokines. By definition, the term adipokine is given to any biologically active substance produced by adipocytes or the adipose tissue including non-adipocyte components (e.g. macrophages), which can act in an autocrine, paracrine or endocrine manner [127, 128]. The emergence of adipose tissue as an endocrine organ began with a pinnacle study by Zhang *et al*. in which the mouse *Ob* gene and its human homologue, were cloned and sequenced from adipocytes, identifying a highly conserved secreted protein leptin [129]. Although morbidly obese mice, referred to now as Ob/Ob mice, had been first identified decades earlier, the exact genetic and molecular determinants of their

associated obesity, hyperphagia, hyperglycaemia and elevated plasma insulin were unknown prior to this study. Ob/Ob mice were found to be homozygous recessive for a mutated version of the *Ob* gene, which resulted in a non-functional variant of leptin. Db/Db mice in contrast had a truncated non-signaling leptin receptor, which resulted in both morbid obesity as well as severe insulin resistance [130, 131]. Leptin has since been well-characterized as a potent regulator of satiety by binding to the Ob-Rb (leptin receptor-B) within the arcuate nucleus of the hypothalamus [132].

The discovery of leptin was the first evidence to support Kennedy's lipostatic hypothesis. This hypothesis proposed adipose tissue secretes hormonal signals in proportion to its overall mass conveying information on nutritional status and controlling energy metabolism [133, 134]. Furthermore, the morbid obesity resulting from leptin or Ob-Rb deficiency supported the concept that alterations in adipose tissue signals could alter local and systemic metabolic processes thereby contributing to the development of obesity and its related comorbidities.

Leptin was, accordingly, the first adipokine explored as a target for pharmaceutical intervention. Human obesity caused by a genetic mutation in leptin or the leptin receptor is, however, extremely rare [135-138]. Paradoxically most obese patients have heightened leptin levels and are considered to be in a state of "leptin resistance" [139]. Preliminary evidence suggested that peripheral leptin administration could overcome this resistance, however Phase I-II clinical trial of subcutaneous injections of recombinant leptin had no effect on body weight or metabolic parameters observed [140]. These results identified that while the vast majority of obesity could not be explained by a single mutation in leptin, continued work to identify new adipokines and their roles in

basic adipose tissue biology and obesity is fundamental to our understanding of the disease pathology.

Following Zhang *et al*.'s study, over 50 adipokines have been identified (**Table 5**) [58, 141, 142]. Adipokines belong to a number of biologically active familial groups including cytokines, growth factors, proteases, proteins of the alternative complement system, proteins involved in vascular homeostasis, regulation of blood pressure, lipid metabolism and glucose homeostasis [141]. Adipokines, therefore, have regulatory roles in numerous aspects of adipocyte metabolism, adipose tissue remodeling (including adipogenesis, adipocyte metabolism, immunocyte recruitment and inflammation) and systemic functions. Adipokines mediate these changes in metabolism through autocrine and paracrine effects in adipose tissue and endocrine effects in tissues such as the vasculature, immunocytes, brain, muscle, pancreas and liver (**Figure 3**).

The remainder of this section will focus on three adipokines that are most relevant to the present dissertation; MCP1, TNFα and adiponectin. These adipokines will be described in the context of their roles in macrophage infiltration, inflammation and insulin sensitivity. This information will provide support for the working model of adipokine-mediate adipose tissue and systemic metabolic dysfunction.

1.11.00: ADIPOKINES AND MACROPHAGE INFILTRATION

After tissue damage, immunocytes are attracted to the injured tissues by low molecular weight peptides called chemokines. One notable chemokine involved in tissue inflammation and repair is MCP1. MCP1 is a polypeptide chemokine produced by a variety of cells in response to inflammatory stimuli and plays a crucial role in the

recruitment of monocytes, neutrophils, and memory T-lymphocytes into tissues by binding to the chemokine receptor-2 (CCR2) [143-145]. Sartipy *et al.* first theorized a possible role for MCP1 in obesity with the discovery that both circulating concentrations and adipose tissue expression of MCP1 are high in Ob/Ob obese mice. Given adipose tissue had ~100–fold higher *MCP1* expression compared with the liver, kidney and lungs it was suggested that adipose tissue specifically comprised the modifiable source of the elevated circulating chemokine. Further *in vitro* analysis of 3T3-L1 adipocytes identified that insulin induced *MCP1* expression under both normal and insulin resistant states. Sartipy *et al.* thus hypothesized that MCP1 may alter adipocyte function and metabolism contributing to the development of insulin resistance by increasing the number of infiltrated macrophages [145]. Studies in transgenic mice with adipocyte-specific over-expression of MCP1 and MCP1 null mice supported this hypothesis [146, 147]. Mice with adipose tissue over-expression of MCP1 had increased numbers of infiltrated macrophages and corresponding insulin resistance. Conversely, mice lacking MCP1 had a reduction in the number of infiltrated macrophages with a corresponding decrease in insulin resistance [146]. The original results from MCP1-null mice are somewhat contested, as latter studies have not established a reciprocal relationship between MCP1 expression and adipose tissue infiltration of macrophages [148, 149]. Differences in genetic background, sampling time, age and environment may have contributed to the documented differences in the mouse phenotype [148]. The variability in MCP1-associated immunocyte recruitment suggests that additional adipokines must also play a role in adipose tissue immunocyte infiltration. In late-stage adipose tissue remodeling, unfortunately, the identity of these adipokines is largely unknown.

1.12.00: ADIPOKINES, INFLAMMATION AND INSULIN RESISTANCE

Inflammatory mediators are regulators of a broad range of cellular responses including cell migration, DNA replication, cell turnover and immunocyte proliferation which collectively contributes to the inflammatory response. Inflammatory mediators are consequently important mediators of pathogen elimination and wound healing. [150]. The connection between obesity and inflammation has been documented in humans as early as 1966 [151-153]. The contribution however of adipose tissue derived inflammatory factors to the obese phenotype was not recognized until the revolutionary publications by Hotamisligil and colleagues decades later [151-153]. In their landmark study, Hotamisligil *et al.* investigated whether adipose-derived TNFα was altered in obesity-associated T2DM. TNFα is an inflammatory cytokine released in response to stressors such as injury and infection, subsequently mediating associated inflammatory response [150]. Adipose tissue expression of *TNFα* was found to be 5- to 10-fold higher in the diabetic animals versus lean controls. Adipose tissue fractionation concluded that the majority of this elevated *TNFα* expression in obese adipose tissue was derived from infiltrated macrophages [117, 128]. Administration of a TNFα neutralizing antibody to obese leptin deficient rats (Fa/Fa) was used to both confirm the adipose inflammation and investigate whether heightened adipose tissue *TNFα* levels influenced glucose homeostasis and insulin-resistance. Neutralization of circulating TNFα was found to decrease insulin resistance and peripheral glucose utilization rates [154]. Hotamisligil *et al.* concluded a direct relationship between the levels of adipose tissue derived TNFα, inflammation and insulin resistance. In further support of this idea, TNFα null mice placed on a high fat diet were found to have ~4-fold lower fasting insulin levels with

corresponding heightened glycaemic response to insulin (decreased insulin resistance) when compared to the wild type counterparts. TNFα deficiency therefore inhibited the development of obesity-associated inflammation and resulting insulin resistance [155]. These results were replicated in Ob/Ob mice lacking TNFα. Despite these findings, TNFα as the single mediator of obesity associated inflammation and insulin resistance is refuted. Although TNFα deficiency did confer higher insulin sensitivity in the Ob/Ob mice lacking TNFα, they still remained more insulin resistant than wild type (lean) controls [155]. Multiple inflammatory factors are consequently thought to govern obesity associated inflammation and insulin resistance associated with obesity. Increases in inflammatory adipokines including IL-6, IL-1β and IL-4 have consequently been causally related to adipose inflammation and insulin resistance [146, 156-158].

In humans, BMI, visceral obesity and markers of insulin resistance are associated with increased adipose tissue *TNFα* expression and circulating concentrations of TNFα [159, 160]. The contributory role of TNFα in insulin resistance was seemingly a good target for pharmacological intervention in an obese population with metabolic syndrome. While etanercept a TNFα neutralizing antibody, was found to significantly decrease the levels of secondary inflammatory markers like C-reactive protein, fibrinogen, and IL-6, it had no effect on insulin-resistance [161-163]. It has been speculated that the lack of effect in humans may result from an inability of the neutralizing antibodies to reach the cell surface and interfere with the local paracrine effects of adipose derived TNFα [164].

1.13.00: ADIPOKINES AND INSULIN SENSITIVITY

The majority of currently known adipokines, including TNFα, IL-6, MCP1 and PAI-1 are positively correlated with BMI, inflammation and/or fat mass and are thought to mediate obesity-associated inflammation and T2DM. Adiponectin, in contrast, is significantly reduced in obesity (particularly visceral obesity), insulin resistance and T2DM [58, 146, 165-170]. Adiponectin was first sequenced and cloned by four separate research groups and is the first adipokine to show beneficial effects by reducing insulin resistance [157, 165, 171-173]. *In vitro* studies using 3T3-L1 adipocytes first identified that chronic insulin treatment or treatment with TNFα, which model the early and late stages of adipocyte insulin resistance and inflammation, significantly down-regulated adiponectin expression [174]. In contrast when treated with the glucose-sensitizing thiazolidinediones (TZD), 3T3-L1 adipocytes increased expression and secretion of adiponectin [175]. Both overweight humans and obese mice treated with a TZD, have a similar heightened concentration of circulating adiponectin. Combined these results suggested adiponectin may combat insulin resistance. Mice deficient in adiponectin support this role. Adiponectin-deficient mice have higher adipose tissue and plasma TNFα concentrations with resulting increased insulin resistance, glucose intolerance and serum triglyceride levels [157, 176]. In contrast Ob/Ob mice which over-expresses adiponectin (Adip-Ob/Ob) had decreased serum FFA, insulin resistance, and glucose intolerance. Adip-Ob/Ob mice also showed increased expression of molecules involved in fatty acid oxidation (e.g acyl-coA oxidase) and energy dissipation (e.g uncoupling protein-2) [177]. Mice given a high-fat meal and supplemented with recombinant adiponectin likewise had blunted postprandial increases in plasma FFAs, glucose and

triglycerides while also increasing FFA oxidation in hind limb muscle. [176]. This protective function of adiponectin has also been demonstrated in humans carrying a gain of function polymorphism for adiponectin (+45G-allele) or one of the adiponectin receptors AdipoR1 (-3882T>C). These individuals are less insulin-resistant and less glucose-intolerant then non-carriers [178, 179]. Jointly, cellular, animal and human studies corroborate a protective role for adiponectin in the development of insulin resistance validating a model in which decreased insulin sensitizing adipokines contribute to the development of insulin resistance. Consequently therapeutic interventions aimed at increasing circulating adiponectin may prevent and/or treat the development of metabolic syndrome. The *in vivo* animal data, however, does not support adiponectin as the exclusive insulin-sensitizing adipokine. Rather, the moderate and variable dysfunctions reported in adiponectin knockout mice suggest that additional insulin sensitizing adipokines must also exist [180, 181].

1.14.00: MODEL OF ADIPOKINE-MEDIATED ADIPOSE TISSUE AND SYSTEMIC METABOLIC DYSFUNCTION.

Based on the aforementioned findings a working model of adipokine-mediated adipose tissue and systemic metabolic dysfunction has been developed (**Figure 4**). This model can be roughly divided into two stages. The first involves immunocyte infiltration and inflammation, the second insulin resistance and T2DM.

1.15.00: STAGE #1: ADIPOKINE-MEDIATED IMMUNOCYTE INFILTRATION AND INFLAMMATION.

As adipose tissue depots grow in coincidence with a high-fat/high-caloric diet and sedentary life style the resulting hypertrophic and hyperplastic adipocytes differentially secrete adipokines. These adipokines activate and recruit neutrophils and T-lymphocytes to the adipose tissue initiating the acute phase of infiltration [20, 110, 116, 182]. Infiltrated neutrophils and T-lymphocytes subsequently secrete factors (e.g. IFγ), which in combination with adipocyte-derived factors (e.g. MCP1), initiate the late phase infiltration of adipose tissue by macrophages and contribute to early stages of insulin resistance. The infiltrated macrophages surround adipocytes resulting in increased secretion of pro-inflammatory adipokines (e.g. TNFα). *De novo* pro-inflammatory molecules both increase recruitment of new immunocytes and contribute to local inflammation.

1.16.00: STAGE #2: ADIPOKINE-MEDIATED LEPTIN RESISTANCE, INSULIN RESISTANCE AND T2DM

With continued weight gain comes a corresponding increase in adipose tissue expression and secretion of insulin-resistant and pro-inflammatory adipokines (e.g. MCP1 & TNFα) along with a decrease in insulin-sensitizing adipokines (e.g. adiponectin). This alteration in adipokine secretion contributes to a systemic inflammatory, insulin resistant and leptin resistant state. Inflammation, insulin and leptin resistance contribute to systemic dyslipidemia and altered glucose metabolism. Together

these endocrine abnormalities mediate the onset of obesity-associated comorbidities, most notably T2DM.

Although adipokines as therapeutic targets including leptin and TNFα have been unsuccessful clinically, adipokines remain an important area of research. Continued work to understand the basic physiology surrounding novel adipokines is fundamental to determining their contribution to obesity and their possibility as therapeutic targets.

Figure 2: Late phase of preadipocyte differentiation. Prior to differentiation *in vitro* preadipocytes undergo several rounds of proliferation (1) until contact inhibition and quiescence is reached (2). Differentiation is subsequently initiated through exposure to a hormonal cocktail of insulin, dexamethasone and isobutylmethylxanthine. Once induced, preadipocytes undergo two rounds of cell division called clonal expansion (3). During clonal expansion the sequential expression of transcriptional factors C/EBPβ & δ induce the expression of C/EBPα, PPARγ and SREBP1c (4) which increase the expression of genes involved with adipocyte function and lipid metabolism resulting in adipocyte maturation and lipid accumulation (5).

Figure 2

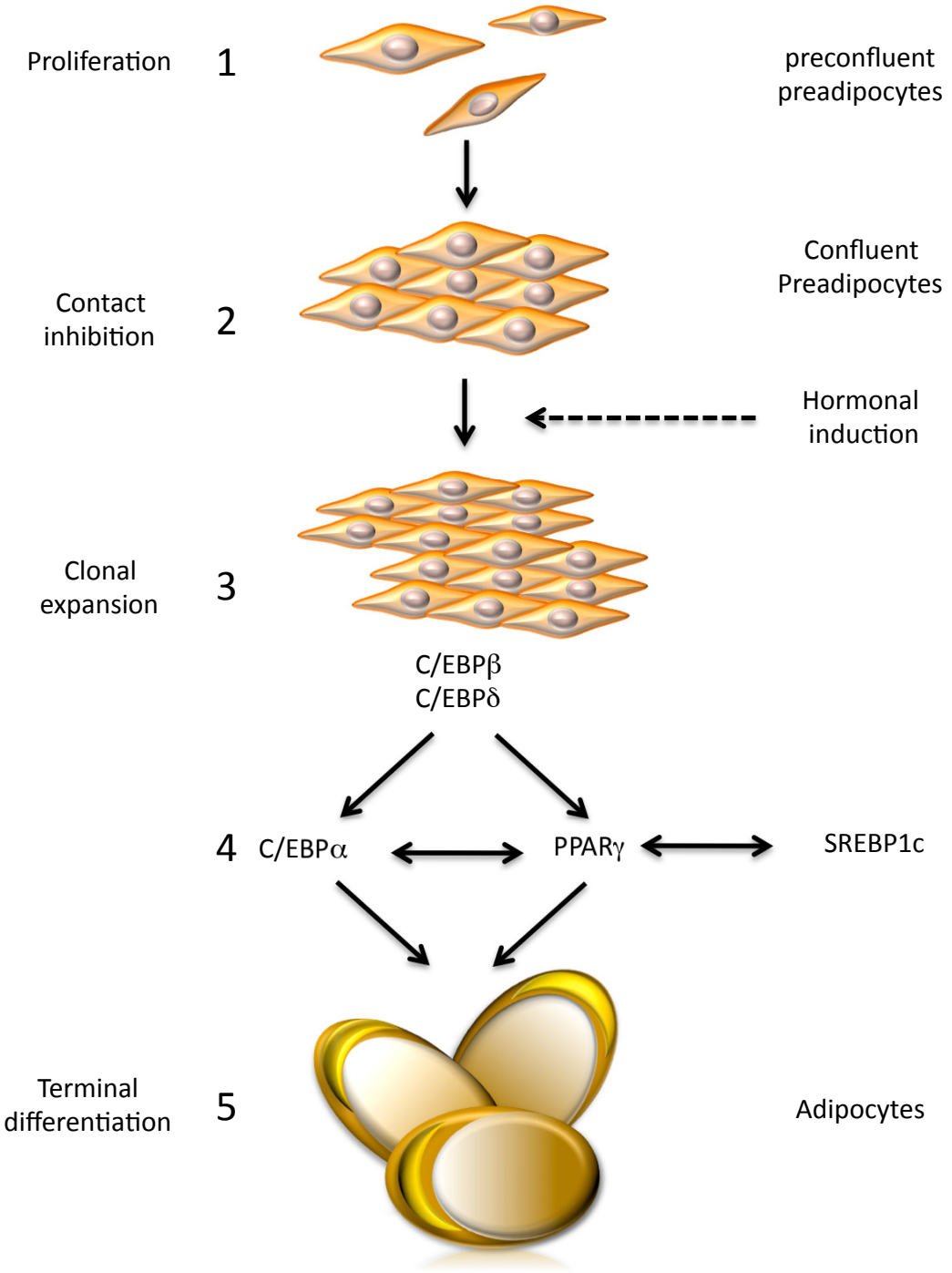

Proliferation
1
preconfluent
preadipocytes
Contact
inhibition
2
Confluent
Preadipocytes
Hormonal
induction
Clonal
expansion
3
C/EBPβ
C/EBPδ
4
C/EBPα
PPARγ
SREBP1c
Terminal
differentiation
5
Adipocytes

Table 5: Example of adipokines and their identified biological functions	
Biological Role	**Adipokine**
Blood Pressure	Apelin
Metabolism	Leptin, Adipsin, Visfatin, Chemerin
Enzymes	Matrix-metaloproteins (MMP)-1, -7, -9, -10, -11, -14, -15, Lysyl Oxidase
Growth Factors	Fibroblast growth factor, Hepatic Growth Factor, Nerve Growth Factor, Stromal derived Growth Factor, Insulin-Like Growth Factor
Cytokines	IL-1β, IL-4, IL-6, IL-10, TNFα, MCP1
Glucose Homeostasis	Adiponectin, Omentin, Resistin, Retinal binding protein 4, Chemerin
Lipid Metabolism	Adipocyte fatty acid binding protein, Apolipoprotein E, Lipoprotein lipase, Chemerin
Vascular Homeostasis	Angiopoietin 1, Angiopoietin 2
Alterantive Complement System	Adipsin, Acylation-stimulating protein, C3
Adapted from [58, 141, 183-185]	

Figure 3: Adipokines. There are currently 50 known adipokines that belong a numerous families of active proteins and hormones including cytokines, growth factors, proteins of the alternative complement system, proteins involve in vascular homeostasis, regulation of blood pressure, lipid metabolism and glucose homeostasis. These adipokines are thought to mediate their effects on metabolism, and energy homeostasis via autocrine, paracrine, and endocrine signaling in tissues such as the vasculature, brain, muscle, pancreas and liver. (Modified from [184])

Figure 3

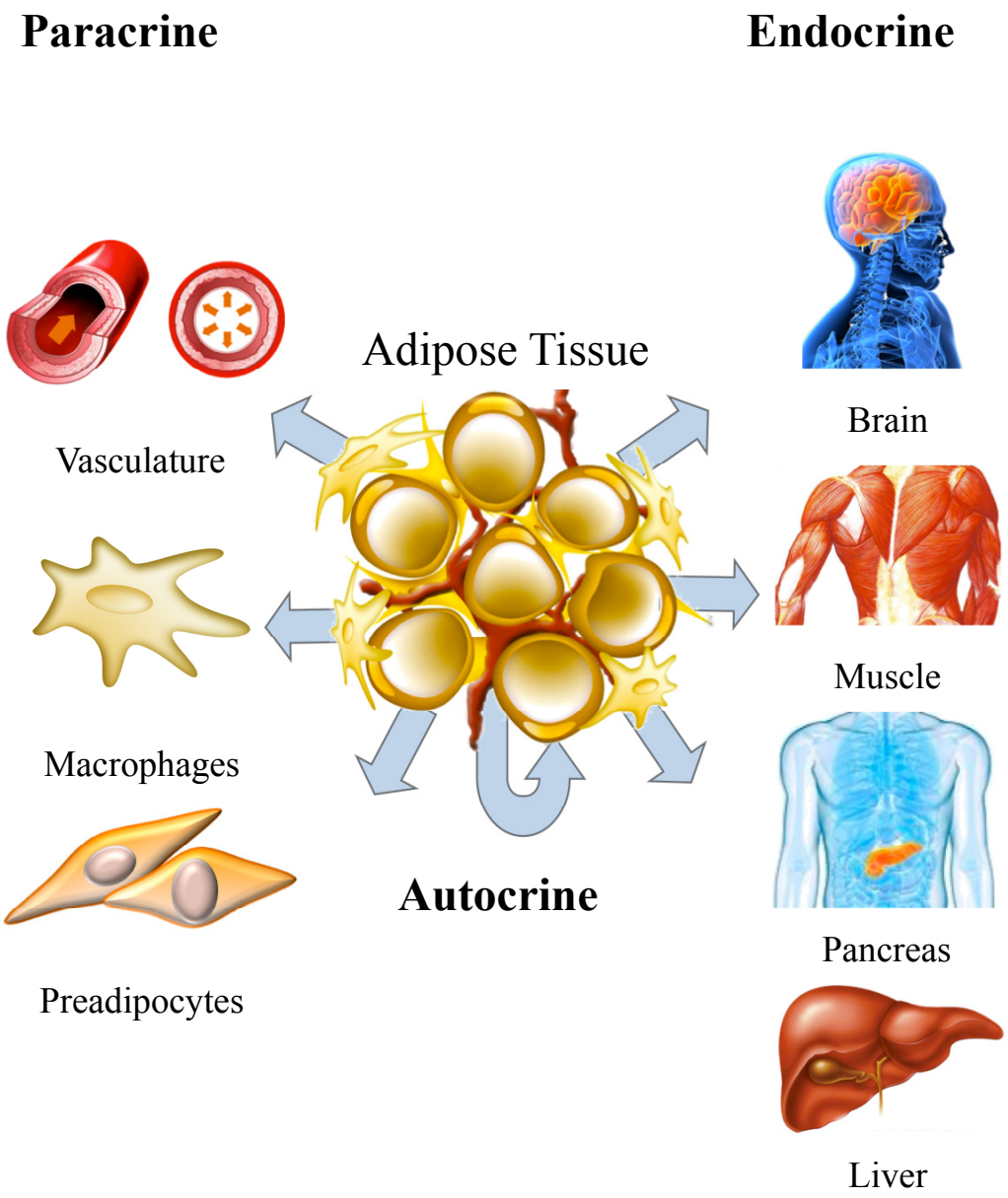
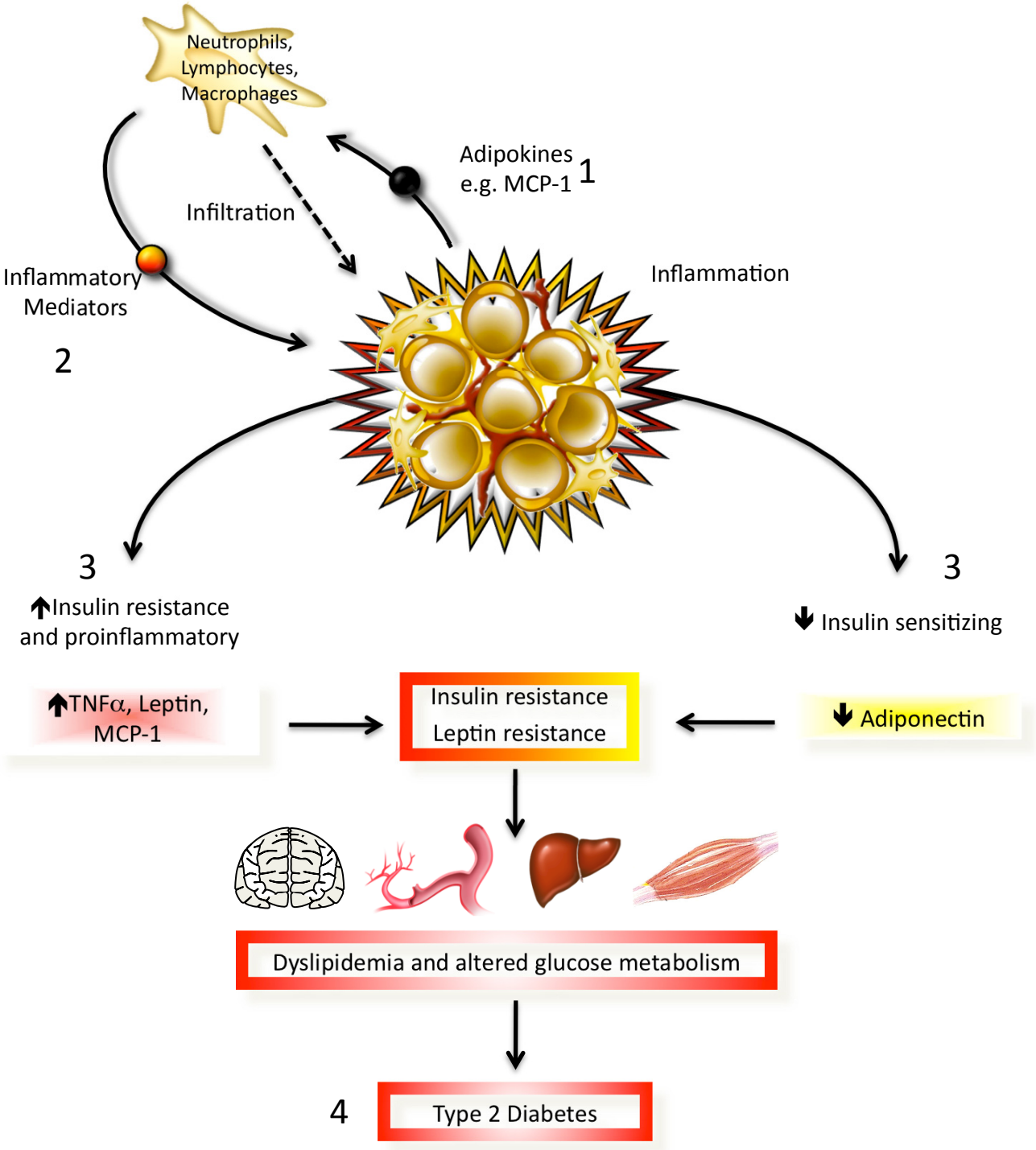

Paracrine
Endocrine
Adipose Tissue
Vasculature
Brain
Muscle
Macrophages
Pancreas
Autocrine
Preadipocytes
Liver

Figure 4: Model of adipokine-mediated adipose tissue and systemic metabolic dysfunction The working model of adipokine-mediated adipose tissue and system metabolic dysfunction proposes adipocytes of obese adipose tissue differentially secrete adipokines (1). These adipokines bind, activate and recruit neutrophils, T-lymphocytes and macrophages. Neutrophils, lymphocytes and macrophages increase the secretion of inflammatory mediators including TNFα (2). As the adipose tissue continues to grow the increased secretion of insulin resistant and pro-inflammatory adipokines and simultaneous decrease in insulin-sensitizing adipokines contribute to systemic insulin and leptin resistance (3). Together these metabolic changes lead to the development of obesity-associated co-morbidities most notably type 2 diabetes (4). This model is explained in detail on page 32.

Figure 4


Neutrophils,
Lymphocytes,
Macrophages
Adipokines
e.g. MCP-1
1
Infiltration
Inflammatory
Mediators
2
Inflammation
3
↑Insulin resistance
and proinflammatory
3
↓ Insulin sensitizing
↑TNFα, Leptin,
MCP-1
Insulin resistance
Leptin resistance
↓ Adiponectin
Dyslipidemia and altered glucose metabolism
4
Type 2 Diabetes

1.17.00: CHEMERIN: A NOVEL ADIPOKINE.

Chemerin (RARES2, TIG2) is a 16-kDA protein first identified in the context of immunology as a mediator between innate and adaptive immunity via the chemokine-like receptor-1 (CMKLR1)[119, 186]. Pioneering work by Goralski *et al*. expanded on this immunological role identifying chemerin as a novel pleiotropic adipokine regulating adipogenesis and adipose metabolism [187]. Considerable advances have been made since 2003, including the discovery of two additional chemerin receptors, GPR1 (G-protein coupled receptor-1) and CCRL2 (chemokine-like receptor-2) as well as the existence of multiple chemerin products each with differing activities. Based on these findings chemerin is becoming recognized as having a far more complex regulatory, signaling, and functional influence in tissues (e.g. adipose) than first anticipated. If chemerin is to be understood in the perspective of normal human biology and disease, a more comprehensive delineation of how these chemerin products are produced and their differential signaling through CMKLR1, GPR1 and CCRL2 must first be fully characterized.

1.18.00: CHEMERIN NOMENCLATURE

Herein the following chemerin nomenclature will be employed. The prefix *m*- and *h*- will designate whether the chemerin product is of mouse or human origin. If the discussed concept applies to both variants no prefix will be used. The word prochemerin will signify the secreted inactive pro-form of chemerin composed of amino acids 21-163 and 21-162 of the translated gene product in humans and mice, respectfully. If the specific chemerin product is known, the terminal amino acid will be identified by a

subscript numeral (e.g. *h*-chemerin$_{157}$). Otherwise the general term chemerin will indicate an unknown chemerin product. Unless indicated the chemerin N-terminal amino acid can be assumed to begin at amino acid 21 of the translated product, which coincides with the predicted N-terminal cleavage site.

1.19.00: CHEMERIN: STRUCTURE AND PROCESSING

Chemerin is produced as pre-prochemerin. Following N-terminal cleavage it is secreted as an inactive 18 kDa precursor prochemerin and subsequently activated into ~16 kDa chemerin by extracellular proteases [119, 186, 188-190]. *Chemerin* is expressed most highly in white adipose tissue and liver with intermediate expression in lung and brown adipose tissue and 80-90% lower expression in all other organs. Based on this expression pattern, adipose tissue and liver are thought to be the major organs of chemerin production *in vivo* [119, 187, 191]. Comparative sequence homology identifies chemerin as a close relative of the cysteine protease inhibitor cystatin and the antimicrobial chemoattractant cathelicidin. A sequence comparison suggests similar structural properties to both molecules. Chemerin, like cystatin, contains a characteristic cystatin structural loop. This loop provides structural stability to cystatin and is presumed to do the same for chemerin. Chemerin, however, contains three, rather than two predicted disulphide bridges for loop stabilization. Chemerin, like cathelicidin, is produced in proform and requires post-translational enzymatic modification for activation. Western blot and mass spectrometry analysis identified that once secreted, *h*-prochemerin is converted by immunocyte, plasma and fibrinolytic-associated proteases into a multitude of full length chemerin products (**Figure 5**) [119, 186, 188-190, 192].

Six separate *h*-chemerin products derived from *h*-prochemerin have been identified *in vitro*. These six chemerin products include *h*-chemerin$_{158}$, *h*-chemerin$_{157, 156, 155, 154}$ and $_{152}$. Each chemerin subtype has both a unique C-terminal amino acid and a differing ability to bind and activate *h*-CMKLR1. Uncertain however, is whether these different binding affinities are equivalent for *h*-GPR1 and *h*-CCRL2 and whether these chemerin products activate differing signaling and functional pathways. The serine proteases plasmin and mast cell tryptase cleave *h*-prochemerin into a form with low activity, *h*-chemerin$_{158}$, by removing 5 C-terminal amino acids [189]. Sequential removal of the remaining C-terminal lysine by plasma carboxypeptidase N or B converts *h*-chemerin$_{158}$ into *h*-chemerin$_{157}$ [188]. *h*-Chemerin$_{157}$ has the highest known activity at the *h*-CMKLR1 receptor [190]. Neutrophil elastase can bypass this two-step process, directly converting *h*-prochemerin into the highly active *h*-chemerin$_{157}$ [186]. *h*-Chemerin$_{157}$ can in turn be inactivated by further cleavage of *h*-chemerin$_{157}$ by either mast cell chymase or angiotensin-converting enzyme into *h*-chemerin$_{154}$ or *h*-chemerin$_{152}$, respectively [190, 193]. With extended exposure, mast cell tryptase, neutrophil elastase as well as proteinase-3 can also directly inactivate *h*-prochemerin by cleaving 8 terminal amino acids, resulting in an inactive/low active *h*-chemerin$_{155}$ [190]. Cysteine proteases neutrophil cathepsin G and K can also convert *h*-prochemerin directly into a highly active *h*-chemerin$_{156}$ by cleaving 7 terminal amino acids [186]. The expression pattern, level and function of these enzymes is therefore of fundamental importance for regulation in prochemerin and/or chemerin processing. Whether or not *h*-prochemerin and *h*-chemerin are processed in this manner by adipocytes *in vitro* or *in vivo* is currently unknown. Mass spectrometry analysis has, however, identified the presence of *h*-chemerin$_{154}$, *h*-

chemerin$_{155}$, and *h*-chemerin$_{157}$ in hemofiltrate, ascitic fluid and human serum, respectively, suggesting differential and localized chemerin processing is pertinent to human circulation and disease [189, 190, 194]. Discovery of these multiple chemerin products has added an extra dimension of complexity to chemerin biology. It suggests that in addition to regulating prochemerin secretion, localized proteolytic processing can drastically alter the functional effects of chemerin signaling. In terms of obesity, it is possible that altered production of prochemerin and these proteolytic enzymes may produce distinctive chemerin products, which in turn may mediate differential signaling cascades.

Chemerin processing occurs on the C-terminus following the structural cystatin fold. The stability that the three disulphide-bond fold provides suggests that the hanging final C-terminal amino acids may reflect a flexible region that could be separated from the full length peptide (**Figure 6**). The separated C-terminal peptide could then potentially maintain its biological activity [190]. In line with this idea, a second chemerin processing theory proposes proteolytic enzymes split the C-terminal ending from full length chemerin, resulting in a circulating small active peptide. This theory was investigated using a luminescence based reporter assay, which produces luminescence in proportion to *h*-CMKLR1 activation. A synthetic peptide corresponding to the last 19-C-terminal amino acids (*h*-chemerin$_{139\text{-}157}$) was identified to have a slightly lower potency (~17 nM) for the *h*-CMKLR1 receptor as the full length active *h*-chemerin$_{157}$ form (~4.5 nM). If this amino acid sequence was further shortened to the final 10 & 9 amino acids (*h*-chemerin$_{148\text{-}157}$ and *h*-chemerin$_{149\text{-}157}$) the peptide maintained its activity with potencies of ~8.2 and ~7.1 nM respectfully. Removal of Tyr^{149} however significantly

decreased the potency (~1.5 μM) while shorter peptides corresponding to chemerin 151-157 did not activate *h*-CMKLR1. Together these results suggested that if a C-terminal peptide was formed *in vivo,* a 9 amino acid peptide would be the minimum requirement for activation of *h*-CMKLR1 [190]. These C-terminal peptides have yet to be identified in either *in vitro* and/or *in vivo* cellular, animal or human models and are therefore questioned for their relevance to human biology but may be of therapeutic importance.

Murine prochemerin shares 63% sequence homology with *h*-chemerin and the final C-terminus (**Figure 5**) [195-197]. Based on this sequence homology, particularly on the C-terminus, *m*-prochemerin has similar predicted proteolytic cleavage sites (**Figure 5**). Findings by Luangsay *et al.* supports this theory. Synthetic chemerin peptides that corresponded to proteolytic cleavage products of *m*-prochemerin and *m*-chemerin were tested for their ability to mobilize intracellular calcium release through *m*-CMKLR1 signaling. *m*-Prochemerin$_{137-162}$, *m*-chemerin$_{137-157}$ and *m*-chemerin$_{140-154}$, like their human counterparts, are devoid of biological activity, while *m*-chemerin$_{137-156}$ activated *m*-CMKLR1 with the same potency as recombinant *m*-chemerin$_{156}$ (pEC_{50} 7.71 vs 9.34 nM). Luangsay *et al* therefore concluded that the activation of *m*-prochemerin like *h*-prochemerin requires accurate C-terminal processing.

Figure 5: Prochemerin Processing. *In vitro* recombinant *h*-prochemerin studies identified *h*-prochemerin is cleaved by immune cell and serum associated enzymes into 6 chemerin products that differ in their C-terminal amino acid and potency for CMKLR1 activation. *h*-chemerin$_{154, 155}$ and $_{157}$ have been found in human hemofiltrate, serum and ascites fluid, respectfully. Given the sequence homology between human and mouse chemerin C-terminus (conserved amino acids are indicated in red), similar *m*-prochemerin protease cleavage is predicted. Only the C-terminal amino acids of the full-length protein are identified within this figure.

Figure 5

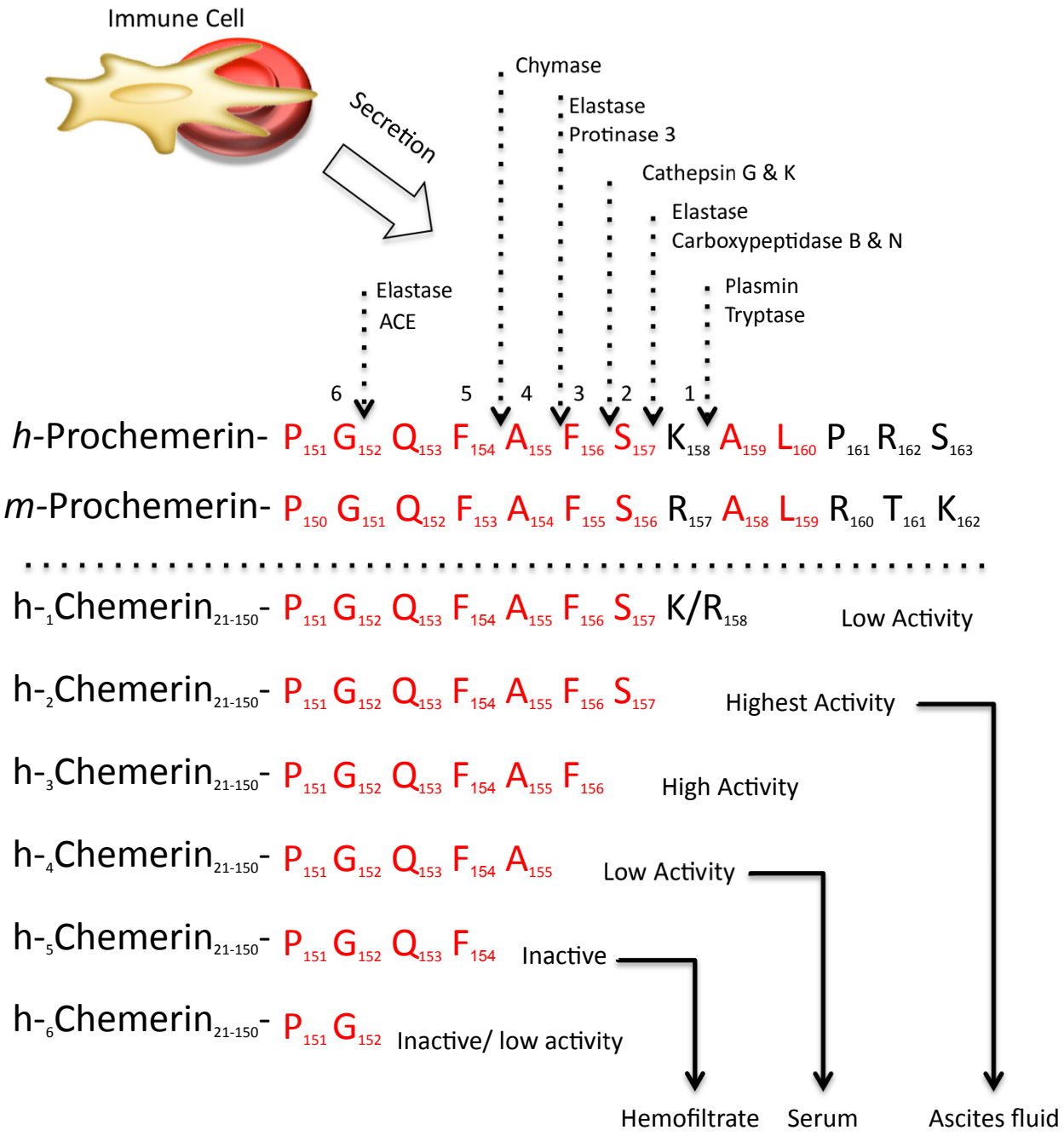
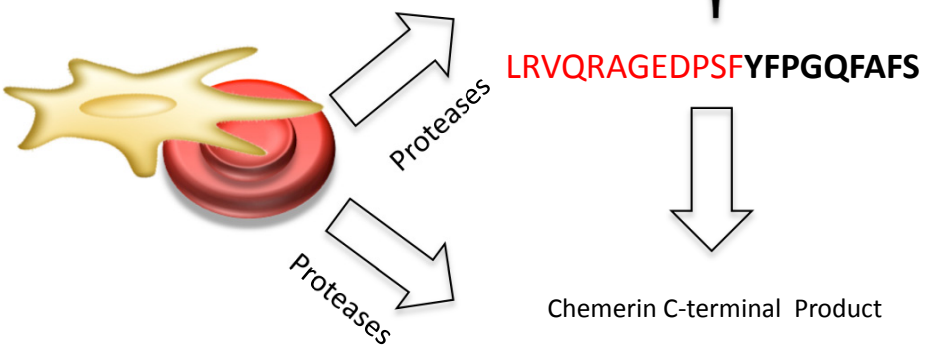

Immune Cell
Secretion
Chymase
Elastase
Protinase 3
Cathepsin G & K
Elastase
Carboxypeptidase B & N
Plasmin
Tryptase
Elastase
ACE
6
5
4
3
2
1
h-Prochemerin- P151 G152 Q153 F154 A155 F156 S157 K158 A159 L160 P161 R162 S163
m-Prochemerin- P150 G151 Q152 F153 A154 F155 S156 R157 A158 L159 R160 T161 K162
h-1Chemerin21-150- P151 G152 Q153 F154 A155 F156 S157 K/R158
Low Activity
h-2Chemerin21-150- P151 G152 Q153 F154 A155 F156 S157
Highest Activity
h-3Chemerin21-150- P151 G152 Q153 F154 A155 F156
High Activity
h-4Chemerin21-150- P151 G152 Q153 F154 A155
Low Activity
h-5Chemerin21-150- P151 G152 Q153 F154
Inactive
h-6Chemerin21-150- P151 G152
Inactive/ low activity
Hemofiltrate
Serum
Ascites fluid

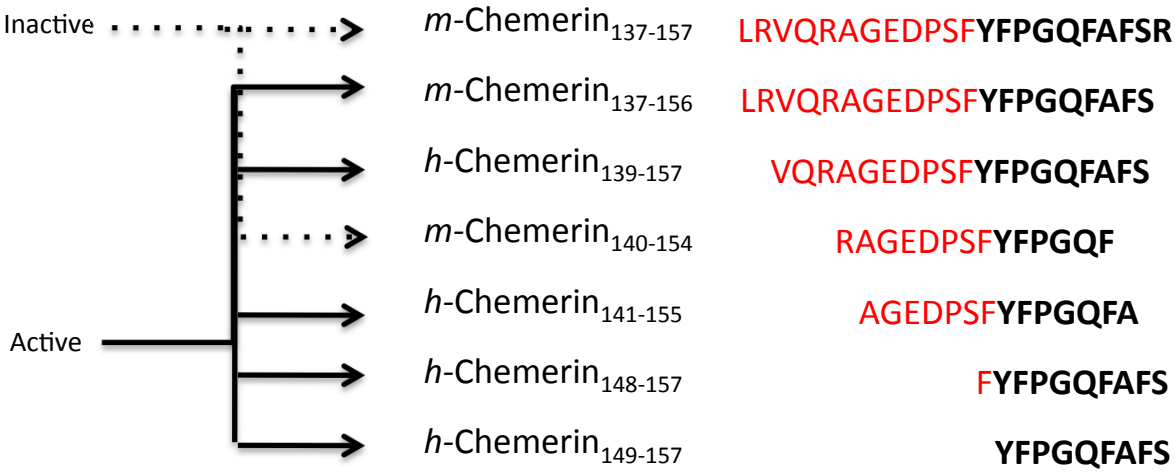
Figure 6: C-Terminal chemerin product. A second chemerin processing theory postulates that the final 21 C-terminal amino acids of *h*-chemerin$_{157}$ or *m*-chemerin$_{156,}$ which follow a disulphide bridge, may be proteolytically cleaved releasing a small chemerin fragment that retains its biological activity. Five synthetic active mouse and human chemerin C-terminal fragments have been identified to activate CMKLR1. The Cs labeled in blue in chemerin$_{157}$ identifies the cysteine involved in the putative disulfide bonds (**A**). Prochemerin and chemerin are thought to take on a similar cystatin-fold conformation as porcrine protegrin identified in the three dimensional model. The chemerin cystatin fold is believed to situate the C-terminus in such a way to allow proteases access for processing (**B**). (Modified from [198])

Figure 6

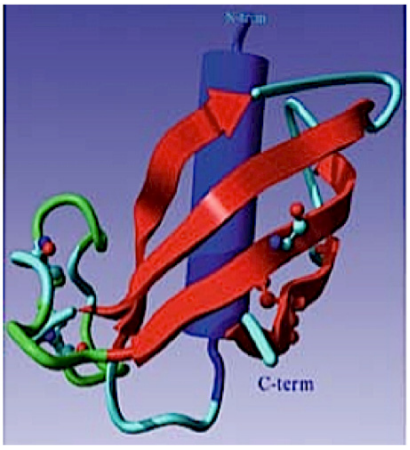
A

h-chemerin$_{157}$ GVAELTEAQRRGLQVALEEFHKHPPVQWAFQETSVESAVDTPFPAG
IFVRLEEFLQQTSCRKRDWKKPECKVRPNGRKRKCLACIKLGSEDKV
IGRLVHCPIETQVLREAEEHQETQCLRVQRAGEDPSFYFPGQFAFS





B



1.20.00: CHEMERIN RECEPTORS

Chemerin is currently known to bind to three receptors CMKLR1, GPR1 and CCRL2. CMKLR1 is the most widely studied as it was the first chemerin receptor identified. Accordingly all of the biological actions attributed to chemerin are currently attributed to CMKLR1 signaling.

CMKLR1 was first isolated from macrophages and immature dendritic cells while investigating chemokine receptors with putative roles as co-receptors for HIV infection [199, 200]. The mouse orthologue of CMKLR1 was simultaneously sequenced and in addition to immunocytes is highly expressed in developing bone, mature parathyroid gland, lung, brain, heart and placenta. Adipocytes are unique in they express high levels of *CMKLR1* in addition to expressing very high levels of its ligand *chemerin* [187, 201, 202]. Analysis of the CMKLR1 protein sequence identified ~38% homology with anaphylatoxin-R, C3a-R and C5a-R chemoattractant receptors yet CMLKR1 represents a novel receptor group given its structural differences, principally its lack of characteristic N-terminal cysteine [200]. Evidence suggests CMKLR1 exists as two separate splice variants CMKLR1a and 1b. CMKLR1a is however the predominant form as 1b is expressed in exceedingly low levels [203]. Current literature has begun to elucidate the manner in which *h*-chemerin$_{157}$ signals through *h*-CMKLR1, although the majority of the CMKLR1 signaling cascade remains elusive. After *h*-chemerin$_{157}$ has bound to *h*-CMKLR1, *h*-CMKLR1 signals via a G_i pertussis toxin sensitive signaling pathway. G_i mediated signaling subsequently increases intracellular calcium, inhibits cAMP accumulation and increases phosphorylation of p42 and p44 MAP kinases (ERK1 & ERK2) [119, 187]. These intracellular processes have been associated with the biological

role of chemerin in monocyte mobilization and adipogenesis [119, 187]. The ability of other chemerin products outside *h*-chemerin$_{157}$ to signal through the aforementioned pathways is yet to be investigated.

G-protein receptor-1 (GPR1) is a second GPCR known to bind chemerin. At present no biological function of chemerin has been attributed to GPR1 signaling. *h*-GPR1 is able to mobilize intracellular calcium after *h*-chemerin$_{157}$ stimulation but to a much lower extent (~30%) than *h*-CMKLR1 [204]. GPR1 is a highly conserved mammalian GPCR that shares sequence homology with human C5a anaphylatoxin receptor, δ opioid receptor, formyl-peptide 2 receptor, human angiotensin receptor 1A, and human somatostatin receptor [205]. Comparative sequence homology puts CMKLR1 as the closest relative to GPR1 [204]. Limited northern blot and QPCR analysis has identified that while *GPR1* is expressed in adipose tissue, muscle and liver it is not detected in the putamen, pons, frontal cortex, thalamus, cerebellum, neutrophils, or monocytes [205, 206]. Our unpublished data have also confirmed high *GPR1* expression in 3T3-L1 and bMSC preadipocytes and adipocytes. *h*-Chemerin$_{149\text{-}157}$ has been shown to bind to a single site on GPR1 with a calculated K_d of 5.3 nM, which is comparable to *h*-CMKLR1 (K_d 4.9 nM). In contrast, utilizing a cell based luminescence reporter assay that produces luminescence in proportion to receptor activation, *h*-chemerin$_{157}$ was found to activate *h*-GPR1 with an EC_{50} of 240 pM, compared with 3 nM for *h*-CMKLR1. Taken together this suggests that *h*-chemerin$_{157}$ is able to bind to both *h*-GPR1 and *h*-CMKLR1 with equal potency, although *h*-chemerin$_{157}$ can more easily activate *h*-GPR1 [204]. The difference in CMKLR1 and GPR1 activation and signaling by chemerin may have functional consequences in tissues expressing both receptors. Given that each chemerin

product has an individual ability to activate CMKLR1, one would reason that they might also differentially activate GPR1. The ratio of the differing cleavage products in circulation may then govern the balance of GPR1 to CMKLR1 activation, resulting in vastly different biological effects. Unfortunately only *h*-chemerin$_{157}$ activity at GPR1 and CMKLR1 has been studied; this theory therefore remains speculative at present.

CCRL2 is a GPCR that contains an uncharacteristic sequence in the 2nd intracellular loop rendering it unable to signal [207]. *h*-CCRL2 is expressed by T-cells, neutrophils, monocytes, macrophages and dendritic cells and is upregulated by lipopolysaccharide (LPS) in macrophages. Radioligand binding studies, in which saturating concentration of unlabeled chemerin are used in the presence of labeled chemerin to outcompete the radiolabel variant, confirmed both *m*-chemerin$_{156}$ (EC_{50} 1.6 nM) and *h*-chemerin$_{157}$ (EC_{50} 0.2 nM) bind to *m*- and *h*-CCRL2 with a similar binding affinity as *m*-CMKLR1 (EC_{50} 3.1 nM). CCRL2 is unable to mobilize any intracellular calcium or increase monocyte migration. However, cells over-expressing *m*-CCRL2 treated with *m*-chemerin$_{157}$ are able to increase intracellular calcium in adjacent cells expressing *m*-CMKLR1. CCRL2 is therefore believed to moderate chemerin signaling by acting as a chemerin "trap" increasing local concentrations of chemerin and thereby increasing CMKLR1 and/or GPR1 signaling on adjacent cells. This would have important functional consequences in tissues in which local concentrations of chemerin are normally well below the functional range but express CCRL2.

1.21.00: CHEMERIN: IMMUNOCYTE RECRUITMENT AND INFLAMMATION

The immune response can be divided into the early innate and late adaptive immune responses. The innate immune system provides the immediate (hours to days) protection against an infection by presenting pathogenic antigens to immunocytes involved in the long-term (days to weeks) adaptive immune response [208]. Macrophages and dendritic cells play an important role bridging innate and adaptive immunity. The cellular components of the innate immune system, which include macrophages, dendritic cells, granulocytes, mast cells and natural killer cells sense pathogens and are involved in pathogenic destruction via phagocytosis, antigen presentation and inflammatory cytokine production thereby commencing the adaptive immune response. Neutrophil migration to and activation by invading pathogens is the first step of this innate response. Upon activation, neutrophils release microcidal products including enzymes and inflammatory chemoattractant cytokines. These chemoattractant cytokines subsequently recruit macrophage and dendritic cells to the damaged tissue resulting in the induction of adaptive immunity. The first biological role for chemerin signaling was inferred from the structural homology of CMKLR1 to chemoattractant receptors in addition to the elevated expression pattern of *CMKLR1* in macrophages and immature dendritic cells. Immunocytes including macrophages, dendritic cells, neutrophils and natural killer cells express chemoattractant receptors including CXCR4 and CCR7 that mediate their migration from blood into sites of tissue damage and inflammation. This recruitment is a hallmark of both acute and chronic inflammation. Consequently, like other chemoattractants, chemerin was hypothesized to increase the migration of macrophages and dendritic cells to sites of inflammation and tissue damage [119, 192]. Accordingly

recombinant *h*-chemerin$_{157,}$ *h*-serum chemerin and *h*-plasma chemerin promote migration of CMKLR1^{+} pre-B lymphocytes, monocyte derived macrophages, immature plasmacytoid dendritic cells and natural killer cells in transwell migration assays [119, 192, 202, 209, 210]. Chemerin is therefore a potent leukocyte chemoattractant protein.

Treatment of isolated murine macrophages with pro-inflammatory and anti-inflammatory cytokines has begun to expand on the specificity of chemerin mediated leukocyte recruitment. Antigens associated with M1 macrophages including lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (PolyI:C) which mimic bacterial and viral infection were found to significantly down-regulate macrophage *m-CMKLR1* expression. Natural killer cells similarly down-regulate *h*-CMKLR1 after treatment with inflammatory mediators IL-2 & IL-15 [209]. In contrast anti-inflammatory and M2 associated transforming growth factor β1 or β2 resulted in *m*-CMKLR1 up-regulation. Although LPS decreased macrophage *m*-CMKLR1 expression, neither TNFα nor IFγ (both components of LPS mediated inflammation) alone suppressed *m-CMKLR1* to the same extent. Alternatively, TNFα and IFγ treatment resulted in only modest suppression of macrophage *m-CMKLR1* expression. These results suggest that immunocyte CMKLR1 down-regulation, and therefore chemerin-associated immunocyte recruitment is also dependent on the composition of inflammatory mediators present in the damaged tissue. A role for CMKLR1^{+} immunocyte migration to tissue damage has been supported in humans. h-CMKLR1^{+} natural killer and dendritic cells are localized in inflammatory skin lesions and kidneys of patients suffering from inflammatory diseases including lupus erythematosus, lupus nepheritis and inflammatory oral lichen planus (OLP) [202, 209, 211]. Histological analysis of OLP lesions co-localized chemerin

reactivity along side dendritic and natural killer cells within the inflamed lesion. Likewise patients suffering from osteoarthritis and psoriasis have heightened *h*-chemerin concentrations with corresponding pathological infiltration of neutrophil, natural killer and dendritic cells into the synovial fluid and psoriatic lesions [212-214]. Chronic inflammatory states including Crohn's disease, ulcerative colitis, chronic dermatitis and chronic hepatitis C are also characterized by elevated circulating chemerin concentrations [213, 215]. Together these *in vitro* and *in vivo* studies support a role for chemerin in inflammatory states via immunocyte recruitment.

Given that *CMKLR1* expression is completely lost in mature dendritic and natural killer cells, chemerin is believed to act in the very early stages of the immune response by increasing recruitment of macrophages, dendritic and natural killer cells to damaged or inflamed tissues prior to their activation. Were chemerin to play this role in early macrophage, dendritic and natural killer cell recruitment, its conversion from pro-form would need to occur very early in the innate immune response. Consequently, neutrophils were investigated as mediators of *h*-prochemerin$_{21\text{-}163}$ activation. [186]. *In vitro* studies identified activated neutrophils secrete serine proteases, including cathepsin G and elastase, that potently activate *h*-prochemerin into *two* highly active forms, *h*-chemerin$_{156}$ and *h*-chemerin$_{157}$ respectfully, supporting a role for chemerin in early immunocyte recruitment [186].

1.22.00: CHEMERIN: AN ANTI-INFLAMMATORY MEDIATOR

Anti-inflammatory roles for CMKLR1 and chemerin have also been identified. Resolvins are locally derived anti-inflammatory signaling mediators derived from omega-3 polyunsaturated fatty acids that are generated during the resolution phase of inflammation. Resolvin E1 (RvE1) has been identified as an *h*-CMKLR1 substrate and is as effective as high doses of dexamethasone or aspirin in limiting leukocyte infiltration. RvE1 signaling through *h*-CMKLR1 exerts anti-inflammatory effects by decreasing TNFα-mediated inflammatory signaling, as well as dendritic cell inflammatory IL-12 production [216]. Analysis of RvE1 and *h*-chemerin$_{144-157}$ signaling at *h*-CMKLR1 suggests that although both ligands signal through *h*-CMKLR1, they do so via different mechanisms. *h*-Chemerin$_{144-157}$, in contrast to RvE1, evoked 3-fold higher G_i-protein activation and extracellular acidification rates. Cash *et al.* expanded on these anti-inflammatory effects of CMKLR1 by identifying that a synthetic polypeptide derived from the final 15 amino acids of *m*-chemerin$_{154}$ (chemerin$_{140-154}$) inhibited macrophage production of inflammatory mediators [217]. Mice pre-treated with *m*-chemerin$_{140-154}$ *in vivo* were consequently protected from zymosan induced peritonitis, a model of sterile inflammation, by decreasing the both number of recruited neutrophil and monocytes into the peritoneal fluid and by decreasing pro-inflammatory production (i.e. TNFα, IL-1β and IL-6). *m*-CMKLR1 null mice were not conferred with this same resistance, confirming a *m*-CMKLR1 dependent anti-inflammatory mechanism [217]. *m*-Chemerin$_{140-154}$ is thus proposed to act as an anti-inflammatory by removing the initial pro-inflammatory instigators and by preventing the release of additional pro-inflammatory mediators by the macrophages [218]. Luangsay *et al* corroborated the anti-

inflammatory effect of recombinant *m*-chemerin$_{156}$ in a model of acute lung injury in mice. C57/BL6 mice treated with a combination of LPS and *m*-chemerin$_{156}$ showed decreased lung tissue inflammation, bronchiolar epithelial hyperplasia as well as peribronchiolar, perivascular, and alveolar infiltration by neutrophils when compared with mice treated with LPS alone. *m*-Chemerin$_{156}$ also reduced the LPS dependent increase in inflammatory mediators including TNFα and IL-6 [197]. In unison the abovementioned studies support that the role of CMKLR1 signaling (i.e. pro- or anti-inflammatory) depend on the type of inflammatory insult and the active chemerin product present.

1.23.00: CHEMERIN: A REGULATOR OF ADIPOGENESIS AND ADIPOSE-METABOLISM

A novel role for chemerin was identified by Goralski *et al.* while characterizing the tissues responsible for producing circulating *m*-prochemerin and *m*-chemerin [119, 187, 219]. QPCR analysis of *chemerin* and *CMLKR1* mRNA expression in 19 separate mouse tissues identified that although both the ligand and receptor were ubiquitously expressed throughout the tissues isolated, adipose tissue was unique in that it expressed the highest levels of both *chemerin* and *CMKLR1*. *Chemerin* and *CMKLR1* expression was found to be ~2-fold higher in the adipocyte fraction compared to the stromal vascular fraction of the adipose tissue. These results support adipocytes as the predominant cell type in adipose tissue that both produced chemerin as well as its receptor, suggesting a possible autocrine role for chemerin in adipocytes. Subsequent exploration of *chemerin* and *CMKLR1* expression in 3T3-L1 cells identified *chemerin* expression to be minimal in

preadipocytes, increasing up to ~300-fold upon differentiation into adipocytes. *CMKLR1* shows a similar expression profile increasing ~60-fold through adipogenesis. The total immunodetectable levels of ~16 kDa *m*-chemerin and *m*-CMKLR1, likewise, increased proportionally with their mRNA expression. Functional analysis of the total *m*-chemerin produced by adipocytes using a $CMKLR1^{+}$ pre-B immunocyte migration assay, in which immunocytes migrate in proportion to the concentration of active chemerin, identified the *m*-chemerin secreted was also activated in proportion with adipogenesis [187]. Taken together, the differentiation-dependent increase in active *m*-chemerin and *m*-CMKLR1 suggested *m*-chemerin may be mediating adipogenesis.

A role for chemerin in adipocyte biology was confirmed using adenoviral-administered shRNA knockdown of chemerin or CMKLR1 in preadipocytes and adipocytes. Knockdown of either chemerin or CMKLR1 in 3T3-L1 preadipocytes prior to hormonal-induced differentiation resulted in a significant reduction of adipocyte gene expression including *perilipin*, *hormone-sensitive lipase*, *adiponectin* and *glucose transporter-4* that corresponded with a reduction in oil red-O staining of neutral lipids, a marker of adipocyte differentiation. Histological analysis confirmed 3T3-L1 cells lacking chemerin or CMKLR1 were inhibited from differentiating. The cells that lacked chemerin remained instead in a fibroblast-like state while those that lacked CMKLR1 displayed a mixture of abnormally large cells with multiple lipid droplets and fibroblast-like cells. It has been determined this inhibition of differentiation results from an inability to undergo early clonal expansion [220]. Adipose-derived *m*-chemerin signaling through *m*-CMKLR1 was therefore established as a fundamental regulator of adipocyte

differentiation. This role has since been corroborated in alternative human and rodent adipocyte models including bMSCs [187, 221-223].

Post-differentiation, knockdown of chemerin and CMKLR1, in contrast, has no overt effect on adipocyte morphology. Rather, numerous adipocyte genes involved in adipocyte metabolism were differentially reduced by chemerin and CMKLR1 knockdown including *adiponectin* and *leptin*. Chemerin signaling through CMKLR1 was as a result also recognized as a mediator of mature adipocyte metabolism. Ensuing functional assays identified *m*-chemerin inhibited isoproterenol-induced lipolysis and decreased insulin-stimulated glucose uptake in 3T3-L1 adipocytes [187, 224]. *In vivo* studies have further supported a role for chemerin in mediating insulin resistance. Ob/Ob and Db/Db obese mice that were administered exogenous chemerin had worse glucose intolerance, and decreased tissue glucose uptake into the liver, white adipose and skeletal muscle [206]. *P. Obesus* rats with impaired glucose tolerance or T2DM had analogous heightened *m-chemerin* and *m-CMKLR1* expression in adipose tissue, which was positively correlated with the animals weight and fasting plasma insulin. The role for chemerin in glucose intolerance is, however, somewhat contested as Takahasi *et al.* identified that *m*-chemerin$_{156}$ increased glucose uptake via insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 in cultured adipocytes [225]. This discrepancy may have resulted from the differences in incubation time with *m*-chemerin$_{156}$ (48 vs. 12 hour pre-incubation) and the different concentrations used (~10 μM vs. ~5 μM). Furthermore it must be taken into account that *in vivo,* skeletal muscle rather than adipose is responsible for the vast majority of glucose disposal. The differences in reported effects of chemerin on glucose homeostasis by Takahashi *et al*.

may therefore result from an inability to take into account the chemerin signaling in tissues like muscle or liver that would occur *in vivo* or it may just be tissue-specific effects.

While the metabolic effects of chemerin are not yet entirely understood the collective data indicate a role for chemerin/CMKLR1 signaling in adipogenesis and metabolism [187].

1.24.00: CHEMERIN IN HUMAN INFLAMMATORY DISEASES

Apparent active chemerin concentrations ranging from 33-358 ng mL^{-1} (~2-22 nM) were measured from ascites and synovial fluids of patients with ovary carcinoma, arthritis and osteoarthritis. Apparent chemerin in a sample is a measure of the ability of an unknown sample to activate *h*- or *m*- CMKLR1-relative to nM equivalents of recombinant *h*- or *m*-chemerin. Given that ovarian carcinoma, arthritis and osteoarthritis display a strong inflammatory component; *h*-chemerin was hypothesized to be involved in their disease pathology and inflammation [119]. Clinical studies investigating total *h*-chemerin (measurement of prochemerin and chemerin) concentrations in the context of human inflammatory diseases with immunological and inflammatory associated pathologies (e.g. polycystic ovary syndrome, Crohn's disease and ulcerative colitis) have likewise identified elevated circulating total *h*-chemerin in disease states [213, 215, 226-230]. The exact role total chemerin plays in these diseases is currently unknown. It is however theorized that the dual nature chemerin plays as a pro-inflammatory and/or anti-inflammatory may be exacerbating or diminishing the associated immunological and inflammatory pathologies associated with these diseases. Accordingly, total serum *h*-

chemerin is independently and positively associated with markers of inflammation and immunological pathologies including leptin, C-reactive peptide, TNFα, fibrinogen, thrombocytes and white blood cell count [226, 227].

Obesity and the metabolic syndrome are likewise characterized by an inflammatory component. Total chemerin concentrations were consequently investigated in the context of obesity and metabolic syndrome. Circulating plasma levels of total *h*-chemerin were significantly higher in human subjects with a BMI greater than 30 kg m^{-2} and are positively associated with measures of body fat (e.g. fat mass, weight and waist to hip ratio), metabolic syndrome (e.g. fasting glucose, fasting insulin, plasma triglycerides, blood pressure, macrophage infiltration) and inflammatory mediators (e.g. TNFα, IL-6, CRP and Leptin). Even after adjustment for BMI, chemerin levels remain independently associated with measurements of metabolic syndrome [222, 231-236]. These results have been corroborated in Caucasian and Mexican American populations, suggesting conservation of elevated total *h*-chemerin amongst the obese of geographically and genetically distinct populations. Obese patients that lose weight after gastric-bypass had a corresponding parallel reduction in circulating total *h*-chemerin supporting adipose tissue as a modifiable-source of circulating total *h*-chemerin. Lower total *h*-chemerin concentrations also corresponded to improvements in insulin resistance and glucose intolerance [232]. The positive association of elevated total *h*-chemerin with insulin insensitivity even after sex, age, weight and BMI were taken into account suggests that increased concentrations of total *h*-chemerin may be mediating aspects of the pathological glucose intolerance associated with obesity and metabolic syndrome [231, 237]. Accordingly, Sell *et al* identified *h*-chemerin$_{157}$ increased insulin resistance and

decrease glucose uptake into primary human myocytes [238]. A role for total *h*-chemerin in glucose intolerance is again debated, as a number of studies have not observed elevated chemerin concentrations in T2DM [222, 231, 232]. These differences may result from differences in ELISA assays, variances in population, age or BMI. The positive correlation of two common human chemerin polymorphisms (rs17173608 and rs10278590) to visceral adipose tissue mass, suggests that chemerin may also mediate the deposition of visceral adipose tissue [239]. In accordance with this premise, multiple regression analysis identified visceral adipose tissue deposition as being independently affected by the concentration of circulating total *h*-chemerin [240]. Jointly the current literature *in vitro* and *in vivo* provides compelling evidence to support that heightened total *h*-chemerin is somehow involved in the pathogenesis of obesity and the metabolic syndrome in addition to other inflammatory diseases but its exact functional role remains unclear.

One major limitation existing within currently published human clinical chemerin studies is their use of the ELISA assay to correlate total *h*-chemerin concentrations with aspects of human disease. The ELISA assay is not able to differentiate between inactive prochemerin and active chemerin products nor is it able to identify small active chemerin C-terminal cleavage forms. Rather these studies infer a role for chemerin in disease states based on the assumption that there is parity between total chemerin and its activity at CMKLR1. These studies may be over or underestimating the effects of chemerin activity. It is therefore imperative that rather than exclusively monitoring total serum *h*-chemerin, research into the specific chemerin products, their activity as well as the role chemerin signaling is playing in the context of these diseases be continued. In this way it can be

elucidated as to whether heightened or diminished chemerin activity is an important contributor to inflammatory and immunological diseases including obesity.

1.25.00: OBJECTIVE AND HYPOTHESIS:

By definition an epidemic is a sudden and great increase in the occurrence of a disease within a population [241]. Accordingly, obesity falls well within the definition of being a worldwide epidemic. Unfortunately few functional therapeutic interventions are in place to combat this growing threat. The lack of therapeutic options is largely due to the complicated nature of obesity. Adipose tissue deposition, adipocyte hypertrophy, preadipocyte hyperplasia, infiltration and inflammation (tissue remodeling) all contribute to the endocrine abnormalities of obesity. The exact molecular mechanisms that govern these dysfunctions are for the most part unknown. Adipokines including leptin, MCP1, TNFα and adiponectin are recognized contributors to weight gain, immunocyte infiltration and inflammation and insulin resistance. Yet none of these molecules alone are the single cause of the aforementioned abnormalities. Consequently therapeutic interventions against these targets have been largely unsuccessful. Regardless adipokines as a whole support a model in which altered adipokine secretion mediates the metabolic dysfunctions associated with obesity. Thus, continued research into novel adipokines is fundamental for advancing our understanding of normal and pathological adipose biology and thereby better identifying novel therapeutic targets.

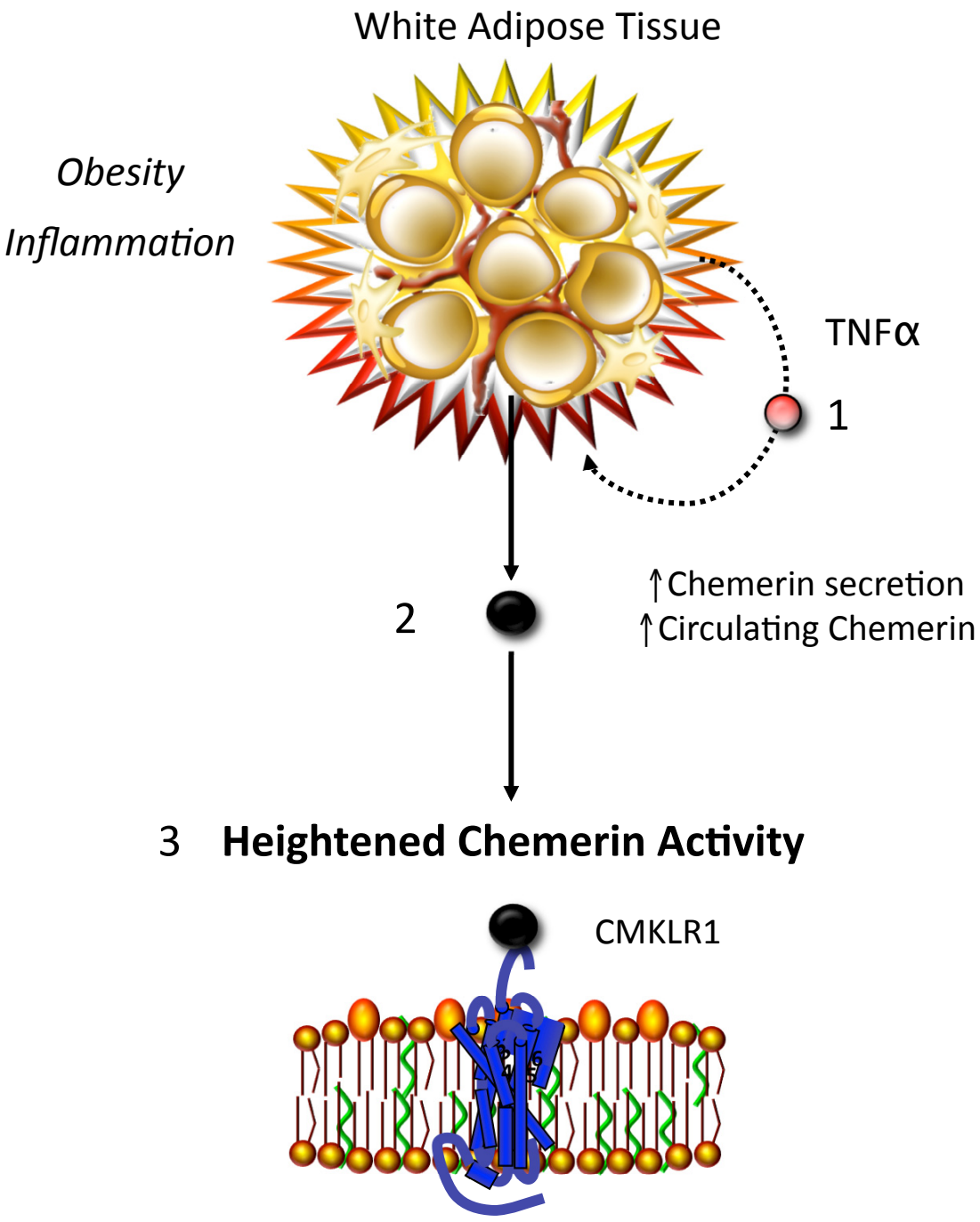
Chemerin is one such novel adipokine. Elevated total *h*-chemerin concentrations are well documented in animal and human models of obesity. The exact role chemerin is playing within obesity is complicated by the pleotropic nature of chemerin signaling.

Adipogenic, inflammatory and anti-inflammatory effects have all been attributed to chemerin signaling. If chemerin is to be truly understood in the context of obesity it is imperative that we move beyond exclusively measuring the concentration of total *h*-chemerin. Alternatively we must first delineate basic chemerin biology including identifying the tissues that produce chemerin, the specific mechanisms of chemerin processing by these tissues and the specific chemerin products produced. In this manner we may define whether chemerin is exacerbating or improving the metabolic abnormalities associated with obesity. Hence, the **overall objective** of my research was to investigate adipocyte-derived chemerin production and activation under basal conditions and following treatment with a component of adipose tissue inflammation. I **hypothesized** that following treatment with an obesity-associated inflammatory mediator (TNFα), adipocyte secretion and activation of prochemerin is altered resulting in elevated circulating active chemerin (**Figure 7**). I sought to investigate this hypothesis in two sequential studies presented in chapter 2 and 3 of this thesis. The general objective of the first study was to identify whether adipocytes are a source of increased active chemerin production and secretion following treatment with TNFα. In addition to chemerin secretion, adipocytes produce abundant active chemerin, so they must also contain the machinery to process prochemerin [187]. The machinery mediating this processing and if it is the same as described for neutrophils is however unknown. The objective of the second study was to define the adipocyte-derived proteolytic mechanisms governing chemerin activation and identify whether it is altered following treatment with TNFα. To investigate the hypothesis and carry out the experimental objectives we opted to use a multifaceted approach. *In vitro* we explored the effects of TNFα on adipose derived *m*-

chemerin production and activation in the well-established 3T3-L1 and primary bMSC derived adipocyte models. While *in vivo* we explored chemerin processing in C57/BL6 wild type, Ob/Ob, Db/Db and TNFα receptor null mice. Most importantly, in attempts to avoid the assumption of parity between total with active chemerin, we measured both total chemerin concentrations by ELISA or western blot in addition to measuring the apparent active chemerin concentration using a cell-based CMKLR1-bioassay. Taken together these studies provide a comprehensive understanding of the multiple processes regulating adipocyte chemerin production, its activation and its resulting activity at CMKLR1 in a pro-inflammatory environment such that would be present in obesity.

Figure 7: Proposed hypothesis I hypothesize that the obesity associated inflammatory-response (as modeled by TNFα treatment) (1) regulates adipose derived chemerin secretion (2), chemerin bioactivation (3), and functional activation of CMKLR1.

Figure 7


White Adipose Tissue
Obesity
Inflammation
TNFα
1
2
↑Chemerin secretion
↑Circulating Chemerin
3 Heightened Chemerin Activity
CMKLR1

CHAPTER 2.00.00: SERUM CHEMERIN LEVELS VARY WITH TIME OF DAY AND ARE MODIFIED BY OBESITY AND TNFα

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MY CONTRIBUTION TO THIS MANUSCRIPT:

As the first author of this publication I conducted all experiments and generated all of the figures in this manuscript. The coauthors provided assistance with conducting the experiments as well as completion of the manuscript.

For copyright approval see **Appendix I**

2.01.00: ABSTRACT

Chemerin is an adipokine with important regulatory roles in adipogenesis. In humans, serum total *h*-chemerin (i.e. *h*-prochemerin plus *h*-chemerin) levels are positively associated with body mass index and metabolic syndrome. However, the mechanisms that increase serum chemerin concentration are unknown. We hypothesized that chronic low-grade inflammation that occurs in obesity promotes chemerin production by adipocytes. Consistent with this, TNFα treatment of 3T3-L1 adipocytes increased bioactive *m*-chemerin levels in the cell media as detected using a *h*-CMKLR1 cell-based bioassay. This effect was blocked by the protein synthesis inhibitor cycloheximide and protein secretion inhibitor brefeldin A indicating that TNFα may enhance *m*-prochemerin synthesis and secretion from adipocytes. *In vivo*, TNFα produced a time-dependent increase in serum total *m*-chemerin and bioactive *m*-chemerin. Bioactive *m*-chemerin was produced by primary mouse adipocytes and hepatocytes. Only primary adipocyte-derived *m*-chemerin was responsive to TNFα regulation implicating adipocytes as a potential source of elevated serum *m*-chemerin following TNFα exposure *in vivo*. In lean mice, serum total *m*-chemerin levels oscillated with peak levels occurring during daytime and trough levels at night. Comparatively, leptin- and leptin receptor-deficient obese mice, which have elevated adipose tissue expression of *TNFα*, displayed elevated serum total *m*-chemerin levels with an enhanced oscillatory pattern. In summary, our novel results identified TNFα as a positive regulator of adipocyte-derived chemerin. We corroborate the finding of elevated chemerin in obese humans by identifying elevated serum levels of total *m*-chemerin in two obese mouse models with a corresponding alteration in the rhythmic pattern of serum *m*-chemerin levels.

2.02.00: INTRODUCTION

Chemerin, a novel adipokine with dual roles in metabolism and immunity is an endogenous ligand of the G-protein coupled receptor, chemokine-like receptor 1 (CMKLR1) [119, 186, 187, 192, 221, 222, 225, 229]. Chemerin is secreted as an inactive 18 kDa precursor protein prochemerin, which undergoes proteolytic processing to pro-inflammatory and anti-inflammatory ligands. Extracellular cleavage of 6 amino acids from the C-terminal end of *h*-prochemerin by serine proteases of the coagulation, fibrinolytic and inflammatory cascades produces a 16 kDa active *h*-chemerin product that binds to *h*-CMKLR1 on immature dendritic cells and macrophages leading to their recruitment to lymphoid organs and sites of tissue injury [119, 186, 188-190, 192, 202]. Further processing of the C-terminal region of *m*-chemerin by cysteine proteases produces a 15mer C-terminal peptide that exerts potent anti-inflammatory effects through *m*-CMKLR1-dependent blockade of pro-inflammatory mediator production by activated macrophages [217].

We identified a uniquely high expression of *chemerin* and *CMKLR1* in mouse and human white adipose tissue [187]. These findings were subsequently corroborated in the obese sand rat indicating a species conservation of *chemerin* and *CMKLR1* expression in adipose tissue [222]. Mouse 3T3-L1 and 3T3-f442A cells express *m*-CMKLR1 and demonstrate a progressive increase in *m-chemerin* expression and production of *m*-chemerin with differentiation into mature adipocytes [187, 221, 225]. Taken together, these data implicate adipose tissue as a primary source of chemerin in the whole organism, which acts as a target tissue for autocrine/paracrine chemerin signaling via the CMKLR1 receptor.

Observations in animals and humans indicate that chemerin and CMKLR1 play an important role in adipogenesis and metabolic homeostasis. When adenoviral-mediated shRNA was used to abolish *m-chemerin* and *m-CMKLR1* expression in 3T3-L1 preadipocytes, differentiation into lipid containing adipocytes was impaired [187]. Accordingly, targeted knockdown of *m-chemerin* or *m-CMKLR1* in either preadipocytes or mature adipocytes reduced the expression of adipocyte genes involved in glucose and lipid homeostasis including *perilipin*, *hormone sensitive lipase* and *glucose transporter-4*. In mature 3T3-L1 adipocytes, *m*-chemerin$_{156}$ treatment blocked isoproterenol-stimulated lipolysis by means of a *m*-CMKLR1-independent mechanism while enhancing insulin-stimulated glucose uptake in adipocytes via increased tyrosine phosphorylation of insulin receptor substrate-1 [187, 225]. Clinical studies have reported elevated serum total *h*-chemerin levels (i.e. *h*-prochemerin and *h*-chemerin) in patients with metabolic syndrome compared to healthy controls [222, 234-236]. In these cases, positive correlations were observed between serum total *h*-chemerin, measures of body fat and a number of markers of metabolic syndrome including body mass index, serum triglycerides and blood pressure [222, 234-236] . An additional study identified a positive correlation between common genetic variations in the *chemerin* gene locus (SNPs rs17173608 and rs10278590) and the development of visceral adiposity[239]. Together these studies reveal that serum total *h*-chemerin levels are dynamic and modifiable in obesity and may have a role in the development and metabolic complications of obesity.

Currently, little is known regarding the regulation of chemerin production by adipocytes and the impact of obesity on serum chemerin levels in animals. The finding that insulin increases whereas metformin decreases adipose production and secretion of

h-chemerin and serum total *h*-chemerin suggests that hyperinsulinemia may play a role [229]. While results identifying that IL-1β up-regulates *m-chemerin* expression 2.5-fold in adipocytes implicates that inflammation, a central feature of obesity, may also have a regulatory role [224]. The positive association between serum total *h*-chemerin levels and TNFα, IL-6 and CRP in humans further supports the regulatory involvement of obesity-associated inflammatory mediators on chemerin [233, 236].

The objective of our study was to determine the effect of the inflammatory mediator TNFα on *m*-chemerin production by adipocytes. TNFα was investigated because of its elevated expression in obese adipose tissue, its involvement in adipose tissue inflammation, its differential regulation of adipokines and positive correlation to serum *h*-chemerin levels [154, 170, 233, 242-245]. We established that TNFα stimulates the production of *m*-chemerin by 3T3-L1 adipocytes and primary adipocytes and elevates serum *m*-chemerin in mice. Consistent with these findings, we found elevated serum total *m*-chemerin levels in leptin-deficient Ob/Ob and leptin receptor-deficient Db/Db mouse models of obesity, which are well known to have elevated adipose tissue expression and secretion of TNFα [154].

2.03.00: METHODS.

2.03.01: ANIMAL PROTOCOL.

The Dalhousie University Committee on Laboratory Animals approved experimental procedures involving mice according to the guidelines of the Canadian Council on Animal Care. Six-week-old leptin-deficient, Lep^{ob}/Lep^{ob} (Ob/Ob), leptin receptor-deficient Lep^{db}/Lep^{db} (Db/Db), wild-type C57BL6/J control mice, TNF receptor superfamily 1a/1b-deficient, $TNFrsf1a^{tm1Imx}/TNFRsf1b^{tm1Imx}$ ($TNFRsf1a^{-/-}/1b^{-/-}$) and B6129SF2/J control mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed in cages lined with pine bedding and had free access to water and Purina mouse chow. The light and dark cycles were 0700-1900 and 1900-0700 hours, respectively.

2.03.02: 3T3-L1 ADIPOCYTE CELL CULTURE.

3T3-L1 preadipocytes were obtained from the American Tissue Culture Collection (Manassas, VA) and were grown and differentiated according to our published protocols [187]. A brief description is as follows; preadipocytes were plated at 200 000 cells per well and allowed to grow to confluence. To differentiate into adipocytes, the preadipocytes were treated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 250 nmol L^{-1} dexamethasone, 0.5 mmol L^{-1} 2-isobutyl-1-metylxanthine, and 100 nmol L^{-1} human insulin for 3 days after which preadipocytes were maintained in DMEM containing 10% FBS and 850 nmol L^{-1} insulin. All media was phenol-red free. For the TNFα experiments, 13-day differentiated adipocytes grown on 12-well plates were rinsed once with 1 mL of phosphate buffered

saline (PBS) then treated with 0.1 –20 ng mL^{-1} recombinant mouse TNFα (R&D systems, Minneapolis, MN) or 0.1 % bovine serum albumin (BSA) in PBS (0.1% BSA/PBS) vehicle in 500 μl of serum free DMEM for a period of 24 h. After the 24 h incubation, the conditioned adipocyte media was collected and the corresponding cellular RNA was isolated. Media and RNA samples were stored at -80 °C until western blots, *m*-chemerin bioassays and gene expression analyses were performed. To determine the effects of TNFα on transcription, translation and secretion of chemerin, 3T3-L1 adipocytes were respectively pretreated with 500 μl of serum free DMEM containing 1 μg mL^{-1} actinomycin D, 1 μg mL^{-1} cycloheximide or 10 μg mL^{-1} brefeldin A (Sigma-Aldrich, Oakville, ON) or corresponding dimethyl sulfoxide (DMSO) (0.1%) or ethanol (0.2%) vehicles [246]. After 2 h, the treatment media was aspirated and replaced with 500 μl of serum free DMEM that contained 20 ng mL^{-1} TNFα or 0.1 % BSA in PBS for 24 h. The conditioned media and RNA was collected and stored as previously indicated.

2.03.03: PRIMARY HEPATOCYTE AND ADIPOCYTE CELL CULTURE.

Primary hepatocytes and bone marrow stromal cell (bMSC) derived adipocytes were isolated and cultured from 8-week-old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) utilizing established methods [223, 247, 248]. Adipocyte differentiation of bMSCs was achieved by treating the preadipocytes for 2 days with induction media containing α-MEM supplemented with 1 μM rosiglitazone, 5 μg mL^{-1} human insulin, 0.1 nM dexamethasone, 50 μg mL^{-1} ascorbic acid, 60 μM indomethacin, 7.5% fetal bovine serum and 2.5 % lot-selected rabbit serum followed by 2 days in maintenance media which consisted of α-MEM containing 1 μM rosiglitazone, 5 μg mL^{-1} insulin, 7.5% fetal

bovine serum, and 2.5% rabbit serum. This was repeated for a total of 3 cycles after which the cells were maintained in maintenance media. TNFα treatments were performed in serum free media as outlined for the 3T3-L1 cells.

2.03.04: RNA ISOLATION AND QUANTITATIVE PCR.

Total cellular RNA was isolated using the RNeasy plus mini kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions and 0.2 μg was reverse transcribed using Stratacript^{TM} Reverse Transcriptase (Stratagene, La Jolla, CA). One μl of the cDNA product was amplified by quantitative polymerase chain reaction (QPCR) using 125 nM of gene specific primers in a total volume of 20 μl with Brilliant SYBR Green QPCR Master Mix using the Stratagene MX3000p thermocycler [187, 249]. Relative gene expression normalized to *Cyclophilin A* expression was calculated using the $\Delta\Delta C_T$ method [250]. The forward (Fw) and reverse (Rv) QPCR primer sequences are identified in **Table 6**.

2.03.05: EFFECT OF TNFα TREATMENT ON SERUM CHEMERIN LEVELS.

We compared the effect of TNFα on serum levels of total *m*-chemerin (i.e. *m*-prochemerin and *m*-chemerin) in TNFrsf1a$^{-/-}$/1b$^{-/-}$ mice and corresponding B6/129SF2/J control mice. At 0800 hours on the day of the experiment, 4 TNFrsf1a$^{-/-}$/1b$^{-/-}$ and 4 control mice were injected with 0.5 μg of TNFα in 200 μl of sterile filtered 0.1% BSA/PBS by intraperitoneal (i.p.) injection. The dose of TNFα was based on a previous study, which found it to be effective at inducing *plasminogen activator inhibitor 1* (*PAI-1*) expression in adipose tissue [243]. An additional 4 TNFrsf1a$^{-/-}$/1b$^{-/-}$ and 4 B6/129SF2/J

control mice were injected with 200 μl of 0.1% BSA/PBS and served as time controls for the experiment. From each mouse, venous blood samples (25 μl) were collected from the saphenous vein into 30 μL non-heparinized hematocrit tubes. The sampling times were 0800, 1000, 1400, 1600, 2000 and 0800 hours. After the blood was collected it was immediately transferred to a 1.5 mL tube and was allowed to clot at room temperature (RT) for 1h. Coagulated blood was centrifuged for 10 min at 800 × g. Serum was collected and stored at -80°C until the chemerin ELISA assay was performed.

2.03.06: EFFECT OF GENETIC OBESITY ON SERUM CHEMERIN LEVELS.

We measured serum levels of total *m*-chemerin in C57/BL/6 wild-type, Ob/Ob and Db/Db mice (n = 5 per group) that had free access to food and water to characterize the effect of genetic obesity on circulating levels of the chemerin protein. Twenty-five μl of venous blood was taken from the saphenous vein of each mouse at 4 hours intervals over a 20-hour period as described in the previous section. The sampling times were 1500, 1900, 2300, 0300, 0700 and 1100 hours.

2.03.07: SERUM CHEMERIN MEASUREMENTS.

The serum levels of total *m*-chemerin were measured in 10 μl serum samples using a mouse chemerin ELISA (EZMCMRN-56K, Millipore, Billerica, MA) according to the manufacturers' instructions. A dose-response curve produced from *m*-chemerin$_{156}$ standards (3.125 to 200 ng mL^{-1}) using a best fit of a 4-5 parameter logistic equation was used to calculate the total serum chemerin concentration.

2.03.08: QUANTIFICATION OF BIOACTIVE CHEMERIN IN ADIPOCYTE MEDIA USING THE CMKLR1 "TANGO" BIOASSAY.

The CMKLR1 Tango bioassay is a cell-based reporter gene assay that specifically and quantitatively measures CMKLR1 activation by chemerin [204]. A brief description of the assay principle is as follows. HEK293T cells, which constitutively express a fusion protein composed of a TEV (tobacco etch virus) protease linked to human β-arrestin2, and a transcriptional-transactivator (tTA)-dependent luciferase reporter gene were transfected with a plasmid (*h*-CMKLR1-TL-tTA) that expresses a fusion construct of *h*-CMKLR1 joined at its C-terminus to a tTA by a TEV N1a protease cleavage site. Chemerin binding to the *h*-CMKLR1-tTA fusion protein recruits the β-arrestin-protease fusion construct to the receptor resulting in tTA cleavage, allowing tTA to pass into the nucleus where it transcribes the luciferase reporter gene. We have adopted the original methods with some modifications to monitor the concentration of bioactive chemerin in adipocyte media and mouse serum [204]. HEK293T cells were maintained in DMEM supplemented with 10 % FBS, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.5 mg mL^{-1} G418, 5 μg mL^{-1} puromycin and 0.2 mg mL^{-1} hygromycin. For the assay, HTLA cells were seeded on 96-well plates at a density of 12,000 cells per well. Twenty-four hours later the cells were transfected by treating each well with 50 μl of serum-free optimem media containing 0.1 μl of polyethylenimine, 25 ng of the CMKLR1-TL-tTA plasmid, 25 ng of a pCMV-β-galactosidase reference plasmid and 50 ng of the carrier plasmid PBSK. After a 24h incubation, the transfection media was aspirated and replaced with 50 μl of 1:10 diluted serum-free 24 h-conditioned adipocyte media, hepatocyte media or serum in duplicate. At the same time, duplicate wells were treated with serum

free media that contained 0, 0.3, 1, 3, 10 and 30 nM recombinant *m*-chemerin$_{156}$ to generate a standard curve from which the apparent sample concentration of *m*-chemerin was calculated. Following a further 24 h incubation, the treatment media was aspirated, the cells were washed once with 100 μl of PBS and incubated for 5 min with shaking (1000 RPM) in 100 μl of reporter lysis buffer (RLT, Promega, Nepean, ON) followed by a rapid freeze/thaw cycle to lyse the cells. For the luciferase assay, 10 μl of each sample lysate and RLT blank was transferred to a 96-well white luminometer plate. Eighty μl of luciferase assay reagent (Promega , Nepean, ON, CND) was auto-injected into each well and the luminescence was monitored for 10 s. For the β-galactosidase assay, 30 μl of each sample lysate and blank was transferred to a clear 96-well plate and incubated with 30 μl of 2x β-galactosidase assay buffer for 15 min at 37°C. The reaction was stopped by addition of 100 μl of 1 M Na_2CO_3 and the absorbance at 420 nM was measured. The luciferase and β-galactosidase measurements were corrected for the respective blanks. Activation of the receptor by each standard and sample was expressed as the ratio of luciferase/β-galactosidase activity. The apparent k_m and V_{max} values were determined by nonlinear regression fit of the standards to the Michaelis-Menten equation using GraphPad Prism (Graph Pad Software Inc., La Jolla, CA):

$$V = V_{max}[S]/k_m + [S] \qquad \text{(eq. 1)}$$

Where V is the measured luciferase/β-galactosidase activity and [S] is the standard *m*-chemerin$_{156}$ concentration. The apparent *m*-chemerin concentration in media or serum was interpolated from this curve. The specificity of *h*-CMKLR1 activation was identified by pre-incubating 10 nM recombinant *m*-chemerin$_{156}$, mouse serum or 3T3-L1 adipocyte media with 1-10 μg mL^{-1} of a primary neutralizing anti-mouse chemerin antibody (R&D

biosystems, Minneapolis, MN) for 1 hour prior to treating the HTLA cells. The anti-mouse chemerin antibody blocked greater than 99% of the bioassay response to the recombinant chemerin, serum and 3T3-L1 media thereby confirming the specificity of *h*-CMKLR1 activation by *m*-chemerin (**Figure 8**).

2.03.09: SDS-PAGE "WESTERN" BLOTTING.

One hundred μl of 24 h conditioned adipocyte media were added to 20 μl of 6x SDS loading buffer. Fifteen μl of the solution was separated on 12.5 % polyacrylamide gel and transferred overnight to nitrocellulose membrane. A polyclonal rabbit anti mouse chemerin antibody generated in house (**Figure 9**) or an R&D Systems mouse chemerin affinity purified polyclonal antibody (**Figure 12**) was used to detect total *m*-chemerin on the western blot according to our previously published methodology [187].

2.03.10: STATISTICAL ANALYSIS.

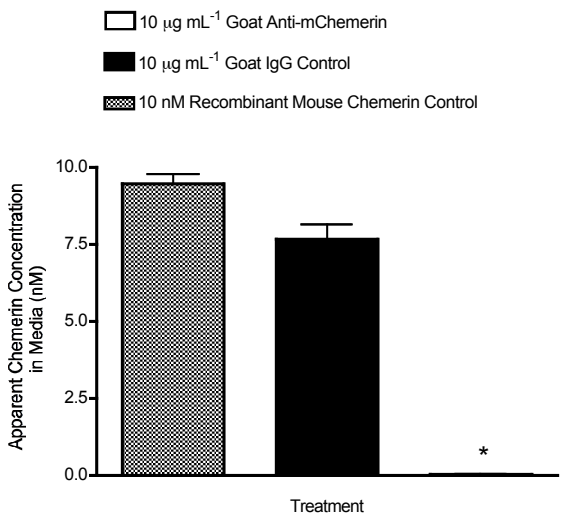
All data are expressed as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism. A two-way analysis of variance (ANOVA) with repeated-measures for time was used for comparing time-dependent effects of TNFα treatment or genetic obesity on serum *m*-chemerin levels. A one-way ANOVA was used for other multiple comparison procedures with one independent variable. A Tukey's or Bonferroni test was used for *post-hoc* analysis of the significant ANOVA. A difference in mean values between groups was considered to be significant when $P \leq 0.05$.

Table 6: Quantitative-PCR primer gene identification, sequences and product sizes			
Mouse Gene	**Identification**	**Sequence**	**Product Size**
chemerin	NM_027852	TACAGGTGGCTCTGGAGGAGTTC	195 bp
		CTTCTCCCGTTTGGTTTGATTG	
CMKLR1	NM_0081153	CAAGCAAACAGCCACTACCA	224 bp
		TAGATGCCGGAGTCGTTGTAA	
IL-6	NM_031168	TAGTCCTTCCTACCCCAATTTCC	75 bp
		TTGGTCTTAGCCACTCCTTC	
TNFα	NM_013693	CCCTCACACTCAGATCATCTTCT	60 bp
		GCTACGACGTGGGCTACAG	
PAI-1	NM_008871	ACGTTGTGGAACTGCCCTAC	247 bp
		GCCAGGGTTGCAACTAAACAT	
NFκB	NM_009045.4	TTCCTGGCGAGAGAAGCAC	139 bp
		AAGCTATGGATACTGCGGTCT	
cyclophillin A	X52803.1	GAGCTGTTTGCAGACAAAGTTC	124 bp
		CCCTGGCACATGAATCCTGG	

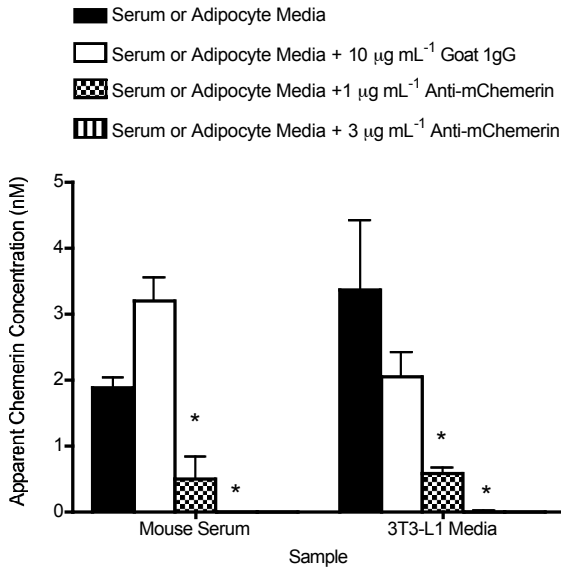
Figure 8: The CMKLR1 "Tango" bioassay is specifically activated by *m*-chemerin in mouse serum and 3T3-L1 adipocyte media. 10 μg mL^{-1} of anti-chemerin antibody significantly neutralized *h*-CMKLR1 activation by 10 nM recombinant *m*-chemerin$_{156}$ compared to the IgG control antibody (**A**). Similarly, 3 μg mL^{-1} and 10 μg mL^{-1} (not shown) concentrations of the anti-chemerin antibody completely neutralized the activation of *h*-CMKLR1 by adipocyte media or serum (**B**). In comparison, the goat IgG control antibody did not significantly affect the apparent *m*-chemerin concentration in adipocyte media or mouse serum compared to the untreated control samples (**B**). All bars represent the mean ± s.e.m. of 3 samples, and are representative of at least 2 independent experiments * $P < 0.05$ compared to the control (goat IgG), ANOVA, followed by Tukey's *post hoc* test.

Figure 8

A


10 μg mL-1 Goat Anti-mChemerin
10 μg mL-1 Goat IgG Control
10 nM Recombinant Mouse Chemerin Control
Apparent Chemerin Concentration in Media (nM)
10.0
7.5
5.0
2.5
0.0
*
Treatment

B


Serum or Adipocyte Media
Serum or Adipocyte Media + 10 μg mL-1 Goat 1gG
Serum or Adipocyte Media +1 μg mL-1 Anti-mChemerin
Serum or Adipocyte Media + 3 μg mL-1 Anti-mChemerin
Apparent Chemerin Concentration (nM)
5
4
3
2
1
0
*
*
*
*
Mouse Serum
3T3-L1 Media
Sample

2.04.00: RESULTS

2.04.01: TNFα STIMULATES *M*-CHEMERIN PRODUCTION BY CULTURED 3T3-L1 ADIPOCYTES.

QPCR analysis identified that TNFα elicited a dose- and time-dependent increase in *m-chemerin* mRNA expression in 3T3-L1 adipocytes (**Figure 9A and B**). The maximum stimulatory effect (3 to 4-fold) occurred at 14-24 h and with 20 ng mL^{-1} TNFα treatment. To determine if total *m*-chemerin concentration in the adipocyte media increased correspondingly, we performed western blotting analysis under denaturing conditions, of serum-free adipocyte media, 24 h after treatment with 1-20 ng mL^{-1} TNFα. The single band detected by the chemerin antibody corresponded to the molecular weight of the 16 kDa recombinant *m*-chemerin$_{156}$ (**Figure 9C**). The relative densitometry measurements from the western blots, show that the immunodetectable levels of *m*-chemerin in adipocyte media were significantly elevated by the 1.0 ng mL^{-1} TNFα but not the higher TNFα concentrations (**Figure 9D)**. We then examined if TNFα treatment increased the apparent concentration of bioactive *m*-chemerin in adipocyte media through the use of a cell-based human CMKLR1 reporter gene bioassay [187, 204]. Recombinant mouse chemerin$_{156}$ activated *h*-CMKLR1 in a saturable fashion as shown by the representative standard curve with an apparent k_m = 6.8 ± 1.9 nM (**Figure 9E**). The activation of the *h*-CMKLR1 reporter assay by 24 h-conditioned adipocyte media was increased in a dose-dependent fashion by TNFα treatment. Based on interpolation from the standard curve, the apparent concentration of bioactive *m*-chemerin in 24 h conditioned adipocyte media increased from 14.5 ± 4.6 nM in the vehicle-treated controls to 107.0 ± 7.4 nM with the 20 ng mL^{-1} TNFα dose (**Figure 9F**). The results from the

CMKLR1 reporter gene bioassay confirmed that TNFα enhanced the 3T3-L1 adipocyte production of bioactive *m*-chemerin.

To address the mechanism whereby TNFα increased the apparent concentration of bioactive *m*-chemerin in adipocyte media, additional experiments were performed in which 3T3-L1 cells were pretreated with inhibitors of mRNA synthesis (actinomycin D), protein synthesis (cycloheximide) and protein secretion (brefeldin A) prior to the application of TNFα. The combination of TNFα with actinomycin D was cytotoxic to the 3T3-L1 cells; thus, we were unable to determine if TNFα induced apparent concentration of bioactive *m*-chemerin through transcriptional mechanisms. This result is consistent with other observations that TNFα combined with actinomycin D induces apoptosis [251, 252]. Pre-treatment of 3T3-L1 adipocytes with cycloheximide (**Figure 10A**) or brefeldin A (**Figure 10B**) completely reversed the TNFα-mediated increase in the apparent concentration of bioactive *m*-chemerin in adipocyte media without significantly affecting the apparent concentration of bioactive *m*-chemerin in the control adipocyte media. Together these results suggest that TNFα-mediated increase in bioactive chemerin production requires both protein synthesis and protein secretion mechanisms.

2.04.02: TNFα INCREASES SERUM TOTAL *M*-CHEMERIN LEVELS IN WILD-TYPE BUT NOT TNFRS1A$^{-/-}$/1B$^{-/-}$ MICE.

The next objective was to determine if the effect of TNFα on *m*-chemerin production in adipocytes translated to regulation of blood serum levels of total *m*-chemerin (*m*-prochemerin and *m*-chemerin) in the whole animal. Our approach was to treat TNFRS1a and 1b receptor deficient (TNFRS1a$^{-/-}$/1b$^{-/-}$) and corresponding wild-type

(B6/129SF2/J) mice with TNFα and monitor changes in serum concentration of total *m*-chemerin over a similar time frame as performed in the cell culture studies. Serum samples obtained from each mouse at t = 0, 2, 4, 8, 12 and 24 h following i.p. injections with 0.5 μg of TNFα or the 0.1 % BSA/PBS vehicle were analyzed for total *m*-chemerin using an ELISA assay. Two-way repeated measures ANOVA identified a significant effect of time ($p<0.001$), treatment ($p< 0.001$) and interaction between time and treatment ($p< 0.001$) on serum levels of total chemerin. A Tukey's *post-hoc* test revealed that serum levels of total *m*-chemerin were significantly elevated 12-24 h following TNFα treatment in the wild-type mice as compared to the TNFα-treated TNFRS1a$^{-/-}$/1b$^{-/-}$ mice and the vehicle-treated wild-type and TNFRS1a$^{-/-}$/1b$^{-/-}$ mice (**Figure 11A**). To determine the overall serum *m*-chemerin exposure for the 24 h period, we calculated the area under the serum *m*-chemerin versus time curve from 0-24 h (AUC_{0-24}). An increase in serum *m*-chemerin exposure is reflected by an increased serum *m*-chemerin AUC_{0-24} in the TNFα-treated wild-type mice compared to vehicle-treated wild-type mice and the TNFα- and vehicle-treated TNFRS1a$^{-/-}$/1b$^{-/-}$mice (**Figure 11B**).

2.04.03: TNFα INCREASES BIOACTIVE *M*-CHEMERIN LEVELS IN MOUSE SERUM AND PRIMARY ADIPOCYTE MEDIA.

Subsequently we determined if the elevation in total serum *m*-chemerin levels following TNFα treatment corresponded to a heightened activation of the *h*-CMKLR1 receptor by serum. C57BL6 mice were injected i.p. with 0.5 μg TNFα or 200 μl of 0.1% BSA/PBS vehicle. Blood was collected 4 or 12 h after the treatment by cardiac puncture. The resulting serum was then analyzed for the apparent *m*-chemerin concentration using

the *h*-CMKLR1 "Tango" bioassay. After 4 h, the apparent concentration of bioactive *m*-chemerin in serum was similar in the control (2.0 ± 0.2 nM) and TNFα-treated (1.7 ± 0.2) mice (**Figure 12A)**. After 12 h, the apparent concentration of bioactive *m*-chemerin in serum of the TNFα-treated mice (3.1 ± 0.3 nM) was about two-fold higher than the corresponding time controls (1.61 ± 0.39 nM) (**Figure 12A)**.

To establish that this dose of TNFα produced an inflammatory response in adipose tissue, we examined the mRNA levels of the transcription factor *NFκB* and the adipokines *IL-6* and *PAI-1* that are induced by TNFα. There was a significant induction of adipose tissue *NFκB* and *PAI-1* mRNA expression after 4 h and IL-6 after 12 h (**Table 7**). Despite the activation of three positive control genes, TNFα had no effect on adipose tissue expression of *chemerin* at either time point (**Table 7**). Similar to the *in vivo* experiments, treatment of mouse bMSC-derived primary adipocytes for 24 h with 20 ng mL^{-1} TNFα significantly increased the immunodetectable and apparent concentration of bioactive *m*-chemerin in the adipocyte media compared to controls (**Figure 12B & C)**. Likewise, TNFα produced a significant induction of *IL-6* but not *chemerin* mRNA expression in the primary adipocytes (**Figure 12D)**. Together these data suggested that increased *m*-prochemerin secretion from adipocytes but not increased *chemerin* expression could contribute to the TNFα-mediated increase in the apparent concentration of bioactive chemerin in serum.

The liver expresses similar levels of *m*-chemerin as compared to white adipose tissue and could therefore potentially contribute to the increase in bioactive *m*-chemerin levels in serum following TNFα treatment. However, the 0.5 μg mL^{-1} TNFα dose did not induce the hepatic expression of any of the positive control genes nor *chemerin* (**Table**

7). We demonstrated for the first time that primary mouse hepatocytes produced bioactive *m*-chemerin that was detectable in the cell culture media using the *h*-CMKLR1 reporter assay (**Figure 12C**). Similar to the *in vivo* results no significant changes in immunodetectable (**Figure 12B**) and bioactive *m*-chemerin in hepatocyte media (**Figure 12C**) or *chemerin, NFκB* and *IL-6* expression (**Figure 12E**), was found 24 h after treating primary hepatocytes with 20 ng mL^{-1} TNFα compared to control.

2.04.04: SERUM *M*-CHEMERIN LEVELS ARE ELEVATED IN OBESITY AND OSCILLATE IN A DIURNAL-LIKE FASHION.

The final objective was to determine if serum levels of total *m*-chemerin were elevated in the obese leptin (Ob/Ob) and leptin-receptor deficient (Db/Db) mice, which have substantially elevated adipose tissue expression of *TNFα* (**Figure 13A**). Two-way ANOVA with repeated measures for time revealed that the serum concentration of total *m*-chemerin was affected by mouse genotype ($p < 0.001$), time ($p < 0.001$) and an interaction between mouse genotype and time ($p < 0.001$) (**Figure 13B**). In lean mice, a diurnal-like pattern was observed where serum levels of total *m*-chemerin were higher (2.6 – 2.7 nM) during the initial daylight hours (1500 – 1900 h), dropped significantly to 1.7-1.9 nM at nighttime and returned to basal levels the following morning (1100 h). The Db/Db mice displayed an initial peak serum concentration of 5 nM at 1500 h, which was followed by a significant decline to a trough concentration of 2.2 nM at 0700 and then an increase to 3.6 nM at the 1100 h (**Figure 13B**). In comparison, the serum concentration of total *m*-chemerin in the Ob/Ob mice appeared to have two cycles of oscillation with peak concentrations of 3.9-4.4 nM and a significantly lower trough *m*-chemerin concentration

of 2.6 nM at 0300 h (**Figure 13B**). The $AUC_{0\text{-}20}$ calculated from the serum concentration of total *m*-chemerin versus time was 40 % higher in Ob/Ob and Db/Db mice compared to the controls – reflecting higher *m*-chemerin concentrations in serum over time in these animals (**Figure 13C**). The wild-type mice showed the least oscillation between maximal and minimum serum levels of total *m*-chemerin (**Figure 13D**). In comparison, the differences between the maximal and minimal serum levels of total *m*-chemerin were 2- and 3-fold larger in Ob/Ob and Db/Db mice, respectively, compared to the control mice.

Figure 9: TNFα stimulates *m*-chemerin production by 3T3-L1 adipocytes. *Chemerin* mRNA expression was measured by QPCR in 13-day differentiated 3T3-L1 adipocytes 24 h after treating with 0.1-20 ng mL^{-1} TNFα (**A**) or at various times after treatment with 20 ng mL^{-1} TNFα (**B**). For determination of relative gene expression by QPCR, the control adipocytes (0 ng mL^{-1} TNFα) served as the reference (expression = 1) to which all other samples were compared. *m*-Chemerin in 24-h conditioned adipocyte media and recombinant (bioactive) mouse *m*-$chemerin_{156}$ (CE) were detected by western blot using an anti-mouse chemerin antiserum as shown by the representative blot (**C**). The relative band intensities (n = 3 per group) were determined by densitometry (**D)**. *h*-CMKLR1-reporter gene activation (luciferase/β-galactosidase activity) by recombinant mouse chemerin fitted to the Michaelis-Menten equation (**E**). The apparent concentrations of bioactive *m*-chemerin in serum free adipocyte cell culture media after 24 h treatment with 1-20 ng mL^{-1} TNFα (**F**) were determined from the sample luciferase/β-galactosidase activity in the *h*-CMKLR1-reporter gene assay and the corresponding k_m and V_{max} values from the assay standard curve (**E**). All bars represent the mean ± s.e.m. of 3 samples and are representative of at least 3 independent experimental replicates. * $P < 0.05$ compared to the control (0 ng mL^{-1} TNFα), ANOVA, followed by Tukey's *post hoc* test.

Figure 9

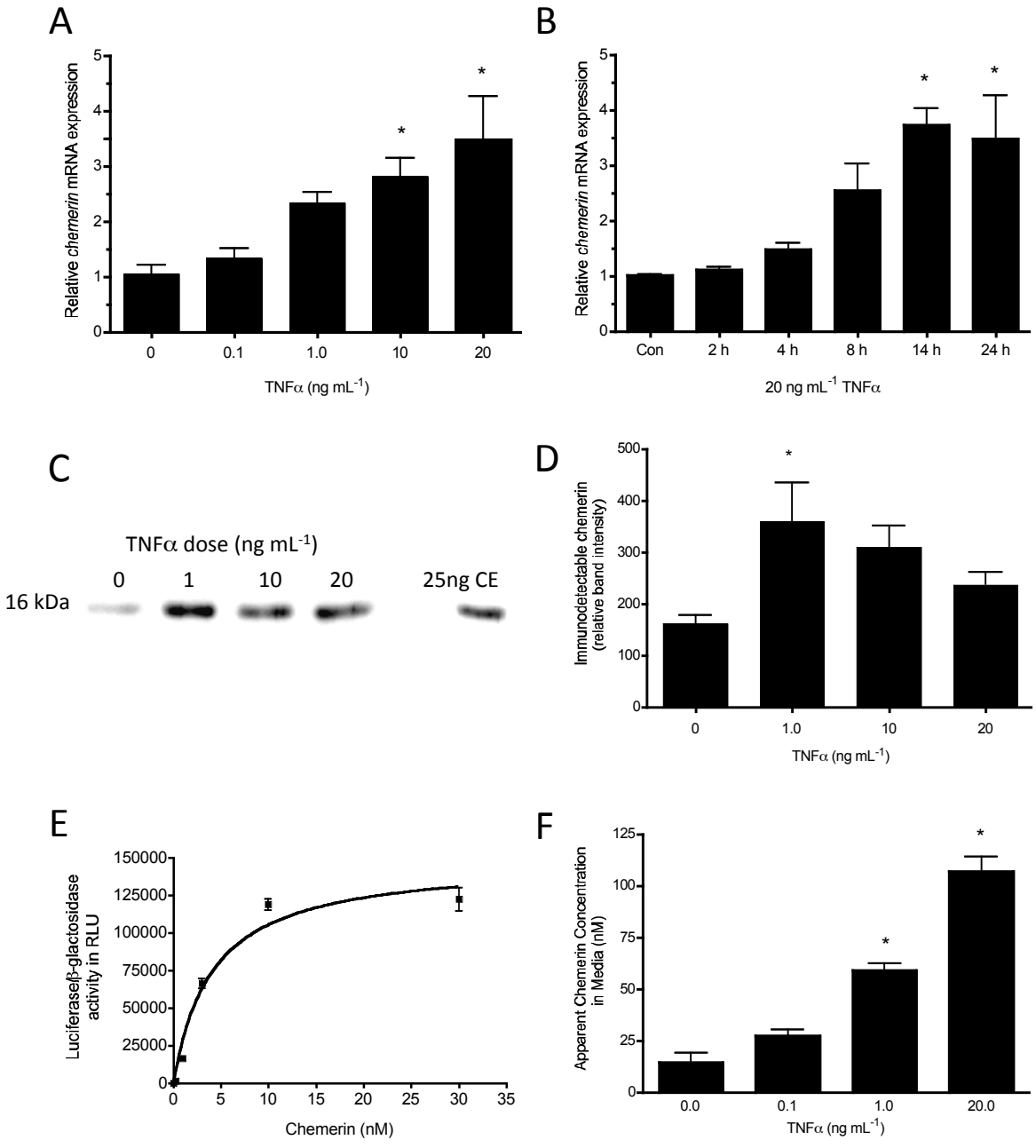
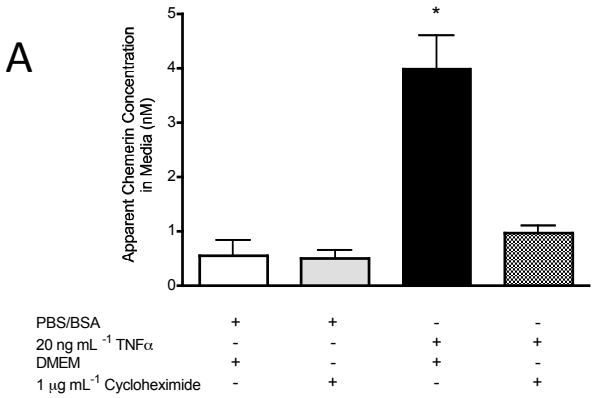

A
Relative chemerin mRNA expression
0
1
2
3
4
5
*
*
0
0.1
1.0
10
20
TNFα (ng mL-1)
B
Relative chemerin mRNA expression
*
*
Con
2 h
4 h
8 h
14 h
24 h
20 ng mL-1 TNFα
C
TNFα dose (ng mL-1)
0
1
10
20
25ng CE
16 kDa
D
Immunodetectable chemerin (relative band intensity)
0
100
200
300
400
500
*
0
1.0
10
20
TNFα (ng mL-1)
E
Luciferase/β-glactosidase activity in RLU
0
25000
50000
75000
100000
125000
150000
0
5
10
15
20
25
30
35
Chemerin (nM)
F
Apparent Chemerin Concentration in Media (nM)
0
25
50
75
100
125
*
*
0.0
0.1
1.0
20.0
TNFα (ng mL-1)

Figure 10: TNFα increases bioactive *m*-chemerin in adipocyte media through a brefeldin A-sensitive mechanism. 3T3-L1 adipocytes were pre-treated with adipocyte media that contained 1 μg mL^{-1} cycloheximide (**A**), 10 μg mL^{-1} brefeldin A (**B**), DMEM vehicle or 0.2% ethanol vehicle (control) for 2 h. The treatment was then removed and replaced with 500 μl of serum-free adipocyte media that contained 20 ng mL^{-1} TNFα or equivalent volume of 0.1% BSA/PBS for 24 h at which point samples were collected. The apparent concentration of bioactive *m*-chemerin (**A & B**) in the media was analyzed via the *h*-CMKLR1 bioassay. All bars represent the mean ± s.e.m. of 3 samples and are representative of 2 independent experimental replicates. * $P < 0.05$ compared to the DMEM/BSA/PBS (white bar) or ethanol/BSA/PBS-treated controls and the DMEM/ TNFα-treated group or ethanol/TNFα-treated groups (black bars), 1-way ANOVA, followed by Tukey's *post-hoc* test (**A & B**).

Figure 10


A
Apparent Chemerin Concentration in Media (nM)
0
1
2
3
4
5
*
PBS/BSA + + - -
20 ng mL -1 TNFα - - + +
DMEM + - + -
1 μg mL-1 Cycloheximide - + - +

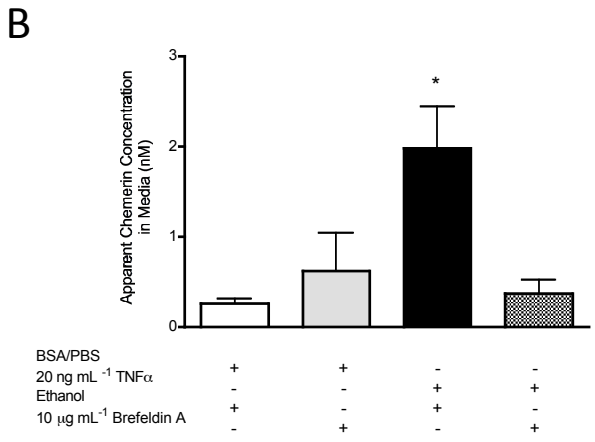
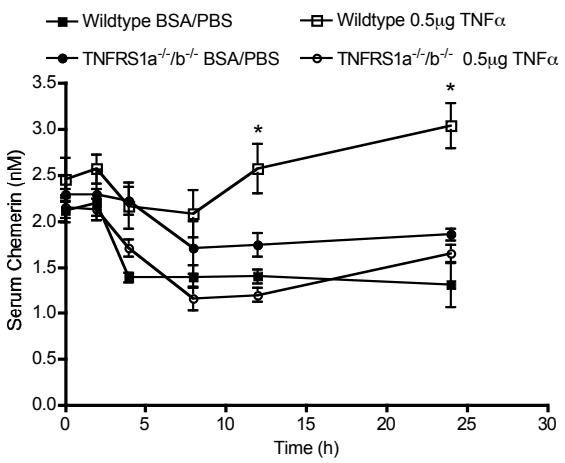

B
Apparent Chemerin Concentration in Media (nM)
0
1
2
3
*
BSA/PBS + + - -
20 ng mL -1 TNFα - - + +
Ethanol + - + -
10 μg mL-1 Brefeldin A - + - +

Figure 11: TNFα increases serum levels of total *m*-chemerin in wild type but not TNFRS1a$^{-/-}$/b$^{-/-}$ mice. Wild type (n = 4) or TNFRS1a$^{-/-}$/b$^{-/-}$ mice (n =4) were administered a single dose of TNFα (0.5 μg) or an equivalent volume of vehicle (0.1% BSA/PBS) by i.p. injection. Blood samples were drawn periodically over a 24 h period from each mouse for the measurement of serum levels of total *m*-chemerin using a mouse chemerin ELISA (**A**). The area under the serum *m*-chemerin concentration time curve (AUC_{0-24}) was calculated using the trapezoidal method (GraphPad Prism) (**B**). Each symbol or bar is the mean ± s.e.m. of 4 mice. *P < 0.05 compared to all other groups at the specific time-points (**A**) and compared to all other groups (**B**), two-way repeated measures ANOVA, followed by Bonferonni *post-hoc* analysis (**A**) and 1-way ANOVA followed by Tukey's *post-hoc* test (**B**).

Figure 11

A


Wildtype BSA/PBS
Wildtype 0.5μg TNFα
TNFRS1a-/-/b-/- BSA/PBS
TNFRS1a-/-/b-/- 0.5μg TNFα
Serum Chemerin (nM)
Time (h)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
5
10
15
20
25
30
*
*
*

B

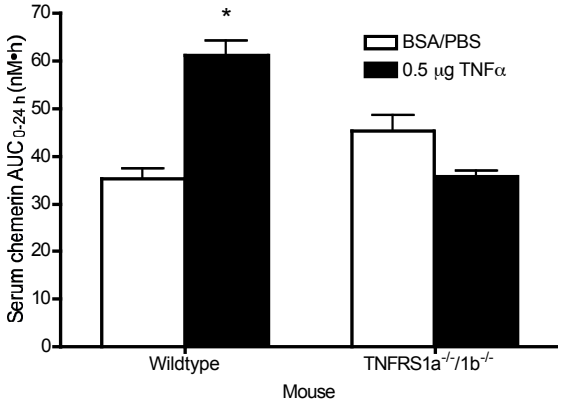

Serum chemerin AUC0-24 h (nM•h)
BSA/PBS
0.5 μg TNFα
Wildtype
TNFRS1a-/-/1b-/-
Mouse
0
10
20
30
40
50
60
70
*

Figure 12: TNFα increases the apparent concentration of bioactive *m*-chemerin in serum and primary adipocyte media. The *h*-CMKLR1 "Tango" bioassay was used to measure the apparent concentration of bioactive *m*-chemerin in serum of C57/BL6 mice 4 or 12 h following the i.p. injection of 0.5 μg TNFα or 0.1 % BSA/PBS (control) (**A**). bMSC-derived primary adipocytes and primary hepatocytes were treated with 500 μl of serum free media that contained 20 ng mL^{-1} TNFα or 0.1 % BSA/PBS vehicle for 24 h after which time the cell media was collected and cellular RNA was extracted. The total immunodetectable levels of chemerin in conditioned adipocyte or hepatocyte media were determined by western blot using an R&D antichemerin antibody (representative of four samples per group and two independent experiments) (**B**) while the respective apparent concentration of bioactive *m*-chemerin was determined using the *h*-CMKLR1 bioassay (**C**). QPCR was use to measure relative c*hemerin, NFκB* and *IL-6* expression in the primary adipocytes (**D**) and hepatocytes (**E**). For each gene, the 0.1% BSA/PBS control (**D, E**) served as the respective reference (expression = 1) to which the TNFα-treated group was compared. Each bar is the mean ± s.e.m. of 4 mice (**A**) or 3 samples which are representative of 3 independent experimental replicates (**C-D)**. ***** $P < 0.05$ compared to the 12 h (**A)** or 0.1% BSA/PBS (**C-E**) control group, using two-way ANOVA with followed by Bonferroni's *post-hoc* analysis (**A**) or a student's t-test respectively (**C-D**).

Figure 12

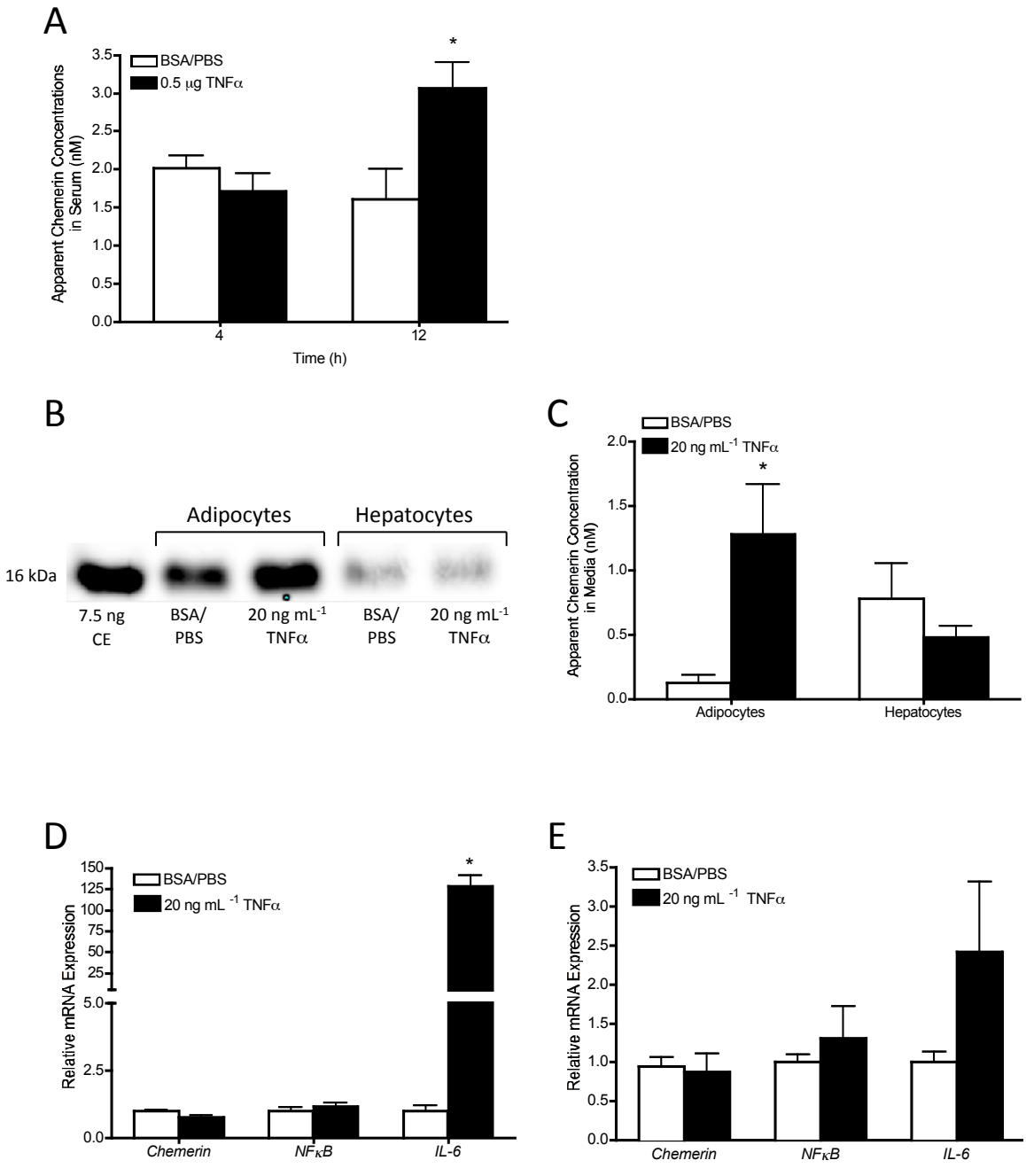

A
BSA/PBS
0.5 μg TNFα
Apparent Chemerin Concentrations in Serum (nM)
Time (h)
B
Adipocytes
Hepatocytes
16 kDa
7.5 ng CE
BSA/ PBS
20 ng mL-1 TNFα
BSA/ PBS
20 ng mL-1 TNFα
C
BSA/PBS
20 ng mL-1 TNFα
Apparent Chemerin Concentration in Media (nM)
Adipocytes
Hepatocytes
D
BSA/PBS
20 ng mL -1 TNFα
Relative mRNA Expression
Chemerin
NFκB
IL-6
E
BSA/PBS
20 ng mL -1 TNFα
Relative mRNA Expression
Chemerin
NFκB
IL-6

Figure 13: Ob/Ob and Db/Db obese mice have increased serum levels of *m*-chemerin. The relative expression of *TNFα* mRNA in white adipose tissue from obese Ob/Ob and Db/Db mice compared to wild-type lean control mice (**A**). 25 μl of blood was collected from the saphenous vein of wild-type, Ob/Ob and Db/Db mice every 4 h over a 20-h period. Serum was analyzed for total *m*-chemerin using a mouse chemerin ELISA assay (**B**). The serum level of total *m*-chemerin was significantly affected by the variables: time, mouse genotype and interaction between time and genotype ($p < 0.001$), two-way repeated measures ANOVA (**B**). Subsequently, each mouse group in **B** was analyzed for time dependent effects on serum total *m*-chemerin using a one-way repeated measure ANOVA with Tukey's post-hoc analysis. † $P < 0.05$, within the wild-type group, the trough *m*-chemerin concentration at 0700 was significantly lower compared to the 1500, 1900 and 1100 time points. ‡ $P < 0.05$, within the Db/Db group, the trough *m*-chemerin concentration at 0700 was significantly lower compared to all other time points. $^{\#}$ $P < 0.05$, within the Ob/Ob group, the trough *m*-chemerin concentration at 0300 was significantly lower compared to the 1500, 2300 an 0700 and 1100 time points. The area under the serum *m*-chemerin concentration time curve ($AUC_{0\text{-}20}$) was calculated using the trapezoidal method (GraphPad Prism) (**C**). The difference in maximum and minimum *m*-chemerin concentration was calculated by subtracting the trough *m*-chemerin concentration from the peak *m*-chemerin concentration for each mouse (**D**). Each bar or symbol is the mean ± s.e.m. of 4 or 5 mice. *$P < 0.05$, significantly different and $P < 0.06$, approached significance compared to the wild-type control mice, one-way ANOVA, followed by Tukey's *post hoc* test (**A, C, D**).

Figure 13

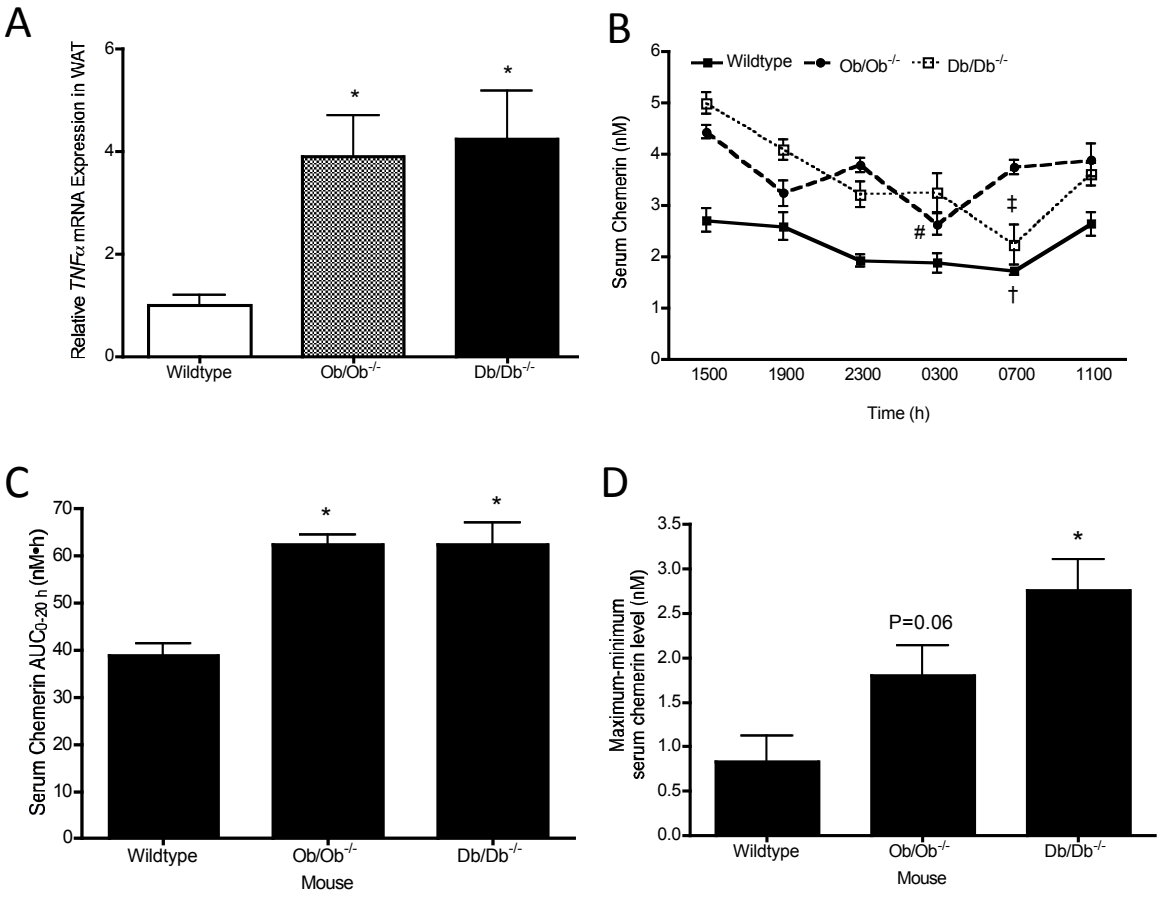

A
Relative TNFα mRNA Expression in WAT
Wildtype
Ob/Ob-/-
Db/Db-/-
B
Wildtype
Ob/Ob-/-
Db/Db-/-
Serum Chemerin (nM)
1500
1900
2300
0300
0700
1100
Time (h)
C
Serum Chemerin AUC0-20 h (nM•h)
Wildtype
Ob/Ob-/-
Db/Db-/-
Mouse
D
Maximum-minimum serum chemerin level (nM)
P=0.06
Wildtype
Ob/Ob-/-
Db/Db-/-
Mouse

Table 7: Relative expression levels of TNFα regulated genes and *chemerin* in adipose tissue and liver. C57/Bl6 mice were treated with 0.5 μg of TNFα or an equivalent volume of 0.1 %BSA/PBS vehicle. White adipose tissue and liver tissue were harvested after 4 or 12 hours for gene expression analysis. For determination of relative gene expression by QPCR, the control adipose tissue served as the reference (expression = 1) to which all other samples were compared. Each value is the mean ± s.e.m. of 4 individual mice. $P < 0.05$, significantly different compared to the control, ANOVA followed by Tukey's *post-hoc* test.

Table 7: Relative expression levels of TNFα regulated genes and *chemerin* in adipose tissue and liver				
A) Gene expression 4 hours post-treatment				
Gene/Tissue		0.1 % BSA/PBS Control	0.5 μg TNFα	P-Value
		Mean ± s.e.m.	Mean ± s.e.m.	
NFkB	Adipose tissue	1.00 ± 0.13	1.89 ± 0.33	**P<0.05**
	Liver	0.37 ± 0.21	0.37 ± 0.15	P>0.05
IL-6	Adipose Tissue	1.00 ± 0.31	2.22 ± 0.23	P>0.05
	Liver	3.79 ± 0.91	2.50 ± 0.32	P>0.05
PAI-1	Adipose tissue	1.00 ± 0.30	6.04 ± 0.75	**P < 0.05**
	Liver	0.01 ± 0.003	0.08 ± 0.03	P > 0.05
Chemerin	Adipose tissue	1.00 ± 0.18	0.91 ± 0.09	P>0.05
	Liver	2.08 ± 0.28	2.363 ± 0.16	P>0.05
B) Gene expression 12 hours post-treatment				
NFκB	Adipose Tissue	1.00 ± 0.06	0.78 ± 0.03	P > 0.05
	Liver	0.37 ± 0.13	0.32 ± 0.07	P > 0.05
IL-6	Adipose Tissue	1.00 ± 0.18	7.66 ± 0.69	**P < 0.05**
	Liver	0.87 ± 0.17	0.57 ± 0.13	P > 0.05
PAI-1	Adipose Tissue	1.00 ± 0.32	2.61 ± 1.28	P > 0.05
	Liver	0.003 ± 0.001	0.014 ± 0.003	P > 0.05
Chemerin	Adipose Tissue	1.00 ± 0.09	1.18 ± 0.08	P > 0.05
	Liver	1.59 ± 0.15	1.80 ± 0.19	P > 0.05

2.05.00: DISCUSSION

The rates of obesity have increased drastically recently with over 315 million adults worldwide now considered obese [253]. Chronic low-grade inflammation is a central feature in obesity and the development of obesity related diseases. Thus, understanding the interrelation between inflammatory mediators and endocrine function of adipose tissue will provide insight into mechanisms that lead to obesity-related pathologies. Several clinical studies have demonstrated positive correlations between plasma or serum levels of total *h*-chemerin and measures of body fat and inflammation [222, 233-236]. Higher levels of total *h*-chemerin in media of adipose tissue explants of obese versus lean humans has been reported indicating that increased adipose tissue production of *h*-prochemerin may elevate circulating levels of the bioactive chemerin protein [238]. Herein, we identified that the obesity-associated inflammatory mediator TNFα is a potent regulator of chemerin. Our results corroborated recent human data, identifying a significantly increased serum amount of total *m*-chemerin in two genetic mouse models of obesity with increased adipose *TNFα* mRNA levels. Furthermore, our findings are the first to indicate that serum levels of total *m*-chemerin follow an oscillatory pattern, which is dependent on the lean versus obese state of the mouse.

Chemerin is produced as pre-prochemerin, which requires N-terminal cleavage before it is secreted as prochemerin. Extracellular processing of prochemerin involves numerous proteases and results in multiple chemerin products, which act as ligands for CMKLR1 and are involved in the initiation or resolution of inflammation [119, 186, 188-190]. To improve our understanding of the regulation of chemerin by TNFα we used an array of assays that would provide information regarding chemerin expression, its

secretion by adipocytes and resulting levels of the bioactive protein in adipocyte media. The finding that TNFα induced *m-chemerin* mRNA levels in 3T3-L1 adipocytes indicated that TNFα could regulate the production of bioactive *m*-chemerin at the mRNA level in these cells. However, the cell toxicity resulting from treatment of 3T3-L1 cells with actinomycin D prohibited confirmation as to whether transcription was a contributing mechanism that lead to elevated media levels of bioactive *m*-chemerin after TNFα treatment. Although we cannot discount transcription in 3T3-L1 cells, we found that cycloheximide and brefeldin A completely reversed the TNFα-mediated increase in adipocyte media concentration of bioactive *m*-chemerin. This could indicate that increased *m*-prochemerin synthesis and secretion contributed to higher adipocyte media levels of bioactive *m*-chemerin following TNFα treatment. However, it is also possible that cycloheximide and Brefeldin A block the synthesis and secretion of extracellular proteolytic factors that convert *m*-prochemerin to *m*-chemerin.

In contrast to the dose-dependent increase in *h*-CMKLR1 activation by 3T3-L1 adipocyte media, the immunodetectable levels of *m*-chemerin in 3T3-L1 adipocyte media followed a bell shaped curve with escalating dose of TNFα. This divergence between the immunodetectable and bioactive *m*-chemerin suggests that TNFα may also regulate *m*-chemerin through extracellular processing mechanisms. One possible mechanism is that TNFα activates extracellular proteolytic processes that degrade bioactive *m*-chemerin into smaller peptides that retain biological activity on the CMKLR1 receptor, but are not detected by western blot. This is possible since several peptides between 8-19 amino acids in length that correspond to the C-terminus of human or mouse chemerin are active ligands of *h*-CMKLR1 or *m*-CMKLR1, respectively [190, 217]. Differential extracellular

proteolytic cleavage of between 5-8 amino acids from the C-terminal end of prochemerin can lead to chemerin variants with distinctive abilities to bind to and activate the *h*-CMKLR1 receptor [119, 188-190, 197]. Thus, a second possibility is that immunodetectable *m*-chemerin in adipocyte media represents a mixture of *m*-chemerin products that would not be distinguished by western blot given their similar molecular weight.

Given that immunodetectable and bioactive *m*-chemerin levels were elevated in the media of 3T3-L1 adipocyte cells, we hypothesized that TNFα treatment would correspondingly increase serum concentration of total *m*-chemerin in mice. An important aspect of our *in vivo* experimental design was to collect blood from each animal at multiple time points allowing for between and within subjects comparisons and greater statistical power through repeated measures analysis. Consistent with the 3T3-L1 adipocyte experiments we observed that TNFα administration elevated serum total *m*-chemerin levels in mice with intact TNFα receptor expression versus their respective time controls. The lack of effect in the TNFα receptor deficient mice indicated that a TNFα receptor-dependent mechanism was involved. Identification that TNFα increased serum *m*-chemerin concentration provides a potential explanation for the previously observed positive association between serum TNFα and total *h*-chemerin levels in humans [233]. Similarly, serum total *m*-chemerin analysis over time in Ob/Ob and Db/Db obese versus lean mice identified elevated serum levels of total *m*-chemerin in obesity. These results corroborated for the first time the recent human studies, which have demonstrated positive associations between body mass index and serum total *m*-chemerin measured by chemerin ELISA [222, 234, 235].

A limitation of the chemerin ELISA assay is that it detects prochemerin and bioactive chemerin (i.e. total chemerin). Through the use of the *h*-CMKLR1 reporter gene assay we detected an increase in the apparent concentration of bioactive *m*-chemerin in serum of TNFα-treated (3.06 ± 0.34 nM) compared to control (1.61 ± 0.39 nM) C57/BL6 mice after 12 hours. This was similar to the elevation in ELISA-detectable *m*-chemerin in TNFα-treated (2.57 ± 0.27 nM) versus control (1.39 ± 0.08 nM) B6129SF2/J mice. Together these findings confirmed that the TNFα-mediated increase in total serum chemerin detected by ELISA reflects an increased level of bioactive *m*-chemerin. In bMSC-derived primary adipocytes we found that TNFα treatment increased the apparent concentration of both immunodetectable and bioactive *m*-chemerin in the adipocyte media. Thus, the increased apparent concentration of bioactive *m*-chemerin in serum following TNFα treatment could involve enhanced *m*-chemerin production by adipose tissue. TNFα induced an inflammatory response in adipose tissue and bMSC-derived primary adipocytes as measured by enhanced expression of *IL-6*, *NFκB* or *PAI-1*. In contrast, there was no such increase in *m-chemerin* mRNA expression within the adipose tissue or primary adipocytes following TNFα treatment. This dissociation indicated that the increased apparent concentration of bioactive *m*-chemerin in serum or adipocyte media following TNFα treatment was not due to enhanced adipose tissue expression of *m-chemerin* mRNA. Rather the results would indicate that TNFα regulation of *m*-chemerin production by adipocytes and serum *m*-chemerin by post-transcriptional mechanisms is important in the whole animal. While not entirely examined in the present study, potential mechanisms whereby TNFα could regulate serum *m*-chemerin levels include increased secretion of prochemerin from intracellular stores, activation of serum

proteases, which convert prochemerin to *m*-chemerin and/or inhibition of proteases that degrade bioactive *m*-chemerin. The liver is known to express high levels of *m-chemerin* and to release the chemerin protein [187, 236]. However, TNFα did not induce the expression of *m-chemerin* in the liver or primary hepatocytes nor did it increase immunodetectable or bioactive *m*-chemerin levels in the hepatocyte media. Thus, increased production of *m*-chemerin by hepatocytes is not likely to contribute to the increased apparent concentration of bioactive *m*-chemerin in serum following TNFα treatment.

Adipokines including adiponectin and leptin are subject to circadian and pulsatile regulation, which can be further modified by degree of adiposity, inflammatory mediators and hormonal factors [254-258]. Through our repeated measures experimental design in lean versus Ob/Ob and Db/Db mice we observed that serum *m*-chemerin in wild-type mice oscillates with peak and trough periods that approximately corresponded to the day-night cycle, respectively. This novel occurrence would have otherwise been missed using a single time point analysis and indicates that timing of treatments and sample collection will be of paramount importance for any future studies that examine circulating *m*-chemerin levels. This importance is demonstrated in our analysis of TNFα effects on serum *m*-chemerin levels *in vivo*, whereby proper time controls were imperative for controlling for natural daily serum *m*-chemerin fluctuations. The greater amplitude or frequency of serum *m*-chemerin oscillation in Ob/Ob and Db/Db would suggest that the pattern of *m*-chemerin production/degradation is dependent on the nutritional status (i.e. lean versus obese state) of the animal. Alternatively, given the leptin and leptin-receptor deficient status of the obese mice and recent reports of a positive correlation between

serum *h*-chemerin and leptin levels, the involvement of leptin signaling cannot be ruled out [233].

In summary, we provide compelling evidence that serum levels of total *m*-chemerin vary with time of day and are modified by obesity and TNFα. Obesity has a profound effect on the rhythmic pattern of metabolic processes including adipocyte function and adipokine secretion [256, 259-262]. In turn, disruption in molecular rhythms may contribute to obesity and obesity-related metabolic disorders [260, 263]. Given these relationships, future research aimed at understanding the oscillatory pattern of chemerin and how it is regulated by adiposity-dependent and independent mechanisms will provide insight regarding the known associations between this adipokine and obesity, inflammation and metabolic syndrome in humans [222, 233-235, 239]

CHAPTER 3.00.00: ELASTASE AND TRYPTASE GOVERN TNFα MEDIATED PRODUCTION OF BIOACTIVE CHEMERIN BY ADIPOCYTES

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MY CONTRIBUTION TO THIS MANUSCRIPT:

As the first author of this publication I conducted all experiments and generated all of the figures in this manuscript. The coauthors provided assistance with conducting the experiments as well as completion of the manuscript.

3.01.00: ABSTRACT

Chemerin is an adipokine with important immune and metabolic roles. Secreted in an inactive form called prochemerin, chemerin undergoes C-terminal proteolytic cleavage converting prochemerin to active chemerin, a biological ligand for the chemokine-like receptor-1 (CMKLR1). Our previous study identified that adipocytes secrete and activate chemerin. Following treatment with the obesity-associated inflammatory mediator TNFα, adipocyte mechanisms are altered resulting in a highly active *m*-chemerin product. Based on these findings we hypothesized adipocytes produce proteases capable of modifying *m*-chemerin bioactivity. Herein we sought to identify theses proteolytic mechanisms. 3T3-L1 adipocytes expressed mRNA of immunocyte and fibrinolytic proteases known to activate chemerin *in vitro*. Following treatment with a general protease inhibitor, *m*-chemerin bioactivity was amplified 10-fold. When the components of the cocktail were investigated individually, aprotinin, a serine protease inhibitor was found to block ~90% of the TNFα-associated increase in *m*-chemerin bioactivity. The inhibitory actions occurred at concentrations corresponding to the IC_{50} of elastase, tryptase, tissue plasminogen activator and tissue plasminogen activator urokinase. Elastase and tryptase were elevated in adipocyte media following treatment with TNFα and their targeted neutralization recapitulated the aprotinin-mediated effects. Bestatin, an inhibitor of aminopeptidases, was in contrast found to further elevate TNFα-associated *m*-chemerin activity. Overall our results support the concept that adipocyte regulate chemerin bioactivity by serine protease-mediated pathways and deactivation via aminopeptidase pathways. Following TNFα treatment, elevated elastase and tryptase modify the balance between chemerin activation and deactivation, resulting in elevated chemerin bioactivity.

3.02.00: INTRODUCTION

Obesity is now overtaking smoking as the leading cause of preventable death in the United States [264]. Similar trends have been reported in most industrialized and many non-industrialized countries [3]. Preventing and treating obesity is therefore a global health concern. The multifactorial nature of the metabolic abnormalities associated with obesity have however, limited the establishment of efficacious therapeutic interventions. Continued effort to clarify the factors regulating obesity-associated metabolic abnormalities including inflammation and adipose tissue remodelling, is therefore indispensible for establishing the mechanisms governing obesity-related disease progression and identification of novel therapeutic targets.

Chemerin is an adipokine that exists as multiple products each with their own unique abilities to activate CMKLR1 and mediate immune and metabolic functions [186-190, 192, 193, 197, 214, 217, 218, 221, 223, 265]. Positive correlations between serum concentrations of total *h*-chemerin and measurements of the metabolic syndrome including body mass index, markers of inflammation (e.g. TNFα & MCP1), serum triglycerides and blood pressure have implicated a role for *h*-chemerin in the pathophysiology of obesity [233-235, 239].

The currently held belief surrounding chemerin production and activity proposes that chemerin is first secreted as inactive prochemerin. Circulating prochemerin subsequently undergoes proteolytic activation at sites of tissue injury into active chemerin products by immunocyte and fibrinolytic associated enzymes. 6 separate *h*-chemerin products have been identified *in vitro* which differ by the C-terminal amino acid and their ability to activate *h*-CMKLR1. They include; *h*-chemerin$_{158}$, *h*-chemerin$_{157}$,

$_{-156}$, $_{-155,}$ $_{-154}$ and $_{-152}$. The serine proteases plasmin and mast cell tryptase cleave *h*-prochemerin into active *h*-chemerin$_{158}$ [189]. Sequential removal of the C-terminal lysine by plasma carboxypeptidase N or B activate *h*-chemerin$_{158}$ into *h*-chemerin$_{157}$, the chemerin product with the highest known activity at *h*-CMKLR1 [188, 190]. In contrast, neutrophil elastase, cathepsin G and K can directly convert *h*-prochemerin into *h*-chemerin$_{157}$ and *h*-chemerin$_{156}$, respectfully [186, 189]. Proteolytic processing is also responsible for deactivation or degradation of *h*-chemerin. Mast cell chymase or angiotensin converting enzyme convert *h*-chemerin$_{157}$ into inactive *h*-chemerin$_{154}$ or low active *h*-chemerin$_{152}$ [193, 265]. With extended exposure, mast cell tryptase, neutrophil elastase as well as proteinase-3 deactivate *h*-prochemerin by converting it to inactive/very low-active *h*-chemerin$_{155}$ [186, 265]. Fibrinolytic enzymes including tissue plasminogen activator (tPA), plasminogen activator urokinase (uPA) and plasminogen also activate *h*-prochemerin although the resulting chemerin products are unknown [192]. Jointly, the *in vitro* results support that chemerin signaling through CMKLR1 and its resulting biological functions are dependent upon local proteolytic mechanisms capable of activating and deactivating chemerin. These processes could be especially important in obese white adipose tissue, which secretes elevated amounts of chemerin [238].

In our previous study we identified that adipocytes produce active chemerin under basal conditions and are a modifiable source of highly active *m*-chemerin following treatment with an obesity-associated inflammatory mediator TNFα [266]. These results are contrary to the currently held model, which dictates immune cell and fibrinolytic proteases as sole responsible parties moderating chemerin activation. Rather the findings support a novel concept in which adipocytes contain all needed machinery to secrete and

process chemerin to its active products. Currently, however, no documented information is available concerning the mechanisms regulating *m*-chemerin activation by adipocytes alone.

The overall object of the current study is to delineate the mechanisms that control adipocyte derived *m*-chemerin activation under basal conditions and following treatment with TNFα**.** Taken together the findings from our study established that adipocytes produce serine and aminopeptidases capable of mediating opposing proteolytic activation and deactivation of *m*-chemerin, respectfully. Following treatment with TNFα, elevated concentrations of elastase and tryptase contribute to an imbalance in these opposing proteases, resulting in a net elevation in chemerin bioactivity.

3.03.00: METHODS

3.03.01: 3T3-L1 ADIPOCYTE CELL CULTURE.

3T3-L1 preadipocytes were obtained from the American Tissue Culture Collection (Manassas, VA) and were grown and differentiated according to our published protocols [187]. All media was phenol-red free. For the protease expression experiments cell lysates from 3T3-L1 adipocytes grown on 12-well plates were harvested on day 0 (preadipocyte), 3, 5, 8 and 13 following differentiation and the subsequent total cellular RNA was isolated. For the protease inhibitor experiments, 13-d differentiated adipocytes grown on 12-well plates were rinsed once with 1 mL of PBS and then treated with 20 ng mL^{-1} recombinant mouse TNFα (R&D Systems, Minneapolis, MN) or 0.1% BSA in PBS (0.1% BSA/PBS) vehicle in combination with a protease inhibitor or its respective control in 500μl of serum-free DMEM for a period of 24 h. The protease inhibitors included a 1:200 dilution of a proteases inhibitor cocktail (PIC), 0.1-30 μM aprotinin, 1-100 μM E-64, and 2-60 nM bestatin or their respective DMSO, H_2O, or H_2O with 0.9% NaCl and 0.9% Benzyl Alcohol (pH 5.7-6.2) controls (Sigma Aldrich, ON, Canada). After the 24 h incubation, the conditioned adipocyte media or whole cell lysate was collected and the corresponding cellular RNA, was isolated. Media and RNA samples were stored at -80°C until western blots, chemerin bioassays and gene expression analyses were performed.

3.03.02: PRIMARY ADIPOCYTE CELL CULTURE.

Bone marrow stromal cells (bMSC)- derived adipocytes were isolated and cultured from 8-wk-old C57BL6/J mice bred in-house using established methods [223]. The protease inhibitor experiments were performed in serum free media as outlined for the 3T3-L1 adipocytes.

3.03.03: RNA ISOLATION AND QUANTITATIVE PCR.

Total cellular RNA was isolated using the Bio-Rad aurum total RNA mini kit (Bio-Rad, Mississauga, Ontario, Canada) according to the manufactuers' instructions, and 0.5 μg was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen, ON, Canada). 0.5 μl of the cDNA product was amplified by quantitative PCR (QPCR) using 125 nM of gene specific primers in a total volume of 20 μl with iTaq SYBR green supermix with ROX QPCR master mix using the StepOnePlus Real-Time PCR system (Applied Biosystems, Streetsville, Ontario, Canada). Relative gene expression normalized to *Cyclophilin A* expression was calculated using the cycle threshold ($\Delta\Delta C_T$) method [250]. The forward and reverse QPCR primer sequences are identified in **Table 8**.

3.03.04: QUANTIFICATION OF BIOACTIVE CHEMERIN IN ADIPOCYTE MEDIA USING THE CMKLR1 "TANGO" BIOASSAY.

The CMKLR1 "Tango" bioassay is a cell-based assay that specifically and quantitatively measures *m*-CMKLR1 activation by active chemerin. By comparing *m*-CMKLR1 activation by media samples to known concentrations of *m*-chemerin$_{156}$ we are

able to determine the apparent concentration of active *m*-chemerin. A detailed description of the assay procedures has been described previously [204, 266]. To exclude potential non-specific effects of serine, cysteine and aminopeptidase inhibition on the *m*-CMKLR1 "Tango" bioassay, a vehicle control or 30 μM aprotinin, 60 nM bestatin or 100 μM E-64 in 120 μL of serum free DMEM was spiked with 0, 1, 3 or 10 nM recombinant *m*-chemerin$_{156}$ and subsequently analyzed by *m*-CMKLR1 "Tango" bioassay.

3.03.05: NEUTRALIZATION OF *M*-CHEMERIN BIOACTIVITY.

To determine the specific activation of the *m*-CMKLR1 "Tango" bioassay by *m*-chemerin, media from 3T3-L1 or bMSC derived adipocytes treated with 20 ng mL^{-1} TNFα or 0.1% BSA/PBS control for 24 hours were incubated for 1 hour with 10 μg mL^{-1} of goat anti-mouse chemerin neutralization antibody (R&D biosystems, Minneapolis, MN) or a goat 10 μg mL^{-1} IgG control antibody (Invitrogen, Burlington, ON) prior to analyzing the samples in the "Tango" assay.

3.03.06: NEUTRALIZATION OF NEUTROPHIL ELASTASE AND MAST CELL TRYPTASE.

The effects of neutrophil elastase and mast cell tryptase on chemerin activity following treatment with TNFα or a vehicle control was investigated using neutralizing antibodies directed towards the C-terminus and internal region of mouse elastase and tryptase, respectfully. 3T3-L1 adipocytes were preincubated for 1 hr with 10 μg mL^{-1} goat IgG (R&D Biosystems, Minneapolis, MN), 5 μg mL^{-1} goat-anti-mouse neutrophil elastase (Catalogue #sc-9521, Santa Cruz Biotechnology Inc., Santa Cruz, CA) 5 μg mL^{-1} goat-anti-mouse mast cell tryptase (Catalogue #sc-32474, Santa Cruz Biotechnology Inc.,

Santa Cruz, CA) or a combination of 5 μg mL^{-1} of anti-elastase and 5 μg mL^{-1} anti-tryptase antibodies in 250 μL of serum free DMEM. The adipocytes were subsequently supplemented for 24 hours with 40 ng mL^{-1} TNFα or a PBS control in 250 μL of serum free DMEM for a final concentration of 20 ng mL^{-1} prior to harvesting the media and analyzing by the *m*-CMKLR1 "Tango" bioassay.

3.03.07: SDS-PAGE "WESTERN" BLOTTING.

20 μl of 24 h conditioned adipocyte media or 7.5 ng of recombinant mouse $chemerin_{156}$ (R&D biosystems, Minneapolis, MN) was added to 6X sodium dodecyl sulfate loading buffer containing β-mercaptoethanol and incubated at 95°C for 5 min. The resulting solution was separated on a 15% polyacrylamide gel and transferred overnight to a nitrocellulose membrane. The nitrocellulose was subsequently washed with TBS for 5 min and incubated in 10 mL of Odyssey blocking buffer (LI-COR, Lincoln, NE) containing 0.1% tween-20 (called western buffer) for 1 hour. Once blocking was complete, the nitrocellulose was placed overnight in western buffer containing either a 1:200 dilution of goat anti-mouse chemerin (R&D biosystem, Minneapolis, MN), neutrophil elastase (M-18) or mast cell tryptase (G-12) (Santa Cruz, Biotechnology, CA) or a 1:50 dilution of goat anti mouse plasminogen (E-14) or uPA (M-20) (Santa Cruz, Biotechnology, CA). The following day the blot was washed 4X for 5 min in TBS-T and incubated for 1 h in a 1:5000 dilution of a 680 or 800 nm infrared fluorophore conjugated polyclonal donkey anti-goat IgG (LI-COR, Lincoln, NE). The nitrocellulose was finally washed 4X for 5 min in TBS-T and switched into TBS prior to

scanning at 700 or 800 nm at a resolution of 84 μM using a LI-COR Odyssey infrared scanner at an intensity of 5 (chemerin, elastase and tryptase) or 6 (plasminogen and uPA).

3.03.08: STATISTICAL ANALYSIS.

All data are expressed as mean ± s.e.m of 3 samples, and are representative of at least 2 independent experiments. Statistical analysis was performed using GraphPad Prism. A two-way analysis of variance (ANOVA) was used for comparing proteolytic inhibitor-dependent effects on basal and TNFα stimulated apparent media *m*-chemerin concentration. A one-way ANOVA was used for multiple comparison procedures with one independent variable. A Tukey's or Bonferroni test was used for *post-hoc* analysis of the significant ANOVA, with the exception of the *m*-CMKLR1 "Tango" bioassays standard curves. A difference in mean values between groups was considered to be significant when $P \leq 0.05$.

3.04.00: RESULTS

3.04.01: ACTIVATION OF *M*-CMKLR1 BY ADIPOCYTE MEDIA IS *M*-CHEMERIN-SPECIFIC.

Previous *in vitro* studies have shown CMKLR1 activation can be attributed to numerous full length chemerin products as well as non-chemerin products including resolvin E1 [216]. To confirm the specificity of *m*-CMKLR1 "Tango" activation by active *m*-chemerin products following treatment with TNFα or a vehicle control, 24 h conditioned serum free media from 3T3-L1 or bMSC adipocytes was neutralized with an anti-mouse chemerin antibody prior to performing the bioassay. 10 μg mL^{-1} of anti-mouse chemerin antibody neutralized ~99 and 95% of *m*-CMKLR1 activation by conditioned media from 3T3-L1 adipocytes treated with a vehicle control or 20 ng mL^{-1} TNFα in comparison to their respective IgG control (**Figure 14A**). Anti-mouse chemerin antibodies were similarly able to neutralize 99% of *m*-CMKLR1 activation in conditioned media from bMSC adipocytes supplemented with PBS or 20 ng mL^{-1} TNFα respectfully (**Figure 14B**). These results confirm that under basal conditions and following treatment with TNFα *m*-CMKLR1 "Tango" activation by adipocyte media is *m*-chemerin specific.

3.04.02: ADIPOCYTES EXPRESS IMMUNOCYTE AND FIBRINOLYTIC ASSOCIATED ENZYMES.

Immunocyte and fibrinolytic associated enzymes are well known to active *h*-chemerin *in vitro* [119, 186, 188-190, 193, 196, 265]. To determine whether adipocytes express these same genes, 3T3-L1 preadipocytes were differentiated according to standard protocols and analyzed for their expression of *neutrophil elastase*, *mast cell*

tryptase, *tPA*, *uPA*, *angiotensin converting enzyme* and *cathepsin K* at varying days through adipocyte differentiation. Adipocyte expression of the aforementioned proteases had two basic expression profiles. Expression of *neutrophil elastase* **(Figure 15A)**, *mast cell tryptase* **(Figure 15B)**, and *angiotensin converting enzyme* **(Figure 15C)** underwent a differentiation dependent increase in expression with ~100-, ~40- and ~5- fold higher concentrations at days 13 versus day 0 respectfully. In contrast *tPA* **(Figure 15D)**, *uPA* **(Figure 15E)**, and *cathepsin K* **(Figure 15F)** were expressed in the undifferentiated state, with a reduction of ~80-90% at days 3 and ~70-80% at day 5 (early adipogenic stages), regaining their baseline expression by day 8. While angiotensin converting enzyme, cathepsin K, tPA and uPA are known to be produced by adipocytes these are the first results to our knowledge identifying that adipocytes express *elastase* and *tryptase* [267-270]. Together these results support adipocytes express genes that encode for enzymes that activate chemerin.

3.04.03: GENERAL INHIBITION OF ADIPOCYTE PROTEASES INCREASES THE APPARENT CONCENTRATION OF ACTIVE M-CHEMERIN IN ADIPOCYTE MEDIA.

To determine the contribution of adipose-derived enzymatic processing on m-chemerin activity under basal conditions and following treatment with TNFα, 3T3-L1 or bMSC adipocytes were treated with TNFα or a vehicle control in combination with a PIC for 24 hours prior analysis by the m-CMKLR1 "Tango" bioassay. This cocktail contains inhibitors of serine (aprotinin), cysteine (E-64), aspartyl (pepstatin A) and aminopeptidases (bestatin). The apparent m-chemerin concentration in 3T3-L1 adipocyte

media was increased by 5-fold by TNFα and PIC treatment alone. Combined treatment with TNFα and PIC interacted such that there was an even higher m-chemerin bioactivity (~20-fold) compared to control (**Figure 16A**). The effects of TNF and PIC treatments, alone or in combination had similar effects in bMSCs. The apparent bMSC media m-chemerin concentration was increased ~5- and ~30-fold respectfully after treatment with TNFα or PIC and by 300-fold with the combination of TNFα and PIC treatments (**Figure 16B**). Western blot analysis identified TNFα, PIC and combined treatment of TNFα and PIC in 3T3-L1 (**Figure 16C**) or bMSC adipocytes (**Figure 16D**) increased total m-chemerin by 1.25-2-fold versus the vehicle control. The overall data indicates that proteolytic enzymes must play a role in determining bioactive chemerin production under basal conditions and following TNFα treatment.

3.04.04: SERINE AND CYSTEINE PROTEASE INHIBITORS ATTENUATE TNFα-DEPENDENT INCREASES IN THE APPARENT BIOACTIVE CONCENTRATION OF *M*- CHEMERIN IN ADIPOCYTE MEDIA.

On the basis of the findings attained from treatment of adipocytes with a general proteases inhibitor, the subsequent goal was to determine whether the specific inhibitors that individually compose the cocktail modify the apparent bioactivity of *m*-chemerin in adipocyte media. Our first goal was to investigate the serine and cysteine protease inhibitors aprotinin and E-64 owing to the well-documented role of serine and cysteine proteases in *h*-chemerin activation by immune cells. In TNFα treated cells, aprotinin reduced the apparent concentration of *m*-chemerin in 3T3-L1 adipocyte media to basal levels in a dose-dependent fashion (**Figure 17A**). Aprotinin had no effect on basal

(vehicle control) *m*-chemerin activity at any concentration. The highest dose of aprotinin had similar inhibitor effects on the apparent concentration of *m*-chemerin in media from TNFα treated bMSCs. (**Figure 17B**). Like aprotinin, treatment with 100 μM E-64 reduced the TNFα-mediated apparent concentration of *m*-chemerin in 3T3-L1 adipocyte media. (**Figure 17C**). 100 μM had no affect on the basal concentration of apparent *m*-chemerin bioactivity.

Subsequent western blot analysis of media from 3T3-L1 (**Figure 17D, F**) and bMSC (**Figure 17E**) adipocytes treated with TNFα (with or with or without aprotinin or E-64) displayed a ~1.5-fold increase in the total media *m*-chemerin compared to the PBS control.

Neither aprotinin (**Figure 18A)** nor E-64 (**Figure 18B)** was identified to have any significant effect on the biological activity produced by recombinant *m*-chemerin in the bioassay. Thus, aprotinin and E-64 can be implicated in the modification of *m*-chemerin bioactivity produced by the adipocytes rather than exerting a non-specific effect on the bioassay. Taken together, these findings indicate that TNFα-stimulated production of bioactive *m*-chemerin by adipocytes requires the activity of serine and to lesser degree cysteine proteases and that this occurs without an equivalent change in immunodetectable *m*-chemerin.

3.04.05: ELASTASE AND TRYPTASE ARE INCREASED AFTER TREATMENT WITH TNFα.

Approximately 90% of TNFα-mediated apparent *m*-chemerin concentration was inhibited by aprotinin at concentrations ranging from 3-30 μM. These concentrations of

aprotinin corresponded to known IC_{50} values of tryptase, elastase, tissue plasminogen activator (tPA) and tissue plasminogen activator urokinase (uPA) [271, 272]. We next wanted to explore whether the expression or secretion profiles of these enzymes were altered in 3T3-L1 and bMSC adipocytes treated with TNFα compared to the PBS control. QPCR analysis at 0, 2, 4 and 8 hours after treatment with PBS or TNFα showed little effect of either treatment on the expression profile of these enzymes in 3T3-L1 adipocytes with an slight elevation in *uPA* expression and non-significant depression of *elastase* expression at 8 and 2 hours respectively. Western blot analysis in contrast, identified a ~2-fold increase in mast cell tryptase and neutrophil elastase in 24 hour conditioned media from both 3T3-L1 and bMSC adipocytes treated with TNFα compared with PBS control. While no changes in tPA were identified, uPA could not be detected by western blot analysis (**Figure 19A**).

3.04.06: NEUTRALIZATION OF NEUTROPHIL ELASTASE OR MAST CELL TRYPTASE INHIBITS TNFα-DEPENDENT *M*-CHEMERIN BIOACTIVITY.

The elevated levels of elastase and tryptase in 3T3-L1 and bMSC adipocyte media following treatment with TNFα suggested that elastase and tryptase were perchance the serine proteases responsible for the associated heightened apparent bioactive concentrations. In support of this both anti-tryptase and anti-elastase alone inhibited the TNFα mediated apparent *m*-chemerin bioactivity by ~40 and 35% respectively compared to the IgG control in 3T3-L1 cells. Combined, the anti-elastase and anti-tryptase treatments had an additive affect neutralizing ~67% of the inflammatory associated chemerin activity. Under basal conditions tryptase and elastase either separately or

together neutralized ~85% of the apparent chemerin activity. Adipose derived elastase and tryptase are therefore partial mediators of both basal and inflammatory associated *m*-chemerin bioactivity (**Figure 19B**).

3.04.07: BESTATIN AMPLIFIES TNFα-ASSOCIATED *M*-CHEMERIN BIOACTIVITY IN ADIPOCYTE MEDIA.

In vitro studies have identified that post-translational C-terminal modification by proteases as a critical mechanism in the regulation of chemerin activity. A role for the N-terminus in chemerin bioactivity has however been largely ignored. Bestatin was consequently investigated for its effect on the apparent concentration of *m*-chemerin in 3T3-L1 adipocyte media when combined with TNFα or a vehicle control. Bestatin was investigated because it is both an inhibitor of aminopeptidases, proteases that cleave the N-terminus of proteins, and it is a component of the protease inhibitor cocktail. 20 to 60 nM bestatin in combination with TNFα increased the TNFα dependent elevated chemerin activity ~4- and ~7- fold respectively from the TNFα control (**Figure 20A**). Similarly, 60 nM bestatin had a small but non-significant effect on the apparent media concentration of *m*-chemerin under basal conditions. Subsequent Western blot analysis of 3T3-L1 (**Figure 20B**) conditioned media treated with TNFα or a vehicle control identified TNFα increased immunodetectable levels of total chemerin ~1.25-2 fold compared to the PBS control regardless of whether it was combined with 60 nM bestatin. Like aprotinin and E-64, 60 nM bestatin had no affect on the bioactivity of recombinant *m*-chemerin in the CMKLR1 "Tango" bioassay (**Figure 20C**). Bestatin is therefore modifying the apparent concentration of adipocyte *m*-chemerin bioactivity rather then

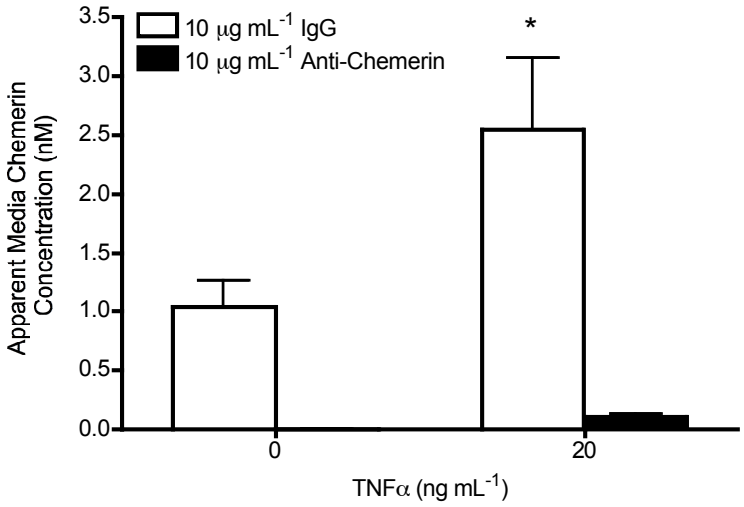
non-specifically altering the “tango” assay. The effect of bestatin on the apparent *m*-chemerin bioactivity implicates aminopeptidases in degradation of active *m*-chemerin.

Table 8: Quantitative-PCR primer gene identification, sequences and product sizes			
Mouse Gene	**Identification**	**Sequence**	**Product Size (bp)**
neutrophil elastase	NM_015779.2	TGTGAACGGCCTAAATTTCC	186
		ACGTTGGCGTTAATGGTAGC	
mast cell tryptase	NM_031187	GAGACCTTCCCCTCAGGAAC	200
		ATGTCCTTCATTCCCAGCAC	
plasminogen	NM_008877	GCTGCCTGTGATTGAGAACA	146
		TCTCGAAGCAAACCAGAGGT	
tPA	NM_008872	GCTGAGTGCATCAACTGGAA	243
		GCCACGGTAAGTCACACCTT	
uPA	NM_008873	AGTGTGGCCAGAAGGCTCTA	279
		GCTGCTCCACCTCAAACTTC	
cathepsin K	NM_007802.3	CAGCTTCCCCAAGATGTGAT	165
		AGCACCAACGAGAGGAGAAA	
angiotensin converting enzyme	NM_001130513	CAGTGTCTACCCCCAAGCAT	101
		GTGAGGGCCATCTTCATTA	
cyclophilin	X52803.1	GAGCTGTTTGCAGACAAAGTTC	134
		CCCTGGCACATGAATCCTGG	

Figure 14: Activation of *m*-CMKLR1 by adipocyte media is *m*-chemerin-specific. Twenty-four hour conditioned media from 3T3-L1 (**A**) or bMSC (**B**) adipocytes treated with 20 ng mL^{-1} TNFα or an equivalent vehicle control were incubated for 1 hour with 10 μg mL^{-1} of goat anti-mouse chemerin neutralization antibody or 10 μg mL^{-1} IgG control antibody, prior to analysis by CMKLR1 "Tango" bioassay. All bars represent the mean ± s.e.m. of 3 samples, and are representative of 3 independent experiments *$P<0.05$ compared to the control (goat IgG), Two-way ANOVA, followed by Bonferroni's *post hoc* test.

Figure 14

A


10 μg mL-1 IgG
10 μg mL-1 Anti-Chemerin
Apparent Media Chemerin Concentration (nM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0
20
TNFα (ng mL-1)

B

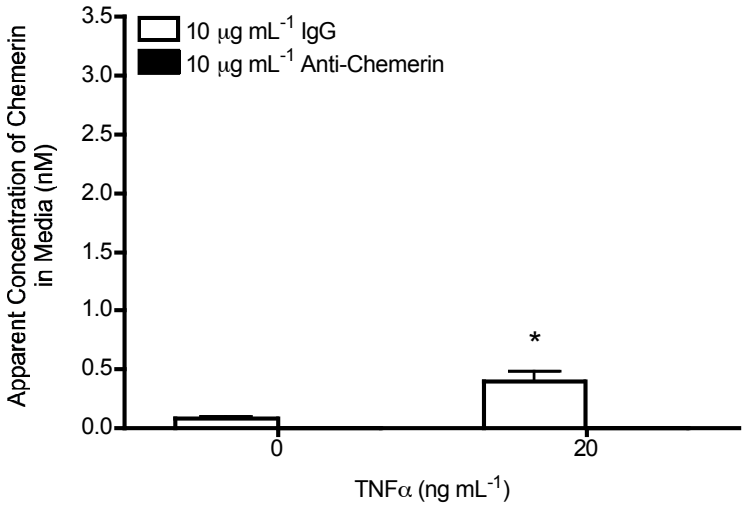

10 μg mL-1 IgG
10 μg mL-1 Anti-Chemerin
Apparent Concentration of Chemerin in Media (nM)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0
20
TNFα (ng mL-1)

Figure 15: 3T3-L1 adipocytes express immunocyte and fibrinolytic associated enzymes. The mRNA expression of *neutrophil elastase* (**A**)*, mast cell tryptase* (**B**)*, angiotensin converting enzyme* (**C**)*, tissue plasminogen activator (tPA)* (**D**)*, tissue plasminogen activator urokinase (uPA)* (**E**) and *cathepsin K* (**F**) were analyzed in 3T3-L1 adipocytes throughout differentiation. All bars represent the mean ± s.e.m. of 3 samples and are representative of 3 independent experiments * $P<0.05$ compared to the control (D0, preadipocytes), 1-way ANOVA, followed by Tukey's *post hoc* test.

Figure 15

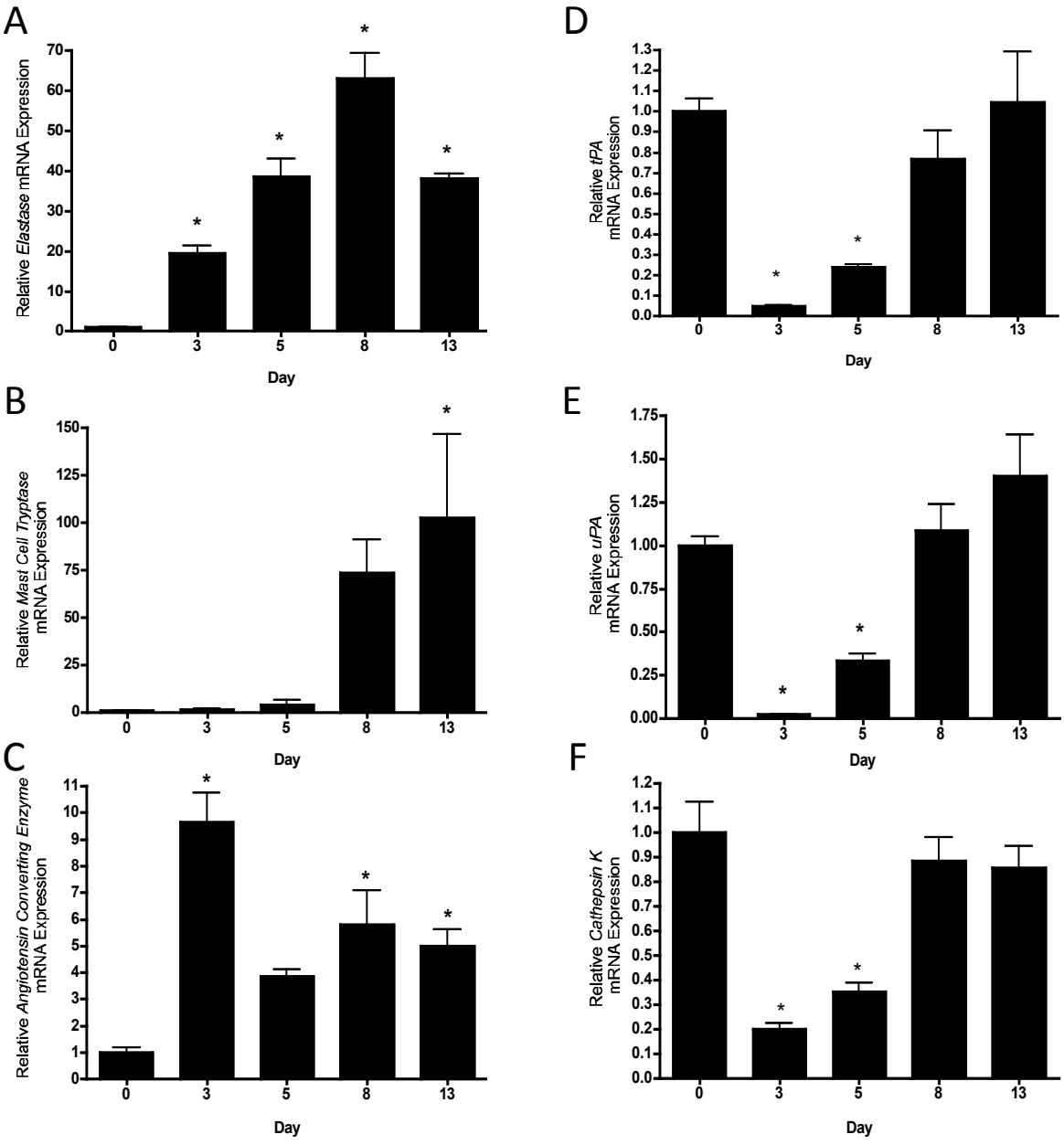

A
Relative Elastase mRNA Expression
Day
B
Relative Mast Cell Tryptase mRNA Expression
Day
C
Relative Angiotensin Converting Enzyme mRNA Expression
Day
D
Relative tPA mRNA Expression
Day
E
Relative uPA mRNA Expression
Day
F
Relative Cathepsin K mRNA Expression
Day

Figure 16: Proteolytic inhibition increases bioactive *m*-chemerin concentrations in adipocyte media. 3T3-L1 adipocytes were treated for 24 hours with 20 ng mL^{-1} TNFα or a vehicle control in combination with a 1:200 dilution of a protease inhibitor cocktail prior to analysis by *m*-CMKLR1 "Tango" bioassay (**A, B**) or western blot (**C, D**). All bars represent the mean± s.e.m. of 3 samples, and are representative of at least 2 independent experiments. Western blot analysis using an R&D anti-chemerin antibody is representative of 4 samples per group and 3 independent experiments. *P<0.05 compared to the TNFα control, two-way ANOVA, followed by Bonferroni's *post hoc* test.

Figure 16

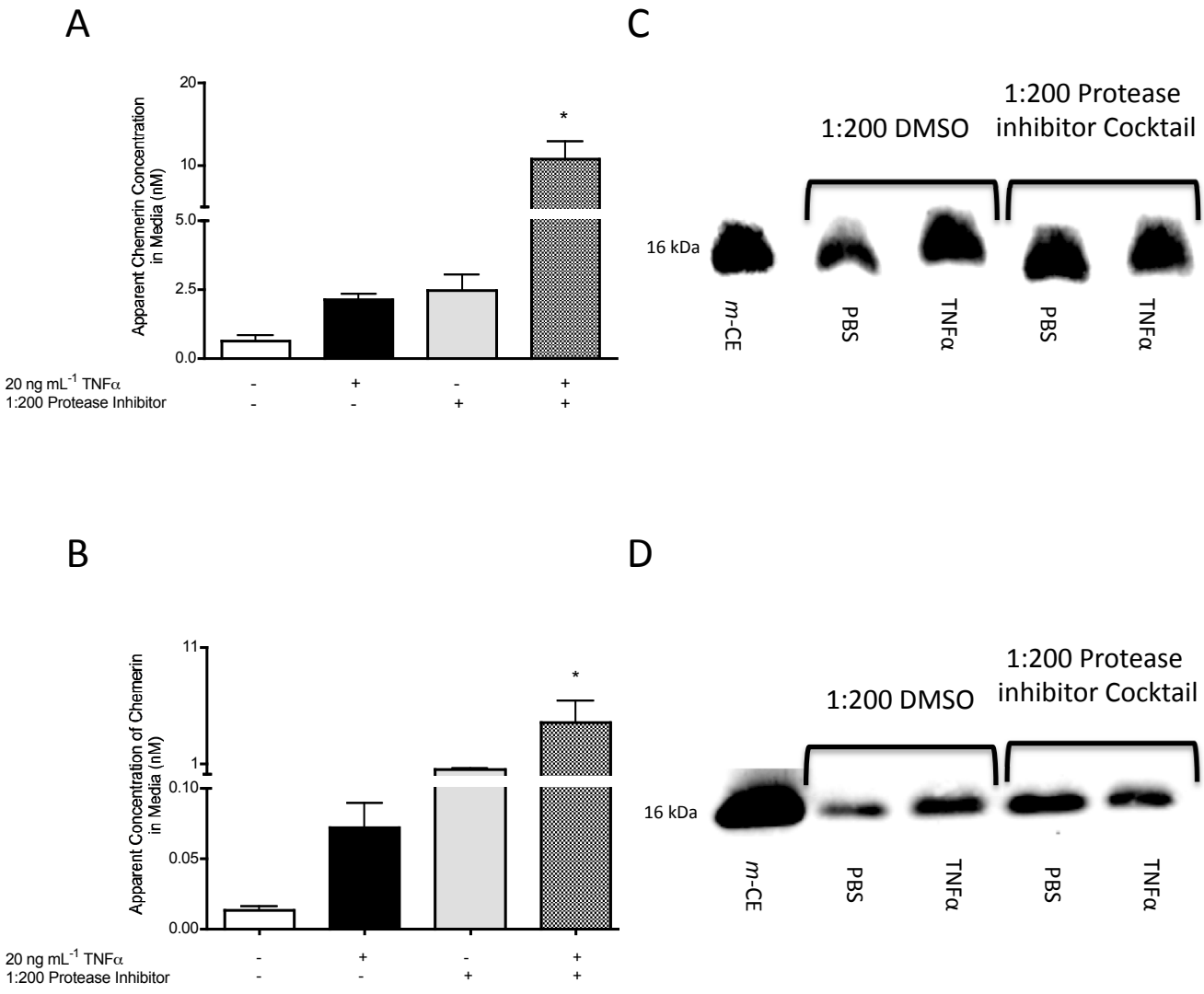

A
Apparent Chemerin Concentration in Media (nM)
20
10
5.0
2.5
0.0
*
20 ng mL-1 TNFα
1:200 Protease Inhibitor
- + - +
- - + +
C
1:200 DMSO
1:200 Protease inhibitor Cocktail
16 kDa
m-CE
PBS
TNFα
PBS
TNFα
B
Apparent Concentration of Chemerin in Media (nM)
11
1
0.10
0.05
0.00
*
20 ng mL-1 TNFα
1:200 Protease Inhibitor
- + - +
- - + +
D
1:200 DMSO
1:200 Protease inhibitor Cocktail
16 kDa
m-CE
PBS
TNFα
PBS
TNFα

Figure 17: Serine and cysteine protease inhibitors attenuate TNFα dependent increases in the apparent bioactive concentration of *m*-chemerin in adipocyte media. A *m*-CMKLR1 "Tango" bioassay and western blot analysis were used to measure the effect of 24 hour treatment of TNFα or a vehicle control in combination with 0-30 μM aprotinin (**A, B, D, E**) or 0-100 μM E-64 (**C, F**) on the apparent (**A-C**) or total (**D-F**) *m*-chemerin concentrations in 3T3-L1 (**A, C, D, F**) or bMSC (**B, E**) adipocyte conditioned media. All bars represent the mean ± s.e.m. of 3 samples, and are representative of 3 independent experiments. Western blot using an R&D anti-chemerin antibody is representative of 4 samples per group and two independent experiments. † $P<0.05$ compared to the TNFα control (**A, B, C**), Two-way ANOVA, followed by Bonferroni's *post hoc* test.

Figure 17

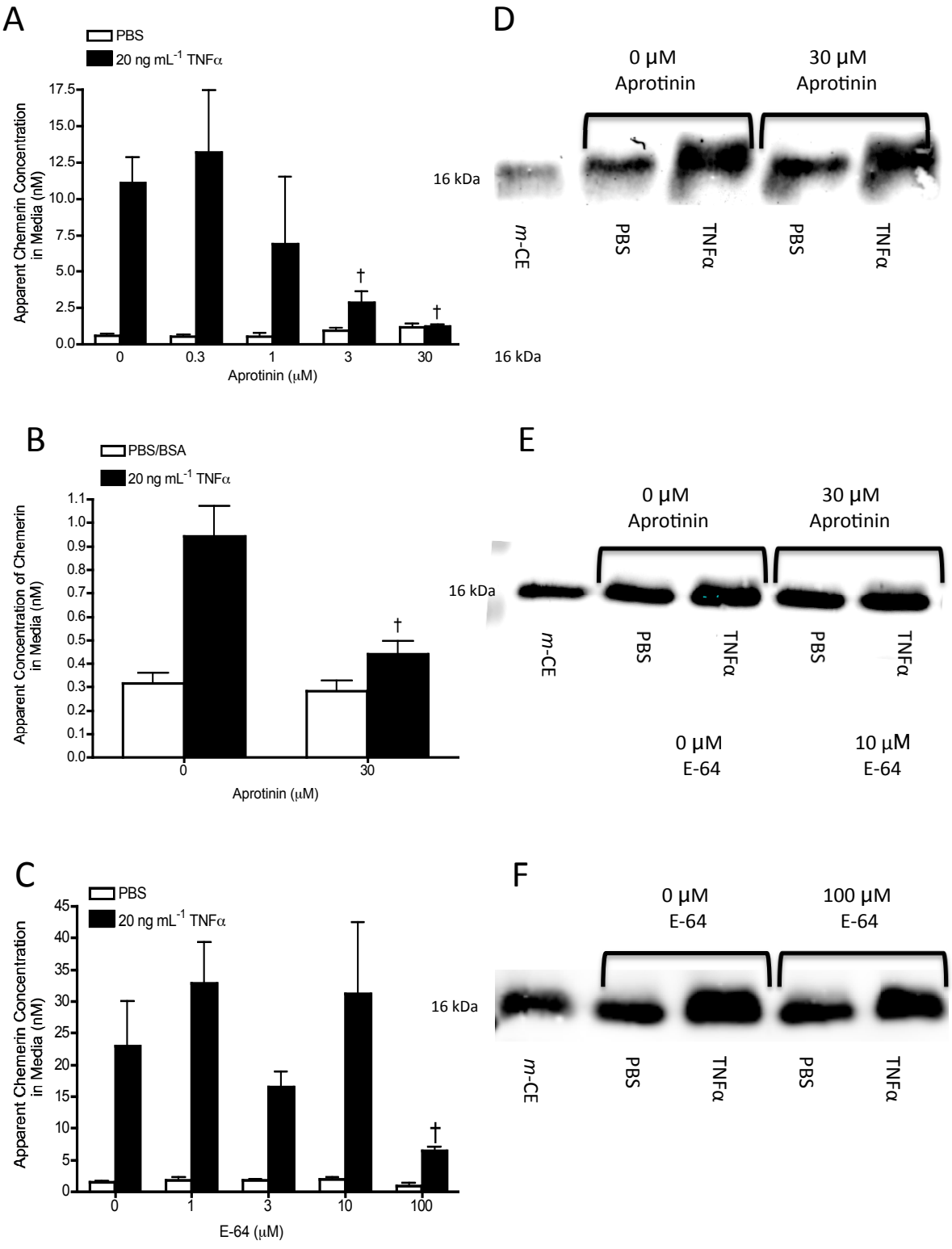
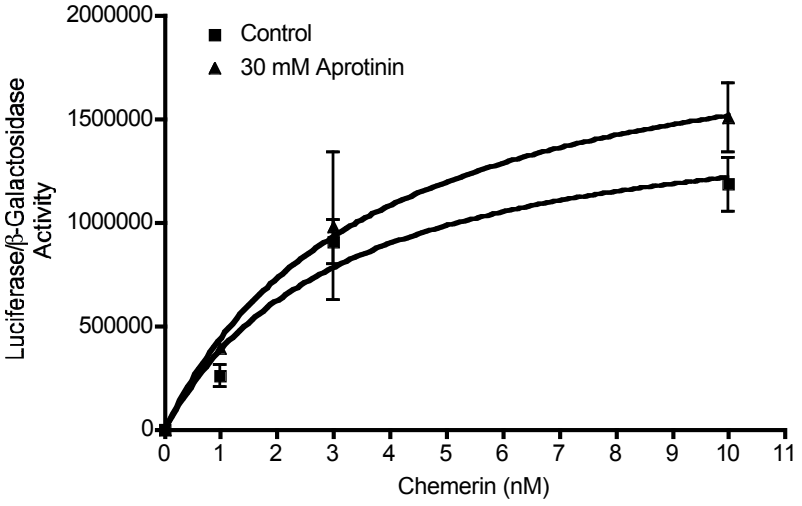

A
PBS
20 ng mL-1 TNFα
Apparent Chemerin Concentration in Media (nM)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0
0.3
1
3
30
†
†
Aprotinin (μM)
D
0 μM Aprotinin
30 μM Aprotinin
16 kDa
m-CE
PBS
TNFα
PBS
TNFα
16 kDa
B
PBS/BSA
20 ng mL-1 TNFα
Apparent Concentration of Chemerin in Media (nM)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
30
†
Aprotinin (μM)
E
0 μM Aprotinin
30 μM Aprotinin
16 kDa
m-CE
PBS
TNFα
PBS
TNFα
0 μM E-64
10 μM E-64
C
PBS
20 ng mL-1 TNFα
Apparent Chemerin Concentration in Media (nM)
45
40
35
30
25
20
15
10
5
0
0
1
3
10
100
†
E-64 (μM)
F
0 μM E-64
100 μM E-64
16 kDa
m-CE
PBS
TNFα
PBS
TNFα

Figure 18: Aprotinin and E-64 do not non-specifically affect the CMKLR1 "Tango" bioassay. To rule out non-specific effects of aprotinin and E-64 on the assay itself, the luciferase/β-galactosidase activity of varying recombinant *m*-chemerin standards combined with 30 μM aprotinin (**A**), 100 μM E-64 (**B**), or their respective controls were analyzed by CMKLR1 "Tango" bioassay. All bars represent the mean ± s.e.m. of 3 samples, and are representative of 2 independent experiments.

Figure 18

A


Control
30 mM Aprotinin
Luciferase/β-Galactosidase Activity
2000000
1500000
1000000
500000
0
0 1 2 3 4 5 6 7 8 9 10 11
Chemerin (nM)

B

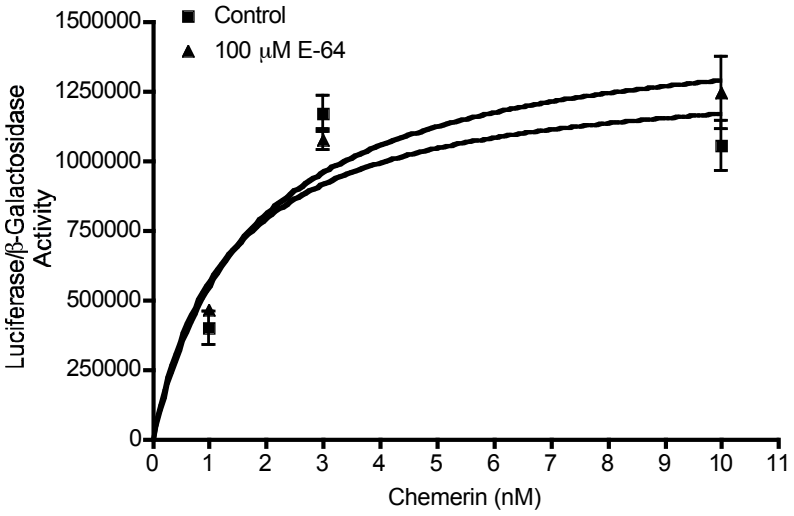

Control
100 μM E-64
Luciferase/β-Galactosidase Activity
1500000
1250000
1000000
750000
500000
250000
0
0 1 2 3 4 5 6 7 8 9 10 11
Chemerin (nM)

Table 9: Relative expression of immune and fibrinolytic enzymes in 3T3-L1 adipocytes following TNFα treatment. 3T3-L1 adipocytes were treated with 20 ng mL^{-1} TNFα or an equivalent volume of 0.1% BSA/PBS vehicle after which they were harvested at 0, 2, 4 and 8 hours after treatment for gene expression analysis. For determination of relative gene expression by QPCR, the 0 time point served as the reference (expression=1) to which all other sample were compared. Each value is the mean ± s.e.m. of 3 samples and representative of 3 independent experiments. $P< 0.05$, significantly different compared to the control, Two-way ANOVA followed by Bonferroni's *post-hoc* test.

Table 9: Relative expression of immune and fibrinolytic enzymes in 3T3-L1 adipocytes following TNFα treatment				
Gene	**Time following Treatment (hrs)**	**Treatment**		**Statistical**
		0.1 % BSA/PBS	**20 ng mL^{-1} TNFα**	**P-Value**
		Mean ± S.E.M.		
Neutrophil Elastase	**0**	1.00 (0.24)	1.00 (0.13)	P > 0.05
	2	2.12 (0.82)	1.02 (0.13)	P > 0.05
	4	1.76 (0.15)	0.80 (0.3)	P > 0.05
	8	0.94 (0.14)	0.19 (0.06)	P > 0.05
Mast Cell Tryptase	**0**	1.00 (0.14)	1.18 (0.83)	P > 0.05
	2	1.43 (0.11)	2.10 (0.37)	P > 0.05
	4	1.89 (0.47)	1.76 (0.53)	P > 0.05
	8	1.43 (0.19)	1.46 (0.15)	P > 0.05
uPA	**0**	1.00 (0.25)	0.75 (0.32)	P > 0.05
	2	0.78 (0.13)	2.95 (0.49)	**P <0.05**
	4	0.88 (0.36)	1.10 (0.2)	P > 0.05
	8	0.75 (0.15)	0.48 (0.12)	P > 0.05
tPA	**0**	1.00 (0.07)	1.01 (0.05)	P > 0.05
	2	1.27 (0.44)	1.10 (0.12)	P > 0.05
	4	0.85 (0.05)	0.95 (0.16)	P > 0.05
	8	0.58 (0.09)	1.12 (0.16)	P > 0.05

Figure 19: Elastase and tryptase are responsible for the TNFα-mediated increase in the apparent concentration of *m*-chemerin in adipocyte media. The concentration of elastase, tryptase, plasminogen, and tPA in 24 hour conditioned 3T3-L1 and bMSC adipocyte media treated with TNFα or a vehicle control were measured by western blot. (**A**). Analysis by *m*-CMKLR1 "Tango" bioassay determined the effect of elastase and tryptase neutralization alone or in combination on 3T3-L1 adipocytes production of active *m*-chemerin following treatment with TNFα (**B**) or a vehicle control (**INSET**). All bars represent the mean ± s.e.m. of 3 samples, and are representative of at least 2 independent experiments. Western blots are representative of 4 samples per group and 2 independent experiments. * $P<0.05$ compared to the TNFα control, or respective vehicle (**INSET**) † <0.05 compared to the TNFα + anti-elastase or anti-tryptase, two-way ANOVA, followed by Bonferroni's *post hoc* test.

Figure 19

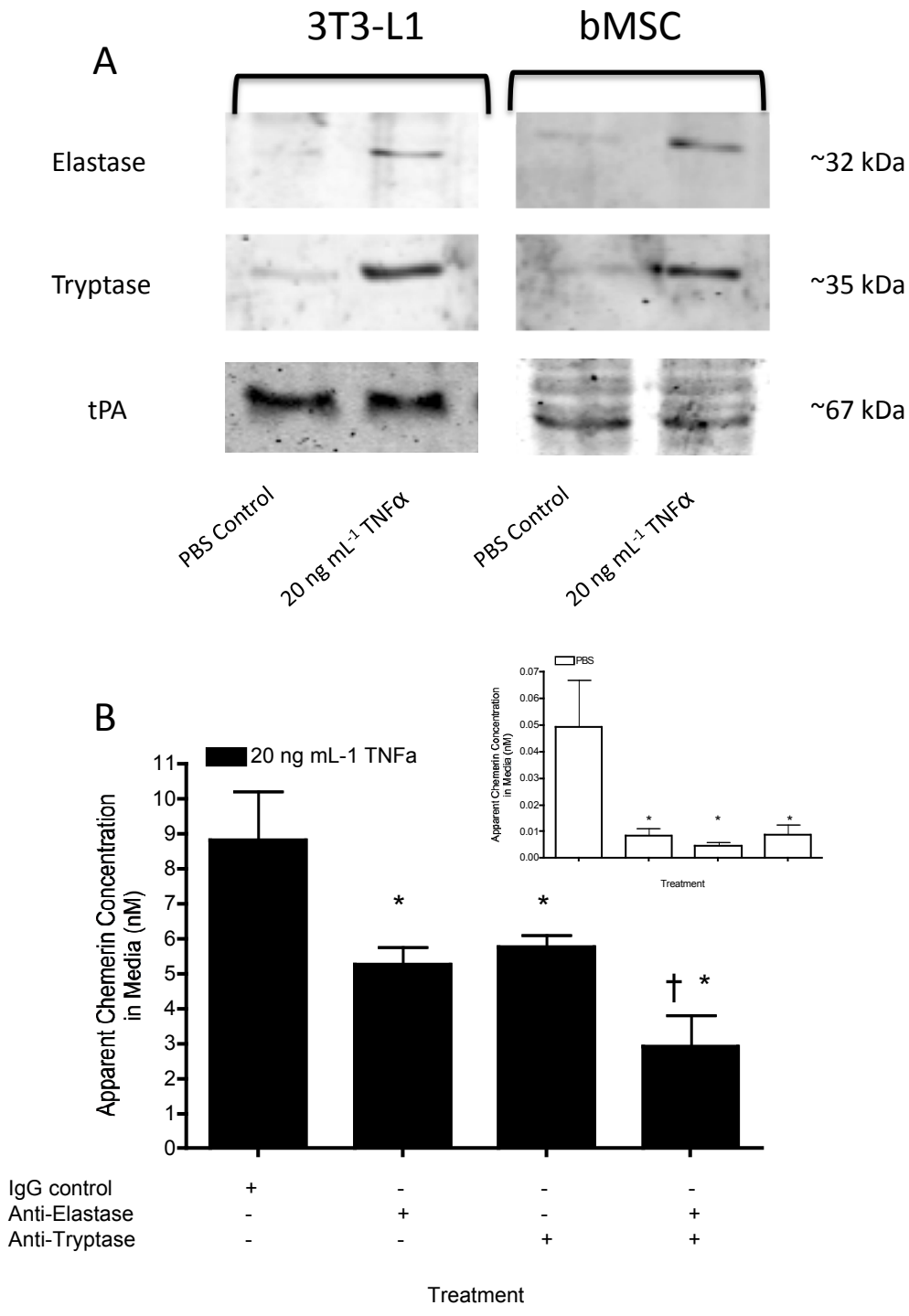
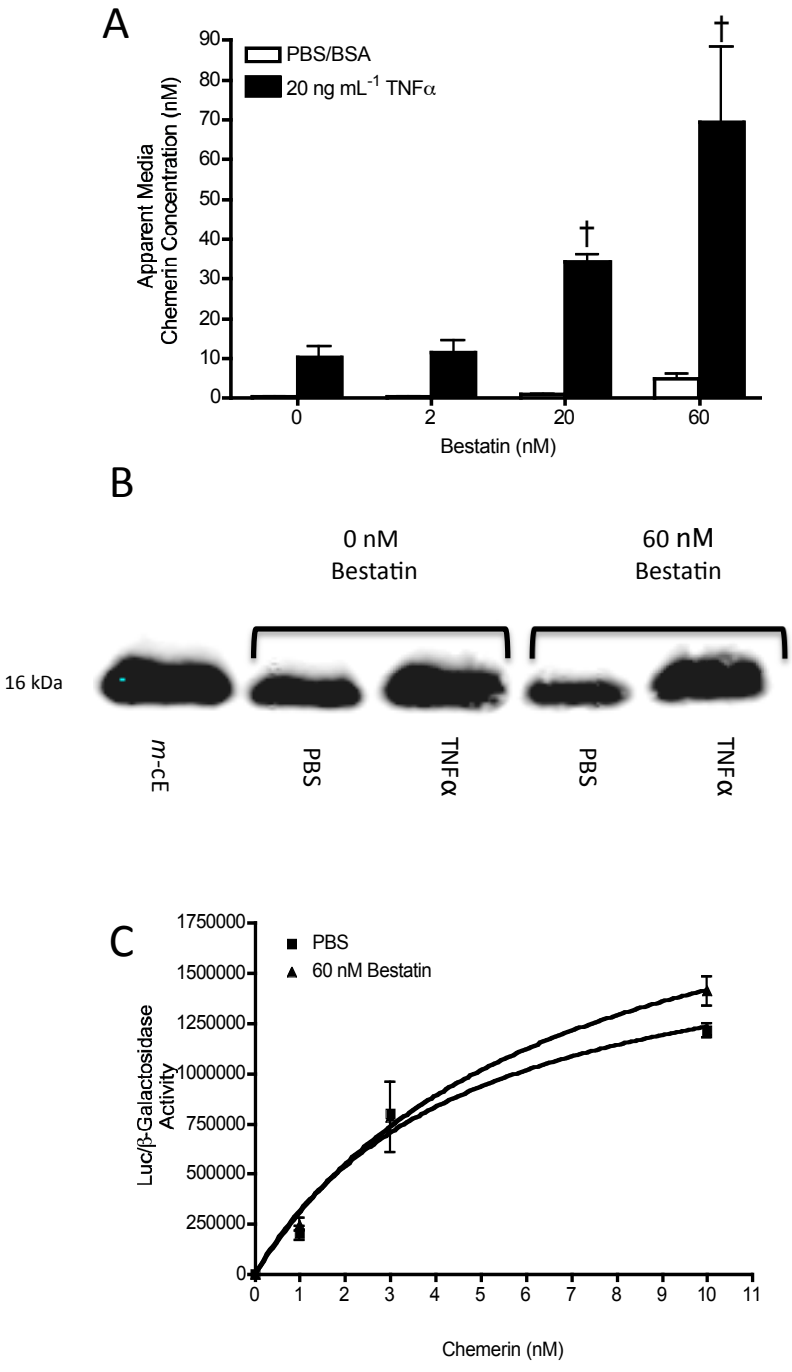

A
3T3-L1
bMSC
Elastase
~32 kDa
Tryptase
~35 kDa
tPA
~67 kDa
PBS Control
20 ng mL-1 TNFα
PBS Control
20 ng mL-1 TNFα
B
20 ng mL-1 TNFa
Apparent Chemerin Concentration in Media (nM)
PBS
Treatment
IgG control
Anti-Elastase
Anti-Tryptase
Treatment

Figure 20: **Bestatin heightens the apparent adipocyte media concentration of *m*-chemerin.** *m*-CMKLR1 "Tango" bioassay (**A, C)** and western blot (**B**) analysis were used to identify the effect of bestatin, an inhibitor of aminopeptidases, alone and in combination with TNFα or a vehicle control on the apparent (**A**) and total (**B**) media *m*-chemerin concentration of 3T3-L1 adipocytes. Non-specific effects of bestatin on the *m*-CMKLR1 "Tango" bioassay were investigated by analyzing the luciferase/β-galactosidase activity of recombinant *m*-chemerin standards combined with 60 nM bestatin or its respective control (**C**). All bars represent the mean ± s.e.m. of 3 samples and are representative of at least 3 independent experiments. Western blot using an R&D anti-chemerin antibody is representative of 4 samples per group and 2 independent experiments. † $P<0.05$ compared to the TNFα control, two-way ANOVA followed by Bonferroni's *post hoc* test (**A**).

Figure 20


A
PBS/BSA
20 ng mL-1 TNFα
Apparent Media Chemerin Concentration (nM)
Bestatin (nM)
B
0 nM Bestatin
60 nM Bestatin
16 kDa
m-cE
PBS
TNFα
PBS
TNFα
C
PBS
60 nM Bestatin
Luc/β-Galactosidase Activity
Chemerin (nM)

3.05.00: DISCUSSION

Chemerin is an adipokine emerging as a critical mediator of immune function and metabolism through CMKLR1 signaling. In our previously published study we identified that adipocytes treated with TNFα increased production and secretion of a highly active chemerin product [266]. Herein we sought to expand on the adipocyte mechanisms that mediate the activation of *m*-chemerin under basal conditions or following treatment with TNFα.

In vitro, six chemerin products with unique abilities to activate *h*-CMKLR1 have been identified. These full length chemerin products are not, however, the only known ligands for CMKLR1 [188-197]. Resolvin E1, an anti-inflammatory mediator derived from omega-3 polyunsaturated fatty acids is also a potent ligand of *h*-CMKLR1 [216]. The ability of a neutralizing chemerin antibody to prevent *m*-CMKLR1 activation by conditioned-adipocyte media indicates that receptor activation in the bioassay is *m*-chemerin specific. Theories concerning chemerin bioactivity suggest in addition to C-terminal post-translational modification, the final 9- or 15-amino acids of chemerin may also represent a flexible region that could be catalytically separated from the full length protein while retaining its bioactivity [217]. The *m*-CMKLR1 "Tango" bioassay inherently cannot differentiate between these full-length and C-terminal chemerin products. The possibility that either chemerin products contribute to the aforementioned basal and TNFα-associated *m*-CMKLR1 activation cannot be discounted. It should be noted, however, that C-terminal chemerin peptides have yet to be identified as endogenous products in cellular, animal or human studies and are therefore questioned for their relevance to *in vivo* chemerin biology.

Mammalian proteases are categorized based on their substrate specificities or mechanisms of catalysis. Proteases function in diverse process including transcription, cell proliferation, differentiation, tissue morphogenesis, tissue remodeling and inflammation by regulating the degradation, localization and activity of proteins [273-275]. Accordingly protease inhibition, over-expression and irregular protease function contribute to the pathophysiology of diseases including rheumatoid arthritis and obesity [276-281]. In obesity, in which adipose tissue can make up ~70% of an individuals body weight; adipocyte production of proteases could be having a number of pathological consequence and yet little is known concerning the expression pattern of many proteases in adipocytes [46]. Our findings are the first to our knowledge to indicate that 3T3-L1 adipocytes express, in a differentiation-dependent manner, a number of immunocyte and fibrinolytic-associated enzymes, including *elastase*, *tryptase*, *uPA* and *tPA,* that modify *m*-chemerin bioactivity. Considering elevated concentrations of total *h*-chemerin are recorded in the obese population it is possible that the localized production of adipocyte-derived proteases have important functional consequences in terms of modifying chemerin bioactivity and resulting biological function. Our findings following adipocyte treatment with a general protease inhibitor cocktail expands on this concept supporting adipocytes are capable of producing proteases that differentially regulate *m*-chemerin bioactivity. In addition to its relation to chemerin biology, the aforementioned adipocyte-derived protease production may also have important functional effects on other adipokines, cytokines and receptors. For instance, proteolytic activation of protease-activated receptors (e.g. PAR-1 and PAR-2) as well as cytokines (e.g. IL-6) can mediate diverse changes in cytokine secretion, integrin activation, cell motility, inflammation, as

well as transcriptional and metabolic responses. Future studies specifying whether adipocyte-derived proteases contribute to these putative roles may elucidate the basic mechanisms involved in adipose tissue biology and the dysfunctions that occur in obesity-associated metabolic abnormalities [273, 278, 282-284].

Serine proteases are well-defined contributors to inflammation. They proteolytically modify chemokines and cytokines increasing both their respective receptor affinities and resulting inflammatory signals [273]. Our results following treatment of adipocytes with increasing concentrations of aprotinin, a serine protease inhibitor, identified for the first time that adipose-derived serine proteases contribute to TNFα-mediated conversion of *m*-chemerin into a highly active product. Despite no reported change in *elastase, tryptase, uPA* and *tPA* mRNA expression following treatment with TNFα, elevations in immunodetectable concentrations of elastase and tryptase were established within the adipocyte media. The heightened concentrations of elastase and tryptase detected in adipocyte media combined with the recapitulation of aprotinin inhibition of *m*-chemerin bioactivation following their targeted neutralization supports adipose-derived elastase and tryptase as specific mediators of the TNFα-associated *m*-chemerin bioactivity. The discrepancy between the profile of *elastase* and *tryptase* mRNA and their resulting protein suggests that adipocytes may be increasing the levels of elastase and tryptase following treatment with TNFα through translational or secretory pathways rather than through increased gene transcription. This is consistent with enzymatic regulation in other cell types, in which pre-formed proteolytic enzymes are rapidly released as a result of cellular activation rather than through increased gene expression [285]. Treating adipocytes with aprotinin and elastase or tryptase antibodies

could not completely inhibit basal or TNFα-associated chemerin bioactivity. Thus, serine proteases are the major but cannot be the exclusive regulators of *m*-chemerin activation. The inhibition of *m*-chemerin activity with treatment of E-64 when co-administered with TNFα suggests that cysteine proteases mediate a minor component of chemerin activation. The concentrations of E-64 used to inhibit chemerin bioactivity were, however, 10-fold higher then those needed to inhibit protease function. A non-specific affect of E-64 can therefore not be ruled out.

Aminopeptidases are proteolytic enzymes distributed ubiquitously throughout tissue and body fluids that are involved in protein inactivation via catalytic cleavage of N-terminal amino acids of designated proteins including IL-8 and enkaphalin [286-289]. Consistent with this role, inhibition of adipocyte aminopeptidases with bestatin significantly increased the TNFα-associated increase in apparent active *m*-chemerin concentration in adipocyte media without altering the total immunodetectable *m*-chemerin concentration. Studies investigating chemerin have exclusively focused on the C-terminus as the predominant foci of activity. Our results are the first to suggest that the N-terminus may also play critical role in moderating chemerin inactivation. However, an indirect effect of aminopeptidases on *m*-chemerin activity cannot be ruled out. Further studies to identify the aminopeptidases and the manner in which they regulate *m*-chemerin activity are therefore needed.

A number of clinical publications provide compelling evidence to suggest elevated chemerin concentrations are playing a role in obesity. Their dependence, however, on the ELISA assay is a limitation for interpretation of the results. Currently available ELISAs provide a measurement of circulating total *h*-chemerin with no

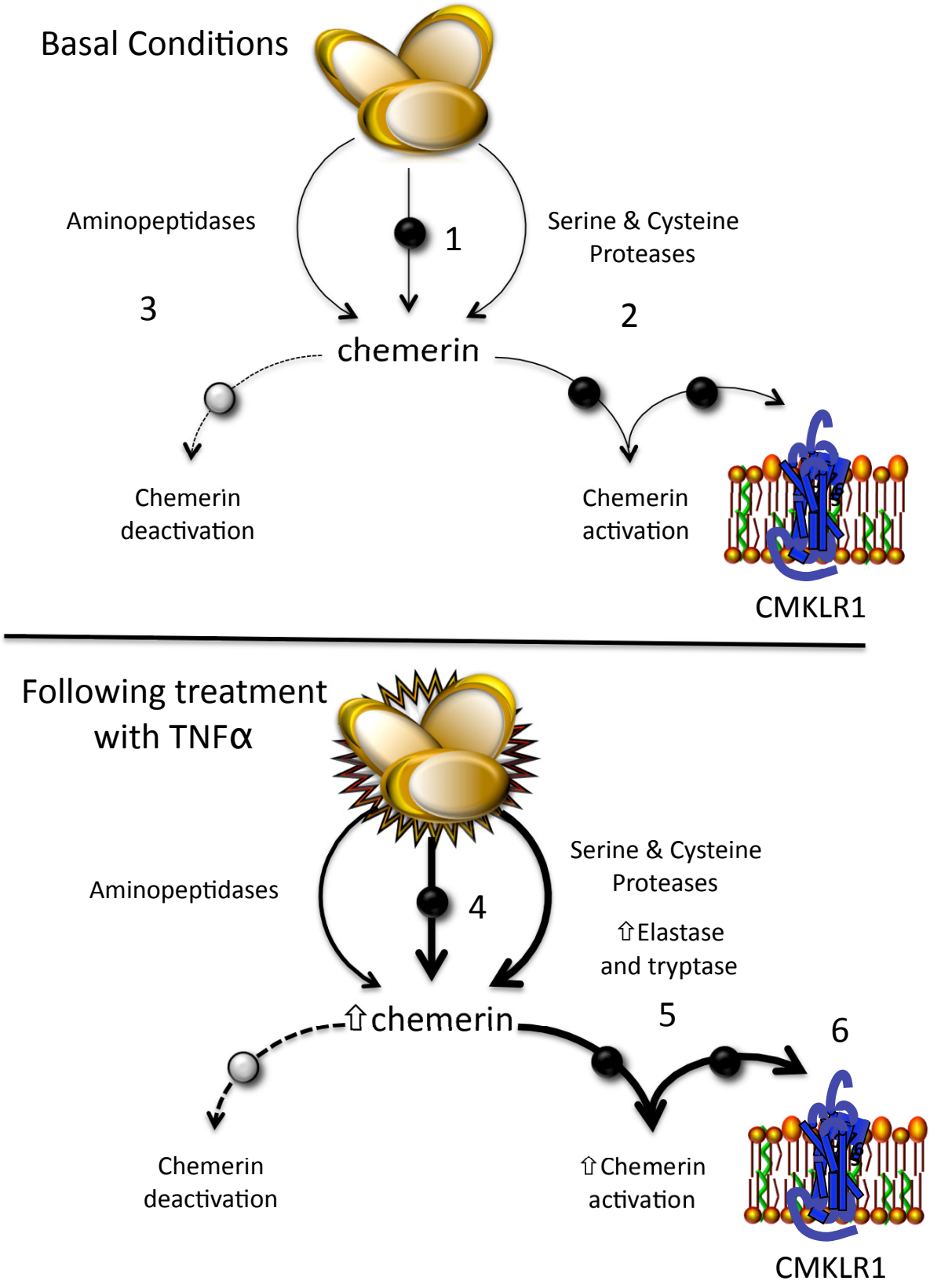
discernable method of differentiating between multiple active chemerin products. Rather clinical studies base their conclusions on an assumption of parity between the concentration of total circulating *h*-chemerin and its activity at *h*-CMKLR1. The findings herein provide documented evidence to suggest the inaccuracy of active to total chemerin parity. TNFα alone produces a 10-fold increase in the apparent bioactive concentrations of *m*-chemerin with only a 2-fold increase in total *m*-chemerin while the addition of aprotinin and E-64 decreased whereas bestatin increased the TNF-mediated change in the apparent *m*-chemerin bioactivity without further altering the total *m*-chemerin concentrations . Current studies based on total protein measurement alone may consequently be under or over-estimating chemerin bioactivity. What remains to be undertaken in future studies is to address the mechanisms mediating the disparity between the total chemerin concentration and its associated bioactivity. Possible explanations include the inability of western blots to resolve single C-terminal or N-terminal amino acid differences that are associated with more and less active *m*-chemerin products. Second, in addition to chemerin primary structure, C- and N- terminal amino acid cleavage may also modify its secondary, tertiary and quaternary structures. These structures can largely change the biological activity of a protein and would, like amino-acid differences, not be picked up by the denaturing conditions of the western blot [290]. A third possibility that cannot be discounted is that with the aforementioned proteases modification of *m*-chemerin bioactivity results from indirect pathways. In this case one could envision proteolytic modification of a secondary protein, which in-turn may enhance or inhibit *m*-chemerin bioactivity via modification of chemerin binding to the secondary protein. In this way chemerin activity at CMLKR1 could either be stabilized

allowing for enhanced signaling effects or inhibited from signaling. Although these processes are important they are well beyond the scope of our current study but will remain vital for understanding chemerin biology.

In summary, we provide persuasive evidence that adipocytes produce serine and cysteine proteases that activate and aminopeptidases that inactivate *m*-chemerin bioactivity. Conservation of these mechanisms in two complementary adipocyte models supports the importance of these adipocyte-derived proteases in controlling chemerin bioactivity. Treating adipocytes with TNFα heightens the affects of both serine and aminopeptidases activation and degradation *m*-chemerin bioactivity, respectively. However, the increased concentration of elastase and tryptase governs an imbalance in these opposing processes resulting in a net greater amount of highly active *m*-chemerin (**Figure 21**). Accordingly, future studies aimed at delineating the profile and regulation of adipocyte-derived proteases, their ability to modify chemerin and the resulting identity and structure of chemerin products will provide needed insight concerning the regulation of chemerin activity and the role elevated total chemerin is playing in obesity.

Figure 21: Working model of adipocyte-derived proteolytic control of *m*-chemerin bioactivity under basal conditions and following treatment with TNFα. Our findings together support a model of adipocyte-derived proteolytic control of *m*-chemerin bioactivity. Under basal conditions the bioactivity of adipocyte-secreted *m*-chemerin (1) at *m*-CMKLR1 is determined by a precise balance between activation by serine and cysteine protease (2) and deactivation by aminopeptidases (3). Following treatment with TNFα, elevated secretion of *m*-chemerin (4) and production of elastase and tryptase (5) alter this balance resulting in a heightened concentration of highly active *m*-chemerin products (6).

Figure 21


Basal Conditions
Aminopeptidases
1
Serine & Cysteine Proteases
3
2
chemerin
Chemerin deactivation
Chemerin activation
CMKLR1
Following treatment with TNFα
Aminopeptidases
4
Serine & Cysteine Proteases
⇧Elastase and tryptase
5
6
⇧chemerin
Chemerin deactivation
⇧Chemerin activation
CMKLR1

CHAPTER 4.00.00: DISCUSSION

To a large extent our comprehension of adipose tissue biology and its contribution to human health has coincided with the escalating proportions of individuals diagnosed as obese in the past 2 decades. Although a great deal of progress has been made in understanding the pathological mechanisms contributing to obesity, the complexity of the disease has limited the efficacy of pharmacological interventions. Continued research on the biology of adipose tissue and pathophysiology of obesity therefore remains our greatest opportunity for better understanding the disease and identifying novel therapeutic targets to treat it.

The diverse metabolic process mediated by adipokines supports adipose tissue as an important endocrine organ. Consequently, the precise balance between excess and insufficient amounts of adipose tissue is crucial as either extreme, exemplified by obesity and lipodystrophy, contributes to deleterious health conditions [58, 184, 291]. In the context of obesity, cellular, animal and human studies have implicated altered adipokine secretion as a causative mechanism in the development of obesity-associated metabolic abnormalities. Abnormalities including weight gain, immunocyte infiltration into adipose tissue, inflammation and insulin resistance have all been attributable to elevated signaling by adipokines including leptin, MCP1, TNFα and diminished signaling by adiponectin. *h*-Chemerin is likewise implicated in the pathogenesis of obesity based on recent findings identifying serum total *h*-chemerin is heightened in patients with a BMI >30 kg m^2 and is positively correlated with a number of markers of body fat, visceral adipose tissue deposition, metabolic syndrome and inflammation including BMI, serum triglycerides, blood pressure, TNFα and IL-6 [231, 233-236, 240].

In spite of these findings, the observational and correlative nature of human chemerin studies combined with their measure of total *h*-chemerin alone, and not active chemerin, preclude the determination of 1) the tissues contributing to increased circulating chemerin; 2) the mechanisms that contribute to elevated chemerin levels; 3) the proportion of circulating chemerin that is active versus inactive; 4) the time of onset of elevated chemerin; and 5) the effect of elevated chemerin on metabolism and inflammation. Our use of *in vitro* murine adipocytes and *in vivo* murine models to study chemerin biology has permitted us to surpass the restrictions attributable to human clinical studies by allowing the monitoring of both active and inactive chemerin under controlled conditions in a species that has highly homologous chemerin processing, CMKLR1 binding and activation when compared to humans. As a result, our findings when taken into consideration with other published works, provide more direct information concerning contributing tissues, mechanisms, onset and the biological function of elevated circulating chemerin. For the remainder of this chapter these aforementioned processes will be discussed in the context of human obesity.

4.01.00: SOURCE AND MECHANISM OF ELEVATED CIRCULATING CHEMERIN.

Adipose tissue and the liver are unique as their relative *chemerin* expression is 80-90% higher when compared to other cells, tissues and organs [187]. Hepatocytes and adipocytes are therefore presumed to be the dominant-contributing partners to circulating chemerin *in vivo*. A complex interplay of adipose tissue inflammation, low-grade systemic inflammation and in some cases hepatic inflammation are contributing

metabolic abnormalities associated with human obesity [292, 293]. Consequently, an initial goal of our studies was to investigate adipocytes and hepatocytes as modifiable sources of active chemerin in response to TNFα, a component of obesity-associated inflammation. Although hepatocytes and adipocytes continuously secrete bioactive *m*-chemerin, following treatment with TNFα, our findings identified adipocytes alone increased bioactive *m*-chemerin production. IL-1β and insulin similarly increase adipocyte-derived and circulating total *m*- and *h*-chemerin [224, 229]. In obesity, adipose tissue remodeling is characterized by late stage infiltration by activated macrophages and sequential elevated production of TNFα, IL-1β and circulating insulin [112, 121, 124-126, 294]. The combination of late stage adipocyte remodeling and increased adipocyte, rather than hepatocyte, production of active *h*-chemerin could then be a conceivable causative factor augmenting circulating total *h*-chemerin in the obese state.

Elevated circulating levels of serum total *h*-chemerin have, however, been identified in patients with an inflammatory non-alcoholic fatty liver disease. These findings support hepatic inflammation as an additional governing mechanism for elevations in circulating *h*-chemerin [230, 295]. It cannot be discounted from our studies that a similar mechanism is not in place in obesity. On the other hand it is just as plausible that liver inflammation may be indirectly mediating distal adipose tissue production of *h*-chemerin. To clarify these possibilities a thorough investigation into hepatic regulation of chemerin is needed.

4.02.00: ONSET OF CHEMERIN ELEVATION.

Studies investigating elevated total circulating *h*-chemerin in human obesity, T2DM and metabolic syndrome have largely focused at later stages of disease progression. The existence of metabolic abnormalities including elevations in fasting glucose, insulin, cholesterol, triglycerides and BMI, places study patients largely into stages 1 or above of the EOSS [235] [222, 231, 234, 237]. By focusing on total *h*-chemerin concentrations only in later stages of disease progression, clinical studies are restricted from identifying the relative onset of elevated chemerin concentrations. Chronicling the relative emergence of elevated chemerin in terms of obesity disease progression or adipose tissue remodeling is fundamental for accurately characterizing the contribution of chemerin to the pathophysiology of obesity. The results from our and others' findings support adipose tissue inflammation and hyperinsulinemia, measures of late stage obesity pathology, as causative mechanisms regulating chemerin production. Accordingly, this theory supports the onset of elevated circulating chemerin as succeeding rather then preceding the initial developmental stages of adipose tissue remodeling. A putative role for elevations in chemerin would, as a consequence, be in the later pathological stages of obesity.

Neutrophils and adipocytes, both components of early stages of tissue remodeling, do, however, secrete proteases capable of modifying *m*-chemerin bioactivity [186]. Considering the variant of chemerin present at CMKLR1 is also a fundamental component to its biological activity, it cannot be disregarded that an early modification of adipose tissue bioactivation of chemerin by neutrophil- and adipocyte-associated enzymes may also contribute to early adipose tissue remodeling. This alteration in

activity would not directly impact total circulating concentrations yet could nevertheless have considerable affects on early adipose tissue function.

4.03.00: VARIANTS OF CHEMERIN AND THEIR CORRESPONDING BIOLOGICAL FUNCTIONS.

Current literature supports distinctive variants of chemerin in three physiological roles. These roles include a pro-inflammatory role increasing $CMKLR1^+$ immunocyte migration, an anti-inflammatory role blocking immunocyte migration and inflammatory mediator production as well as a metabolic role in adipogenesis, glucose homeostasis and lipolysis [187, 192, 197, 206, 217, 218, 221]. Studies suggest that although each of these processes is mediated through CMKLR1, the role of chemerin is dependent upon the particular chemerin product formed and therefore the proteolytic mechanisms through which they are formed. Zabel *et al*. reported pro-inflammatory *h*-chemerin products are dependent on activation by serine proteases [189]. In contrast, anti-inflammatory *m*-chemerin products are described as being dependent on cysteine protease activation [217]. Our current studies identified that the bioactivity of adipocyte-derive *m*-chemerin products under basal conditions are predominantly dependent on the serine proteases tryptase and elastase for activation. These findings would support adipocyte-derived *m*-chemerin products as pro-inflammatory in nature. In line with this concept Goralski *et al.* have previously published that adipocyte-derived *m*-chemerin products increase the migration of *m*-$CMKLR1^+$ pre-B lymphocytes *in vitro* [187]. These same chemerin products were also necessary for adipogenesis, suggesting serine-associated chemerin products may regulate both pro-inflammatory and metabolic processes. Following

treatment with TNFα, a heightened concentration of elastase and tryptase convert *m*-chemerin into a highly active form. Considering TNFα-associated chemerin bioactivity has a similar reliance on serine proteases as basal adipocyte-derived chemerin, their reported differences in CMKLR1 activation could then reflect differences only in the magnitude and not the nature of the biological effect. In accordance with this concept our unpublished data identified conditioned media from 3T3-L1 adipocytes treated with TNFα enhance the migration of $CMKLR1^{+}$ pre-B lymphocyte versus adipocyte media alone. In terms of obesity, these results suggest that elevated production of highly active adipocyte-derived chemerin may be contributing to obesity-associated metabolic abnormalities by increasing immunocyte recruitment and metabolic effects including enhanced adipogenesis.

4.04.00: **OBESITY, ELEVATED CHEMERIN, AND EXACERBATION OF OBESITY-ASSOCIATED COMORBIDITIES.**

Obesity is a known modifiable risk factor for the development of inflammatory comorbidities including Crohn's disease, arthritis, psoriasis, lupus erythematosus and kidney disease [296-301]. Like obesity, observational studies have also inferred a role for heightened circulating *h*-chemerin to the pathophysiology of these aforementioned diseases [119, 202, 211, 302]. The findings from our current studies supports adipocyte derived chemerin as pro-inflammatory. A possibility worth exploring in future studies is to determine if elevated circulating concentrations of *h*-chemerin present in obesity contribute to the higher incidences of arthritis, lupus erythematosus, or kidney disease by governing, or magnifying, the underlying inflammatory component of these disease

states. Histological analysis of skin lesions and kidneys of patients diagnosed with inflammatory lupus erythematosus, lupus nepheritis and oral lichen planus have identified localization of *h*-CMLR1^{+} natural killer and dendritic cells to damaged tissues [202, 209, 211]. Whether or not the infiltration of inflamed tissues by CMKLR1^{+} immunocytes is enhanced in an individual who is also obese is not yet explored. Studies in adults with inflammatory bowel disease do, however, support that concomitant obesity increases long-term morbidity and mortality increasing disease activity, frequency of perianal complications and hospitalization [300, 303]. Similarly, patients with psoriasis, an inflammatory skin disease characterized by elevated CMKLR1^{+} immunocyte infiltration, have poorer long-term prognosis and greater extent of inflammatory skin lesions with increasing BMI [214, 304, 305].

4.05.00: RECOMMENDATIONS FOR FUTURE STUDIES.

Upon consideration of the totality of our presented findings, what requires final highlighting are the important implications our findings have on current and future research practices pertaining to chemerin biology. Under current standard practices ELISA and/or western blot are used to quantify the concentration of total chemerin in biological samples. Following quantification, the concentration of chemerin is correlated to markers of disease pathology inferring a role for chemerin based on these relationships. My findings identify a number of limitations with that type of experimental study design.

Limitation 1: Timing of blood collection

Current reported methodologies provide no standard timing for blood collection. Analysis of total serum chemerin in wild type and two obese mouse models indicate chemerin follows a rhythmic alteration throughout the day and night. Depending on when blood was drawn significant differences in concentrations could be erroneously reported.

Recommendation 1:

My recommendation is to determine whether chemerin follows a similar rhythmic alteration throughout the day and night in humans, following which precise timing for harvesting biological samples should be documented and followed.

Limitation 2: Assumption of parity between total and bioactive chemerin

Following treatment with TNFα, adipocyte-derived chemerin had a 5-10-fold disparity between its apparent total and bioactive concentrations. The total concentration and activity could also be drastically altered using protease inhibitors without equivalent changes to the total protein concentrations. Taking into consideration that all of the diseases explored in the context of chemerin biology have TNFα-associated pathologies, and may also be on medications that modify proteolytic function, similar disparities could exist in humans. By relying on a measure of total chemerin alone, current clinical studies are incapable of differentiating between chemerin products and are therefore potentially under or over estimating the signaling capability and function of chemerin *in vivo*.

Recommendation 2:

My recommendation is to provide a measurement of total chemerin, chemerin bioactivity and the ratio of chemerin products within all biological fluids. In this manner chemerin will be most accurately characterized and a role can therefore be better defined.

Limitation 3: Assumption of equivalent chemerin products in circulation and in individual organs.

Under current working methodologies systemic samples are taken and are assumed to provide an accurate representation of the chemerin products found in all organ systems. Adipocytes and hepatocytes, however, each have a unique disparity between their produced concentration of bioactive and total *m*-chemerin. These findings support independent cellular control of chemerin secretion and proteolytic activation. In terms of disease, localized cellular and tissue proteolytic processing may control regional chemerin activation, inactivation or degradation resulting in distinct chemerin roles at individual tissues and organs.

Recommendation 3:

To avoid the assumption that the chemerin products in circulation are identical to those that signal at individual organs it is my recommendation that chemerin be analyzed via the methods presented above both in circulation and across individual tissue beds. Although this may not be readily feasible in humans, using animal models including mice to explore chemerin products and signaling in the context of each organ systems would provide a comparable framework for understanding chemerin biology in humans.

4.06.00: FINAL SUMMARY

Multiple chemerin products, receptors and biological functions all contribute to the complexity of chemerin biology. This complexity has, however, been largely neglected by current studies resulting from either the chosen focus of their study or the inherent limitations of their experimental design. Our studies presented herein used multiple experimental methodologies *in vitro* and *in vivo* in attempts to avoid some of these previous limitations. Accordingly, our findings identified TNFα as a governing mechanism in the production and proteolytic activation of adipocyte-derived and circulating bioactive *m*-chemerin. In the context of human obesity, our studies suggest adipose tissue inflammation, which is characterized by elevations in TNFα, may in the same manner contribute to the documented heightened concentrations of *h*-chemerin. Like the studies before them, our studies are by no means complete. If, however, future studies are completed to fill the gaps that I have outlined throughout this thesis using the methodological recommendations supported by our findings, it is my belief we will at last have the needed insight to identify the role elevated chemerin is playing in obesity. Only once this information is known should we be confident enough to implicate elevated chemerin in obesity disease pathology and consider it as a therapeutic target.

APPENDIX I: COPYRIGHT APPROVAL

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