ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
UNIVERSITY OF CRETE

Τ.Θ. 1393, 71409 Ηράκλειο, Κρήτη

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ
FACULTY OF MEDICINE

P.O Box 1393, Heraklion, Crete, Greece

Μεταπτυχιακό Πρόγραμμα Σπουδών στην
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Τ.Θ. 2208, 71003 Ηράκλειο, Κρήτη

Graduate Program in
"**The Molecular Basis of Human Disease**"
P.O.Box 2208, 71003 Heraklion-Crete, Creece

"Effects of Substance P on preadipocyte and adipocyte expression"

by

Aristea Sideri

Research Fellow in Dr Charalabos Pothoulakis' Laboratory
Division of Digestive Diseases, IBD Center,
Department of Medicine,
University of California Los Angeles

Υπότροφος του Ιδρύματος: «Αλέξανδρος Ωνάσης»

Ευχαριστίες

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Ειλικρινά,
Αριστέα Σιδερή,
Λος Άντζελες, Καλιφόρνια
Οκτώβριος 2011

Contents:

Α. Περίληψη

Στο παρελθόν ο λιπώδης ιστός εθεωρείτο απλώς ή αποθήκη ενέργειας του οργανισμού: ένας αδρανής ιστός, ικανός απλά να αποθηκεύει και να απελευθερώνει λιπίδια ανάλογα με τις μεταβολικές ανάγκες του οργανισμού. Μέσα στην τελευταία δεκαετία, η επιστημονική κοινότητα έχει αλλάξει ριζικά την άποψή της επί του θέματος. Έχει περίτρανα πλέον αποδειχθεί ότι ο λιπώδης ιστός είναι ένα ιδιαίτερα ενεργό μεταβολικό και ενδοκρινικό όργανο, παράγοντας και απελευθερώνοντας πλήθος ορμονών και κυτταροκινών, τις αδιποκίνες. Οι αδιποκίνες φαίνεται να παίζουν σημαντικό ρόλο στη ρύθμιση της όρεξης (δρώντας στο Κεντρικό Νευρικό Σύστημα), στη ρύθμιση φλεγμονωδών καταστάσεων (καθώς πολλές από τις αδιποκίνες είναι στην ουσία σημαντικά προφλεγμονώδη ή και αντιφλεγμονώδη μόρια), στη ρύθμιση του μεταβολισμού της γλυκόζης.

Οι Ιδιοπαθείς Φλεγμονώδεις Παθήσεις του Εντέρου (ΙΦΕΝ) είναι ένας γενικός όρος παθήσεων με ευρύ φάσμα κλινικών συμπτωμάτων του γαστρεντερικού συστήματος. Πρόκειται για την Ελκώδη Κολίτιδα και τη Νόσο του Crohn’s, δύο διαφορετικές κλινικές οντότητες με κοινά χαρακτηριστικά την έντονη φλεγμονή του γαστρεντερικού συστήματος, κάποια μοριακά χαρακτηριστικά, αλλά και αρκετές διαφορές σε όλα τα επίπεδα. Ένα χαρακτηριστικό της Νόσου του Crohn’s, που κάτι αντίστοιχο δεν έχει παρατηρηθεί στην Ελκώδη Κολίτιδα, είναι η συσσώρευση μεσεντερικού λίπους περιβάλλον το φλεγμαίνον τμήμα του εντέρου (έρπον λίπος). Εδώ και δεκαετίες, οι χειρουργοί χρησιμοποιούσαν αυτό το λίπος ως σημάδι του ορίου της φλεγμονής κατά τις επεμβάσεις. Σε σχέση με την Ελκώδη Κολίτιδα, το μεσεντερικό λίπος δεν έχει μελετηθεί ιδιαίτερα.

Το νευροπεπτίδιο Ουσία Π, το οποίο απαντάται κυρίως στο Κεντρικό και Περιφερικό Νευρικό Σύστημα, στον Εντερικό Νευρικό Σύστημα και στο Ανοσοποιητικό Σύστημα, έχει φανεί να αυξάνεται στον ορό αλλά και στο εντερικό επιθήλιο ασθενών αλλά και ζωικών μοντέλων των ΙΦΕΝ, και ανάλογα με το στάδιο της νόσου να έχει άλλοτε επιβαρυντικές και άλλοτε προστατευτικές και επουλωτικές

δράσεις. Επίσης έχει φανεί ότι νευρικές απολήξεις που απελευθερώνουν Ουσία Π όταν παρεμποδιστούν έχουν ευεργετικά αποτελέσματα στα μοντέλα των ΙΦΕΝ.

Παρά την ύπαρξη αυτών των ευρημάτων και πέρα από την ύπαρξη του υποδοχέα της Ουσίας Π, και της δράσης αυτής σε πρόδρομες μορφές λιποκυττάρων από παχύσαρκους ασθενείς, οι επιδράσεις (αλλά και τα μοριακά μονοπάτια που κρύβονται πίσω από αυτές) της Ουσίας Π στο λιπώδη ιστό από υγιείς και ασθενείς με ΙΦΕΝ, δεν έχουν μελετηθεί. Στόχος της παρούσης εργασίας είναι η μελέτη αυτών των δράσεων.

Χρησιμοποιώντας την κυτταρική σειρά 3T3-L1, μεσεντερικά πρόδρομα λιποκύτταρα από αρουραίους και μεσεντερικά λιποκύτταρα από δείγματα ασθενών με ΙΦΕΝ και υγιών ατόμων, εξετάστηκαν οι δράσεις της Ουσίας Π σε μεταγραφικό επίπεδο σχετικά με τους υποδοχείς της (κυρίως τον NK1R) και διάφορες κυτταροκίνες και σε πρωτεϊνικό επίπεδο σχετικά με μόρια προσκόλλησης και μεταγραφικούς παράγοντες που συμμετέχουν στη διαφοροποίηση. Επίσης, δημιουργήθηκε ένα πρωτοπόρο σύστημα με σκοπό δημιουργία επαγόμενων ιστο-ειδικά διαγονιδιακών ζωικών μοντέλων, το οποίο δοκιμάστηκε επιτυχώς.

Τα αποτελέσματα που απορρέουν από τη μελέτη των 3T3-L1 τα είναι αμφιλεγόμενα, και θεωρήθηκε ότι τα κύτταρα που χρησιμοποιήθηκαν ήταν ιδιαίτερα ασταθή. Από τα πρόδρομα λιποκύτταρα των αρουραίων, φαίνεται ο υποδοχέας NK1R να μην είναι ανιχνεύσιμος, αλλά τα κύτταρα να ανταποκρίνονται με έκφραση κυτταροκινών στη χορήγηση Ουσίας Π. Τα αποτελέσματα από τα ανθρώπινα δείγματα τείνουν να υποστηρίξουν την ύπαρξη διαφορετικών επίπεδων έκφρασης του υποδοχέα αλλά και της απάντησης των κυττάρων ανάμεσα σε πρόδρομα και ώριμα λιποκύτταρα ασθενών και υγιών, ακόμα και ανάμεσα στα στάδια των ασθενειών. Τόσο όσον αφορά στα πρόδρομα λιποκύτταρα από αρουραίους, όσο και στα ανθρώπινης προέλευσης, περισσότερα δείγματα είναι απαραίτητα για ασφαλή συμπεράρματα. Η επιτυχημένη δοκιμή του νέου ιστο-ειδικού λεντι-ιού μας

φέρνει ένα βήμα κοντύτερα στην πραγματοποίηση του υπό σχεδιασμώ επαγόμενου λιπο-ειδικού διαγονιδιακού ζώου που θα είναι χρήσιμο εργαλείο για τις περαιτέρω μελέτες της δράσης του λιπώδους ιστού.

B.Summary

In the past, adipose tissue was considered just the energy storage of the organism: a totally inactive tissue capable only to store and release lipids according to metabolic body needs. Within the past decade though, our knowledge on the functions of the adipose tissue has completely changed the way the scientific community approaches the subject. It has been proven without doubt that the adipose tissue is a particularly metabolic and endocrine organ, able to produce and release to the circulation numerous hormones and cytokines, that are collectively called adipokines. Adipokines seem to play a major role in controlling appetite (through their actions on the Central Nervous System), in modulating inflammatory responses (since most of them can act as pro-inflammatory or even anti-inflammatory molecules), even on glucose metabolism.

Inflammatory Bowel Diseases (IBDs) is a general term that includes diseases with a wide spectrum of symptoms of the Gastrointestinal (GI) Tract. Namely, it includes Ulcerative Colitis and Crohn's Disease, two separate clinical entities sharing the intense inflammation of the GI Tract, some molecular characteristics, but numerous differences in multiple levels as well. One interesting characteristic of Crohn's Disease, without any similar observations in Ulcerative Colitis, is the accumulation of mesenteric fat wrapping the inflamed parts of the intestine, the so called "creeping" fat. This "creeping" fat has been the sign of the limits of the inflammation used for decades by surgeons that had to operate on Crohn's patients. When it comes to Ulcerative Colitis, similar observations of the fat or research on it has not been performed.

The neuropeptide Substance P (SP), which is mostly found in the Central and Peripheral Nervous System, the Enteric Nervous System and the Immune System, has been found to be increased in the blood serum of patients and animal models of IBD, as well as the enteric epithelium at different stages, and has been suspected to have both aggravating and ameliorating effects. It has

also been shown that the nerve endings that release SP seem to be increased in the fat tissue of animal models of IBD.

However, in spite of those data, the effects (and most importantly the mechanisms behind the possible effects) of SP on healthy and IBD patients' adipose tissue has not been dissected. Only the existence of SP's receptor and the effect of SP on obese patients' preadipocytes has been examined in the past. The purpose of the present study is to unravel the previously mentioned possible effects.

Using the cell line 3T3-L1, mesenteric pre-adipocytes from rats, as well as human mesenteric preadipocytes and differentiated adipocytes from human healthy subjects and patients with IBD, the effects of SP treatment on transcriptional (mostly concerning NK1R, the rest of the receptors, adhesion molecules and cytokines) and protein (concerning mostly transcription factors that play role in adipocyte differentiation and adhesion molecules) levels were examined. Additionally, a pioneering system aiming to lead to the creation of one inducible and truly fat-specific animal model was successfully tested.

The results coming from the study of 3T3-L1s are conflicting and the assumption was made that the specific batch used was extremely unsteady for some reason. The results from rat mesenteric preadipocytes suggest response of those cells to SP stimulation, which, however, may not be mediated through SP-NK1R interaction. Human mesenteric preadipocytes´ results tend to support differences in the expression levels of the NK1R, as well as differences amongst the responses to SP between control and IBD samples, undifferentiated and differentiated cells and the stages of the disease. Concerning the rat mesenteric preadipocytes and the human cells, a larger amount of samples will be needed to draw safe conclusions. The successful trial of the new fat-specific lenti-virus brings us a step closer to achieving the creation of the first truly inducible fat-specific animal model which will be a useful tool for the following studies.

C. Introduction

i. Substance P (SP) and Neurokinin Receptors

Substance P is a neuropeptide that was first discovered in 1970 by Chang and Leeman (1). Along with neurokinin A, neurokinin B, neuropeptide K and neuropeptide gamma, it is the most notable member of the tachykinin family of neuropeptides. Tachykinins in humans derivative from two benes namely, *TAC1* (where SP is transcribed from) and *TAC3 (2)*. Recently, an additional *TAC4* gene has been discovered and several additional family peptides have been characterized (e.g. endokinins, HK1) (3-5). Tachykinins were named after their ability to cause rapid contraction to smooth muscle fibers, and they share a common hydrophobic C-terminal region, which is essential for the biological functions of those peptides, via potentiating the interaction of the tackykinins with their receptors (6). The different members of the family are all produced by hydrolysis of precursor peptides (7). All of the family genes (*TAC1, TAC3* and *TAC4*) are located on different chromosomes, numbers 7, 12 and 17, respectively (8).

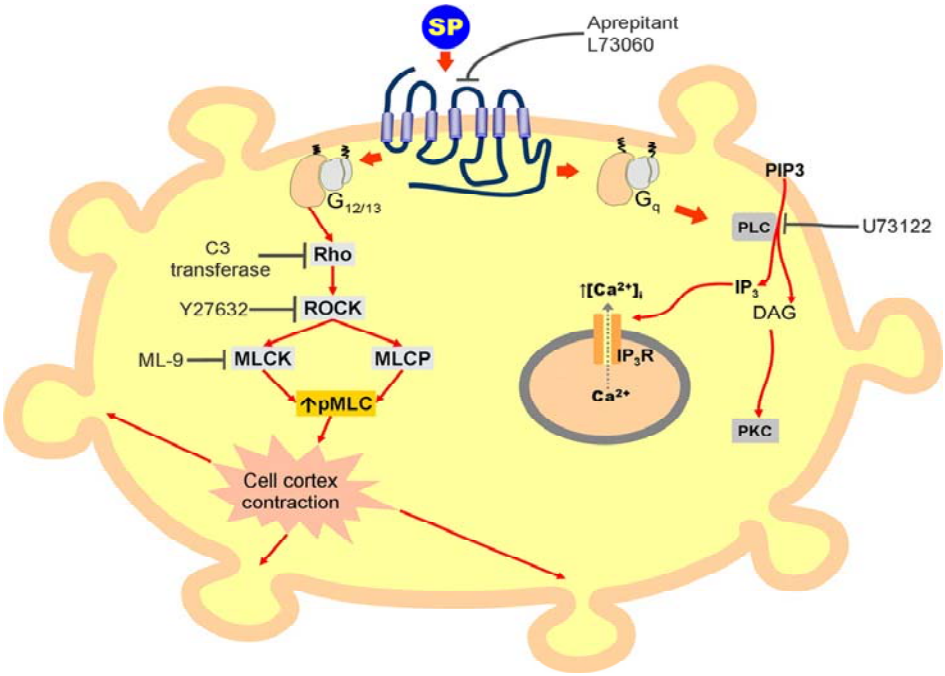
SP was first identified to be expressed in the hypothalamus; however it has been found to also be expressed in numerous other tissues, especially in the central nervous system, the gastrointestinal tract and the immune system (9). It is an 11 amino acid peptide, which as is the case for other neuropeptides, acts as a neurotransmitter and a neuromodulator in the central nervous system. In the peripheral nervous system, it is shown to be released by sensory neurons that take part in pain perception (10), and especially in inflammatory pain (11). It is expressed de novo and stored in relatively large vesicles throughout the cytoplasm of neurons. Its excretion is slow, compared to other neurotransmitters, and its function is long lasting. It exerts its effects through G-coupled receptors- the neurokinin receptors (12) (with higher affinity for NK1R).

The enteric nervous system is considered “the brain in the gut” (13), since it acts in an autonomous way controlling all of the gastrointestinal functions with minor involvement of the central nervous system. The roles of both the enteric nervous system and the immune system in Inflammaroty Bowel Diseases (IBD-namely Crohn’s Disease and Ulcerative Colitis), as well as their interactions in this group of diseases, has been demonstrated in several studies (14-16). SP is present in both systems. In the enteric nervous system, it is excreted by cells of the myenteric plexus, of the submucosal plexus, of the dorsal root ganglia and by the extrinsic/intrinsic sensory neurons (13, 17, 18). In immune cells, SP has shown to be secreted by at least monocytes, macrophages, eosinophils, lymphocytes (19-22) among other cell types.

The neurokinin receptors mentioned above, are NKR1, NKR2 and NKR3. They are derivatives of the *TACR1, TACR2* and *TACR3* genes, respectively, which in turn are located on the chromosomes 2, 10 and 4 chromosomes (8). Neurokinin receptors are G-coupled receptors that consist of 7 transmenbrane helices (23). Recently, a different truncated form of NK1R has been discovered, lacking 96 aminoacids from the C-terminal region that has been implicated in the control of the innate immunity, via the generation of more prolonged responses to SP due to lower affinity and impaired internalization (24). Figure 1 below is a schematic depiction of the intracellular signals induced following activation of the NK1R receptor in response to SP (or another tachykinin), as well as the candidate pharmaceutical interventions to block these downstream events (23).

Pharmacological inhibition of SP-NK1R signaling events became of interest due to the know involvement of these molecules in the pathophysiology of several diseases including IBD. In the central nervous system, SP-NK1R interactions have been implicated in depression (25), anxiety (26), and pain (especially inflammatory pain). SP has also been shown to be involved in several models of lung injury (27), as well as various malignances including melanoma (28) and gastric cancer (29). In

Model for the signaling pathways downstream of NK1R



Meshki, J. et al. J. Biol. Chem. 2009;284:9280-9289

Figure 1. A schematic presentation of the downstream molecular events following the binding of SP on NK1R, and the possible pharmacologic intervention points.

addition, SP signaling is shown to be active in processes involved in the defense against infectious agents (30) (NK1R is present in several immune cell types, see above), and thought to participate in pathological conditions of the urinary tract (31), even in some forms of dermatitis (32). Finally, SP has been shown to play a significant role in the pathophysiology of Inflammatory Bowel Diseases (13, 33), Irritable Bowel Syndrome (34) and recent studies have even suggested a potential role for SP-NK1R interactions in the development of obesity and insulin resistance (35-37). A SP-NK1R antagonist (Aprepitant) is extensively used in the clinic as a drug to prevent chemotherapy induced nausea (38), suggesting that SP-NK1R signaling antagonists may be candidates for future pharmacological applications.

SP has also been demonstrated to induce proliferation in immune cells (both of erythroid and myeloid origin) (39), and preadipocytes (40).

Studies using knockout mouse models of both SP and NK1R have provided extensive insight on the effects of SP-NK1R interactions and the intracellular signaling events that follow (35, 41). Mice that lack NK1R have been shown to gain less weight (at least in part due to decreased appetite) in response to high fat feeding, and demonstrate significantly improved glucose metabolism compared to their WT littermates (35). In animal models of IBD, NK1R knockout mice seem to have less severe symptoms at early stages, similar to Clostridium difficile associated colitis, while they demonstrate worse healing during the later stages of the disease as well as worse overall prognosis (41). Thus it is evident that SP may play a proinflammatory role during the early stages of colitis, but a protective one in the later stages of the disease. In addition, in experiments using NK1R antagonists during the induction of either TNBS or DSS colitis in mice, blockade of SP-NK1R signaling led to improved symptoms and less severe course of colitis in these animals (42, 43).

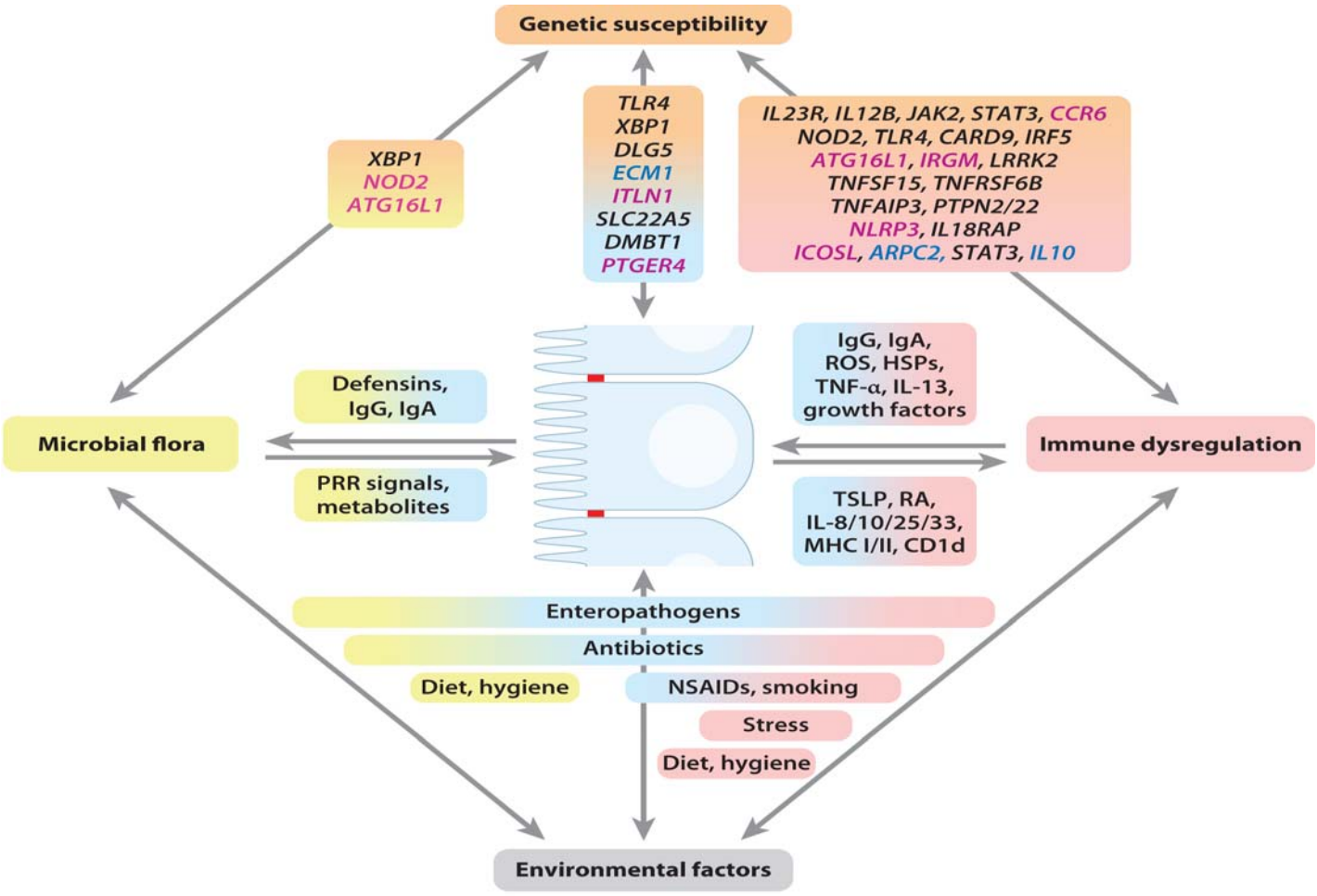
ii. Inflammatory Bowel Diseases (Crohn's Disease and Ulcerative colitis)

Inflammatory Bowel Diseases (namely Ulcerative Colitis and Crohn's Disease-UC and CD), are complex diseases of unknown aetiology that may be affected by an array of different genetic, microbial and immunologic factors. They represent two distinct diseases with chronic progressive and/or relapsing inflammation of the colonic mucosa (UC) or the entire Gastrointestinal track involving all layers of the intestinal wall (CD), with the possible concurrent involvement of other tissues or organ systems.

Genetic susceptibilities, thought to predispose to abnormal and exacerbated immune reactions to microbiota, are shown to be involved, along with various other behavioural and environmental

factors (including nutrition), in the pathogenesis of these diseases. Several genes are considered to play a role in IBD, and while familial cases represent only 5-10% of the total cases, the rest can be attributed to sporadic forms. The evidence for a genetic element in disease pathophysiology seems to be stronger for CD, rather than UC, with monozygotic twins presenting with disease concordance at 50-75% and 10-20% respectively (44). There have been many Genome Wide Association (GWAS) and meta-analysis studies that identified genes that represent possible risk factors for both IBDs, with

significant overlap but also several disease-specific cases (45, 46). While there seem to be less potent disease-predisposing genes identified some of the candidates have demonstrated strong association with both disease incidence as well as severity. For example, autophagy genes (for example ATG16L1, IRGM), NOD-like receptors, and interlectins (ITLN1) are found to be linked specifically to the CD (47) pathophysiology and IL-10 receptor gene variants have been found to be directly linked to an early-onset familial form of CD that presents as a monogenic disease (48). On



Kaser A, et al. 2010.
Annu. Rev. Immunol. 28:573–621

Figure 2. A schematic graph presenting the known interactions between genetic, microbiologic, immune factors and probably environmental factors involved in IBD. In red, CD-specific genes or pathways are shown, in blue UC specific and in black are shown that considered to be involved in both.

the other hand, gene variants of IL-10, ARPC2, IEC, ECM1 and a E3 ubiquitin ligase seem to be more specific for UC (47) susceptibility. Interestingly enough, many of the genes suggested to predispose to IBD have previously been found to predispose to other immune-related diseases (such as type I diabetes, psoriasis, asthma, systematic lupus erythematosus, rheumatoid arthritis) (46, 49, 50). Surprisingly, the combination and frequency of those risk genes vary significantly amongst different populations (51). Additionally, although there is no evidence of autoimmune mechanisms involved in IBD, auto-antibodies cross-reactive with bacterial antigens have been detected (52).

The intestine is inhabited by more than 10^{14} microorganisms, and the immune system must discriminate between the harmful and the commensal microbiota. In some studies, microbiota seem to play an important role in the pathogenesis of IBD, likely as the potential target of an exacerbated immune response (47). Normally, microbiota play an important role in the regulation, the development and the maturity of the intestinal and systemic immune mechanisms (53, 54). Novel research tools have made it possible to scan the whole microbial population of the intestine in order to reveal differences between healthy controls and IBD patients (55).

Several IBD-like colitis animal models, with a different basis for the development of colitis have been well characterized. These models are either genetic or chemical-based. Interleukin 2 (IL-2), Interleukin 10 (IL-10) and T cell receptor mutant mice represent widely used genetic models of IBD-like colitis (56-58). The most commonly used chemically induced models are the DSS (dextran sulfate sodium) and TNBS (2,4,6 trinitrobenzene sulfonic acid) (59, 60). A great deal of the knowledge on the molecular mechanisms of IBD has been based on the studies employing one or different combinations of these models.

As mentioned above, the immune system and the enteric nervous system exhibit an abundance of SP expression compared to other sites in the body. Interestingly, both of these tissue

have been implicated in the development of IBD pathophysiology. In addition, studies on human samples have demonstrated the expression of the main receptor of SP, NK1R, in the Gastrointestinal Track in various cell types apart from the enteric nervous system (61), like the smooth muscle (18), the epithelial and the endothelial (62) cells. Concerning the epithelial cells, some studies show elevation of the levels of NK1R in IBD patients (61, 63, 64), while others do not report such changes (64-66). NK1R expression in humans during UC and CD in the actively inflamed versus the non-inflamed regions seems to vary in those studies as well. The differences between the studies could be attributed to the differences amongst the populations, the variability in disease activity and even differences in ongoing treatments of the patients included in these studies [5]. Overall, these studies suggest an active role of SP in the intestinal epithelial-associated immune responses in IBD patients.

Similar conclusion can be drawn from the results of studies on SP and NK1R null mouse models already described in the previous section (Substance P (SP) and Neurokinin Receptors) of the introduction.

ii. Adipose Tissue (and IBD)

Until the last decade, adipose tissue was considered an inactive tissue, useful merely for the storage of excess energy in the body in the form of lipids. Nowadays the prevalence of obesity in the western society has reached epidemic proportions (67), and the correlation between obesity and numerous metabolic diseases (68), even cancer (69), has been demonstrated decisively. Additionally, it is believed that obesity is associated with a low-grade systemic inflammation and it has been well established that adipose tissue is an active metabolic and endocrine organ able to produce numerous pro-inflammatory and anti-inflammatory cytokines (like Leptin, Adiponectin, Interleukin 8 and TNFα),

termed adipokines (70, 71). Finally, studies have demonstrated the recruitment of immune cells within adipose tissue during adipose tissue expansion that occurs with obesity (71, 72).

Overall, the accumulated knowledge gathered during the last decade on adipose tissue physiology and function leads to the hypothesis that molecular events associated with the development of obesity may play an central role in diseases with a pathophysiology that includes a strong immune component in its aetiology, such as IBD (36). Adding to that, the anatomical proximity of the intestine with the intra-abdominal fat, and the possible interactions between the two tissues can not be omitted (36). In agreement with those suggestions, the observation of the formation of a large fat that surrounds the inflamed intestine in CD has been well documented and used by surgeons as a landmark for the delineation of active disease. This adipose tissue, of mesenteric origin, has been termed “creeping fat” (73, 74), and it has been shown that this new fat mass contains higher levels of Interleukin 6 and TNFα compared to more distal uninvolved mesenteric fat masses from the same patients (75). Recent studies indicate that CD patients with increased Body Mass Index require more frequent hospitalization (76) and have significantly less time between the time of diagnosis and the requirement of surgical intervention (77). Moreover, IBD seem to be correlated with nutritional habits, such as lipids of animal origin and high glucose consumption, both common characteristics of obese populations (78).

Studies on animal models have revealed that diet-induced obese mice develop more severe colitis during DSS-induced intestinal inflammation compared to non-obese controls, and suggested that leptin levels (79) and a possible T cell deregulation (80) may have important roles in the mechanisms driving these responses. A genetically modified mouse model lacking adiponectin has produced controversial results after the induction of chemically induced colitis as one group demonstrated worsening and the other improvement of colitis (81, 82). In a separate study TNBS-

induced colitis promoted increased accumulation of neutrophils in the mesenteric adipose tissue of mice, a development that is likely due to the high levels of cytokines accumulated within these depots (59).

Considering all the aforementioned knowledge on the potential effects of fat tissue-associated responses in the development and severity of outcome of IBD and in combination with studies that demonstrated the proinflammatory effects of neuropeptides (including SP) on fat cells, we will attempt to delineate the potential mechanisms by which the neuropeptide SP may trigger intercellular events in adipocytes that may in turn produce mediators of interest in IBD research. Although, research on the potential involvement of adipose tissue in the development of several diseases has exploded recently, evidence that associate fat tissue function as it relates to IBD is limited. Our studies may generate further evidence on this association and help identify novel targets for potential therapeutic approaches.

D. Materials and Methods

i. 3T3-L1 cell line

3T3-L1 cells were obtained from ATCC (Cat# CL-173), cultured at 37^{o}C, 5% CO_2 (incubator from Thermo-scientific, model 3110) in DMEM medium (GIBCO Cat# 11995) supplemented with 10% FBS (GIBCO 10437-028) and 1% Penicillin/ Streptomycin (GIBCO 15140-122). Medium was changed every 2-3 days.

Cells were passaged when 70-80% confluency was attained, so as to maintain a growing state. In order to passage cells (1 to 5-10 dilution), medium was aspirated and DPBS (GIBCO 14190) was used to wash off the remaining medium. Cells were lifted from culture plates (treated polystyrene culture dishes from Corning Cat# 430167) using incubation in Thrypsin-EDTA (GIBCO 25200114) for 5 minutes at 37^{o}C. Cells were then collected in a 50ml Falcon tube, centrifuged at 1000 rmp for 10 minutes, and cell pellets were re-suspended in medium in order to be plated again.

ii. Treatments of 3T3-L1 cells with SP

Cells were plated in six well plates (BD BIOSCIENCES 353046). Before treatments, cells were starved in DMEM supplemented with 1% FBS and 1% P/S overnight in order for cells to become synchronized. For treatment, cells were washed with DPBS and were subsequently incubated with DMEM with 10% FBS and 1% P/S with or without SP (10^{-8} or 10^{-7} M in TFA 0.1%) for 0, 4, 8, and 12 hours. Control samples were treated with equal volume of TFA 0.1%. TNFa (10ngr/ml) was used as a positive control, since it has been known to increase the expression of adhesion molecules. At designated time points, supernatants were collected and cells were harvested in either Trizol or RIPA buffer for RNA or protein collection, respectively.

iii. Differentiation of 3T3-L1 cell line

3T3-L1 differentiation was induced using the following method:

Cells were grown to 100% confluence in DMEM (DMEM; GibcoBRL-Cat# 11965-084: high glucose, with L-glutamine, with pyroxidine HCl, without sodium pyruvate) supplemented with 10%Calf Serum (GibcoBRL-Cat#16170-078/Lot #1060198), 1% MEM Sodium Pyruvate (100mM; GibcoBRL Cat#11360-070) and 1% P-S-G (100x P/S/G; GibcoBRL Cat#10378-016).

Fourty eight hours after confluence was achieved (designated DAY 0), cells were stimulated with MDI induction medium (DMEM supplemented with 10%Calf Serum, 1% MEM Sodium Pyruvate, 1% P-S-G, 10^{-4} *1.15g/ml IBMX;-Sigma I-7018, 1µg/ml Insulin- Bovine; Sigma I-5500, 1µM Dexamethasone -Sigma D-4902) for another 48 hours (until DAY 2).

On DAY 2, cells were incubated with Insulin Medium (DMEN supplemented with10% FBS-GibcoBRL-Cat# 10437-028/Lot # 1026566, 1% MEM Sodium Pyruvate, 1% P-S-G, 1µg/ml Insulin), for another 48 hours (until DAY 4).

On DAY 4, cells were incubated with basic medium, DMEM supplemented with 10% FBS, 1% MEM Sodium Pyruvate, 1% P-S-G. , Medium was renewed every two days, until full differentiation was achieved (approximately by DAY 8).

iv. Isolation of rat mesenteric preadipocytes

In order to isolate mesenteric (or any other type of intra-abdominal fat) preadipocytes from adipose tissue of Brown Norway rats, the following steps were followed:

Falcon tubes with 10ml of AMEM (INVITROGEN 11095-098) with 10% FBS and 1% P/S were prepared under sterile conditions and weighted. Next, animals were anesthetized and decapitated. All tools used in the following steps were previously autoclaved and handled in aseptic conditions. Using

large tweezers, the lower part of the abdomen was lifted, and a large cut was introduced to the dermis with large scissors, taking care not to penetrate the peritoneum. With the use of scissors again, the dermis was separated from the peritoneum, and was held outwards with the use of hemostats. The lower peritoneum was then held up, a cut was introduced with new scissors and the sides of the peritoneum were held up using new hemostats. Once the opening was wide enough, the corresponding fat pads were dissected and placed into the falcon tubes, using fresh tweezers and scissors.

After removing the desired tissue from the animal, the Falcon tube was reweighted to estimate the weight of fat collected. According to this weight the appropriate volume of 1mg/ml collagenase (Worthington 41B12520) in HBSS (GIBCO 14175) was prepared (3ml of collagenase medium is needed for each gr of tissue collected) and was sterilized by filtration through a 22μm Spritzen/ Syringe filter. The appropriate volume of collagenase was added to the corresponding tissue which is then chopped to fine pieces with the use of sterile scissors and was incubated in shaking water bath at 37 ^{o}C and 100rpm for approximately 1 hour, vortexed every 10 minutes to enhance tissue digestion. After the completion of the digestive step, the tissues were passed through sterile gauzed funnels and kept in new sterile 50ml Falcon tubes. Tubes were centrifuged at room temperature at 1,200 rpm for 10 minutes, supernatants were aspirated and the pellets formed- that contained mostly pre-adipocytes were re-suspended in 10ml AMEM supplemented with 10% FBS and 1% P/S, plated in treated polystyrene culture dishes from Corning (Cat# 430167) and kept overnight at 37 ^{o}C, 5% CO_2.

The following day, cells were inspected for possible contaminations and washed with HBSS to dispose of dead cells or unwanted cell debris. Using this procedure, some immune cells are bound to

be collected as well. In order to maintain the population of preadipocytes as pure as possible, two or three passages are required for those cells to be eliminated.

v. Treatments with SP of rat mesenteric preadipocytes

Rat mesenteric preadipocytes were plated in six well plates. Before the exposure, cells were starved in AMEN supplemented with 2% FBS, 1% P/S, in order for cells to become synchronized. The following day, cells were washed with DPBS and incubated with complete medium (AMEM supplemented with10% FBS, 1% P/S) with or without SP 10^{-7} (control cells were treated with equivalent volume of 0.1% TFA). At 6 hours, supernatants were collected, cells were again washed with DPBS and collected in TRIZOL (INVITROGEN 15596-026).

vi. Isolation of human mesenteric preadipocytes

Human mesenteric fat tissue specimens were collected during intra-abdominal operations of patients conducted for various reasons. Specimens used in this research were kindly provided by the Pathology Core of Ronald Reagan Medical Center (UCLA), Dr Arsenescu from Kentucky Medical Center and Dr Kirkland from Mayo Clinic. Patients had signed an informed consent while no access to patients' personal data was possible with the exception of the information regarding sex, age, disease status and in some cases, the weight and height of the patients.

Isolation of human mesenteric preadipocytes was similar to the isolation of rat mesenteric preadipocytes. The two protocols differed at the following steps. Human tissuew were minced in 1mg/ml Collagenase in HBSS, supplemented with with 4% Fatty Acid Free BSA (CALBIOCHEM 126579). Moreover, following tissue centrifugation to collect preadipocytes, mature adipocytes were kept from the upper layer formed using a sterile pipette, the rest of the supernatant was aspired and

the pellet comprised mostly of human pre-adipocytes was re-suspended in 10ml Erythrocyte Lysing Buffer (ACK Lysing Buffer, GIBCO # A1049), incubated in 100rmp shaking 37 ^{o}C water bath for 5minutes, to dispose of the erythrocytes, and then samples were centrifuged once more at 1000 rpm for 10 minutes and cell pellets were re-suspended in complete AMEM.

It has been our goal to create a unique bank of human mesenteric adipocytes and preadipocytes, in order to explore their interplay in various diseases or conditions. For this purpose, a portion of pre-adipocytes were frozen at the 3rd passage, using freezing medium (50% complete AMEM, 45% FBS and 5% DMSO (SIGMA D2650), under standard freezing techniques.

vii. Differentiation of human mesenteric preadipocytes

Human preadipocytes were incubated with Human Maintenance Medium [1lt DMEM/F-12 (Gibco 12500-062) supplemented with 23mM Hepes salt (Sigma H-4034), 25 mM $NaHCO_3$ (Fisher S233-3), 1% Penicillin/ Streptomycin (Gibco15140-122, 20 X), 10mg/l Transferin (Sigma T-3309), 0.03 mM Biotin (Sigma B-4639) and 2 mM L-glutamine (Gibco 25030-081)] for 24 hours before initiating the differentiation process in order to consume all of the FBS and for the cells to stop multiplying (multiplication and differentiation are two different cell processes that can not take place simultaneously in the case of preadipocytes). The pH was adjusted to 7.4, and the medium was filter-sterilized.

The following day, cells were washed and incubated with Human Differentiation Medium [1lt DMEM/F-12 supplemented with 15 mM Hepes salt, 15 mM NaHCO3, 1% Penicillin/ Streptomycin, 2 mM L-glutamine, 10mg/l Transferin, 0.03 mM Biotin, 0. 5 μM Human-insulin (Sigma I-2059), 17 μM Pantothenate (Sigma P-5155), 0.1 μM Dexamethasone (Sigma D-4902), 2 nM T3 (Sigma T-6397), 540 μM IBMX (Sigma 15879), 1μM Ciglitazone (Bio Mol cat# GR205) and 1mg/ml Fetuin (Sigma

cat#2379). The pH was adjusted to 7.4, using either HCl or NaOH and the medium was filter-sterilized.

Once cells were incubated with Human Differentiation Medium, the medium was renewed every 2-3 days until differentiation was achieved (a period of at least 21days).

viii. Treatments with SP of human mesenteric preadipocytes

There were three different kinds of treatment with SP:

a. Treatment of preadipocytes [in treated polystyrene culture dishes (Corning Cat# 430167) if RNA was collected or six well plates if protein was collected].

After starving cells overnight with AMEM, 2% FBS, 1% P/S in order to synchronize cells without stressing them, they were washed with DPBS and incubated with 3ml of AMEM complete with 10^{-7} or 10^{-8} M SP, or the equivalent volume of 0.01% TFA. After 4, 6, 8, 16, 24 hours (depending on the experiment), supernatants were collected, cells were washed with DPBS and collected in TRIZOL or RIPA with TRITON X 100 plus protease and phosphatase inhibitors for RNA of protein isolation respectively.

b. Treatment of differentiated adipocytes [in treated polystyrene culture dishes (Corning Cat# 430167) if RNA was collected or six well plates if protein was collected].

Given the fact that the differentiated adipocytes can not proliferate, and also that the deprivation of nutrition factors from the medium would probably cause too much cellular stress (taking into consideration our previous findings and the fact that at this point the cells have become too fragile), cell starvation was skipped. On the 22nd day of differentiation, cells were washed with DPBS and incubated with complete AMEM (to discontinue differentiation signaling) with 10^{-7} or 10^{-8} M SP, or the equivalent volume of 0.01% TFA. After 4, 6, 8, 16, 24 hours (depending on the experiment),

supernatants were collected, cells were washed with DPBS and collected in TRIZOL or RIPA with TRITON X 100 plus protease and phosphatase inhibitors for RNA of protein isolation respectively.

c. Treatment of preadipocytes, while differentiating (in six-well plates).

Once the process of differentiation was initiated, half of the cells were treated with 10^{7} M SP in their medium while the rest received the equivalent volume of 0.01% TFA. Media were renewed every 2-3 days and supernatants and cells were collected for protein isolation at DAY 2, 1 WEEK and 3 WEEKS, in order to captivate phenomena taking places in different stages of differentiation.

ix. Real time PCR (qPCR)

Once cells were collected in TRIZOL, RNA isolation was conducted either according to manufacturer's instructions provided by INVITROGEN, or with QIAGEN's miRNeasy kit (127004). RNA concentration was estimated using Spectrophotometer (NanoDrop ND-1000) and 1μg RNA was used to prepare cDNA.

For the preparation of cDNA, nuclease free water was added to 1μgr of RNA to reach a total volume of 11.25μl. 1μl of random decamers (Applied Biosystems AM5722G) and 0.25μl RNAsin (PROMEGA N2615) were added to each sample, and the samples were placed in a heat-block at 70 ^{o} C for 70seconds. The samples were then placed on ice for 5minutes.

In each sample 7.5 μl of a master mix (4μl 5x First Strand Buffer (ROCHE 11581295001), 0.25μl RNAse out, 1μl MMLV-RT (INVITROGEN 28025-013), 0.1μl Nucleotides (Agilent Technologies 28025-013), 2.15μl Nuclease Free H_2O) was added and the samples were placed at 37 ^{o}C heat-block for 1 hour.

Samples were supplemented with an additional 80μl Nuclease Free H_2O and were used for qPCR or were stored at -20 ^{o}C.

For the qPCR, 4.5 μl from the RT reaction above were mixed with 10μl of 2x Master Mix (Applied Boisystems 4352042) 4.5μl of nuclease free water and 1μl of the primer mix corresponding to the gene of interest, and then were amplified using the 7500 Fast Real-Time PCR System (Applied Biosystems) at the Step1 1x 20seconds at 95 ^{o}C, Step 2 40x 3seconds at 95 ^{o}C and 30seconds at 60 ^{o}C programm. Riobosomal 18S RNA or GAPDH was co-amplified as internal control for each reaction.

Numerous primers were used for the purposes of our research, all purchased from Applied Biosystems. Mouse primers used, were: I-CAM1 (Mm00516023_m1), V-CAM-1 (Mm01320970_m1), TACR1 (Mm00436892_m1, Mm00436893_m1), TACR2 (Mm00436898_m1), CXCL1 (Mm04207460_m1). Rat primers used, were: GAPDH (Rn01775763_g1), TACR1 (Rn00562004_m1), TACR2 (Rn00436896_m1), IL-1b (Rn00580432_m1), IL-6 (Rn01410330_m1), BDNF (Rn02531967_s1), TNF (Rn00562055_m1), CXCL1 (Rn00578225_m1), CCL2 (Rn00580555_m1). Human primers were: 18S (conserved amongst species, used for mice and rats too) (Hs03928990_g1), TACR1 (Hs00185530_m1), TACR2 (Hs00169052_m1), TACR3 (Hs00357277_m1), IL-1b (Hs01555410_m1), IL-2 (Hs00174114_m1), IL-6 (Hs00985639_m1), IL-8 (Hs00174103_m1), IL-10 (Hs00961622_m1), IL-12b (Hs01011518_m1), BDNF (Hs01010223_m1), ADIPOQ (Hs00605917_m1), I-CAM1 (Hs00277001_m1), V-CAM1 (Hs00365486_m1), TNF (Hs00174128_m1), Leptin (Hs00174877_m1).

x. Western Blot

Proteins were collected in RIPA TRITON X100 (BOSTON BIOPRODUCTS BP-116TX) supplemented with Protease and Phosphatase inhibitors (1 μM aprotinin, 5 mM benzamidine, 2 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, all from Sigma). The samples were

centrifuged at 1000g for 5minutes at room temperature for unbrocken cells, large unbroken parts of the membrane, nuclei and large cellular comparments to be removed. The protein concentration of the supernatants was measured using the BCA kit (Thermo Scientific 23227), at the SpectraMax M5 (Molecular Devices). 20 or 30 mgr of protein of each sample were separated using SDS-PolyAcrilamide Gel Electrophoresis, according to Laemmli and transferred to polyvinylidene difluoride (PVDF) membranes in 25 mM Tris, 192 mM glycine. Following transfer, membranes were blocked for 2 hours at room temperature with 10% nonfat dry milk in TBS supplemented with 0.05% Tween-20 (TBST) and probed with specific antibodies at 4^{o}C, over night, as indicated. Membranes were washed three times in TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. After three more washes, the membranes were incubated in ECL reagent (PerkinElmer Life Sciences, Waltham, MA) for 1 min and then exposed to a FujiFilm LAS-4000 mini image station. Data analysis was performed using Image Reader LAS 4000.

Protein ladders used were: MagicMark™ XP (INVITROGEN LC5602) and Precision Plus™ Protein Standards (BIORAD 161-0375). Primary antibodies used for the mouse experiments are: goat polyclonal IgG anti-I-CAM1 (Santa Cruz Boitechnology sc-1511, used 1/1000 dilution), goat polyclonal IgG anti-V-CAM1(Santa Cruz Boitechnology sc-1504, used 1/1000 dilution). Human primary antibodies were rabbit IgG anti-C\EBPa (Cell Signaling 2844S, used 1/1000 dilution), rabbit polyclonal IgG anti-C\EBPb (Santa Cruz Boitechnology sc-150, used 1/500), rabbit polyclonal anti-p-C\EBPb IgG (Santa Cruz Boitechnology sc-16994, used 1/500), rabbit anti-PPARg IgG (Cell Signaling 81B8, used 1/500), mouse monoclonal IgG anti-b-Actin (Santa Cruz Boitechnology sc-81178, used 1/2000). The secondary antibodies used were goat anti-mouse IgG-HRP, donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP (all from Santa Cruz Boitechnology with catalog numbers sc-2005, sc-2020 and sc-2004 respectively, and all used 1/2000 dilution).

xi. ELISA measurements

DuoSet ELISA Development kit was used in order to measure the naturally produced human IL-8 (R&D Systems). According to manufacturer's instructions, plates were incubated overnight with Capture Antibody and were subsequently washed and blocked. Plates were incubated with samples, washed and incubated with the secondary antibody at the suggested concentration. Streptavidin-HRP is added for 20minutes, followed by another 20minutes with Substrate solution and Stop solution. Optical density was determined at 450nm at SpectraMax M5 (Molecular Devices).

xii. Transformation, plasmid isolation and transduction

Adiponectin promoter was acquired from Dr Philipp E. Scherer (83). The plasmid was inserted into DH5α bacteria *via* heatshock, subsequently cultured in S.O.C. (Invitrogen 15544-034) medium, at 37^{o}C shaking incubator for 1 hour. Then the bacteria were spread on LB-Agar culture plates with ampicillin. Plates were incubated over night at 37^{o}C and the following day a number of bacterial colonies were picked for the propagation of midi cultures. Plasmid isolation was performed using the EndoFree Plasmid Maxi Kit (Qiagen 12362) and was provided to the UCLA Vector Core to be used for the production of a lentiviruses. One of the lentiviruses produced, bared both GFP and Luciferase tags (Figure 3), making the total viral genome slightly over 8k. There was an unsuccessful attempt to infect human mesenteric preadipocytes with this lentivirus (even after they were differentiated). This failure was attributed to the fact that the viral genome material was above the generally accepted borderline.

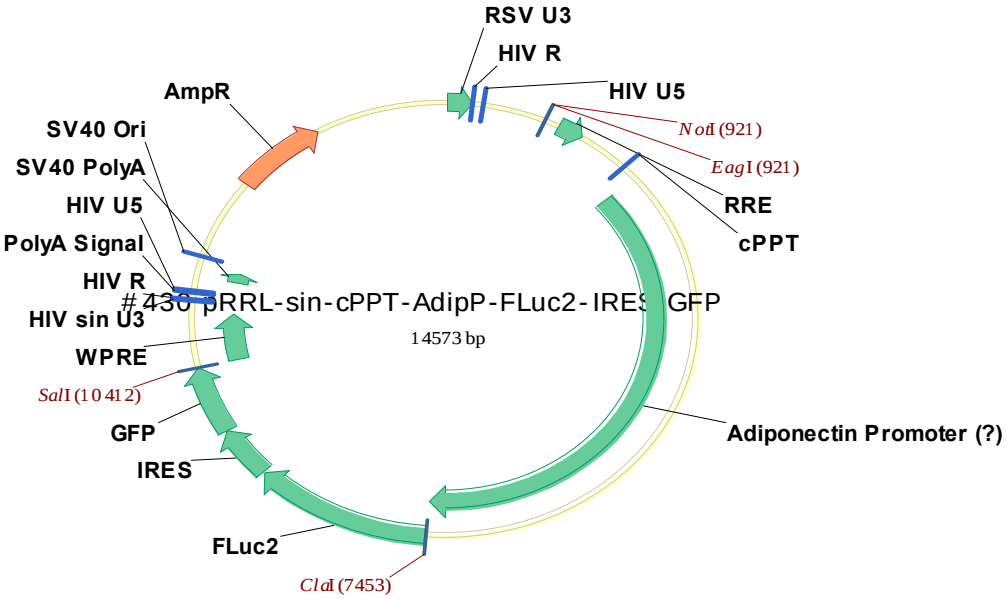


Figure 3. A schematic presentation of the map of the first lenti-virus used.

UCLA Vector Core provided us with a second lentivirus, this time without the Luciferase tag (Figure 4). Human mesenteric preadipocytes were infected with different concentrations of the virus (0.46 µg/ml, 0.23µg/ml, 0.115µg/ml, and 0.46µg/ml) in the presence of Protamine sulfate to assist the viral infection of cells in complete AMEM. , Cells treated just with Protamine sulfate in the absence of the lentivirus served as a negative control. After 8 hours, cells were washed with DPBS and maintained in culture in complete AMEM.

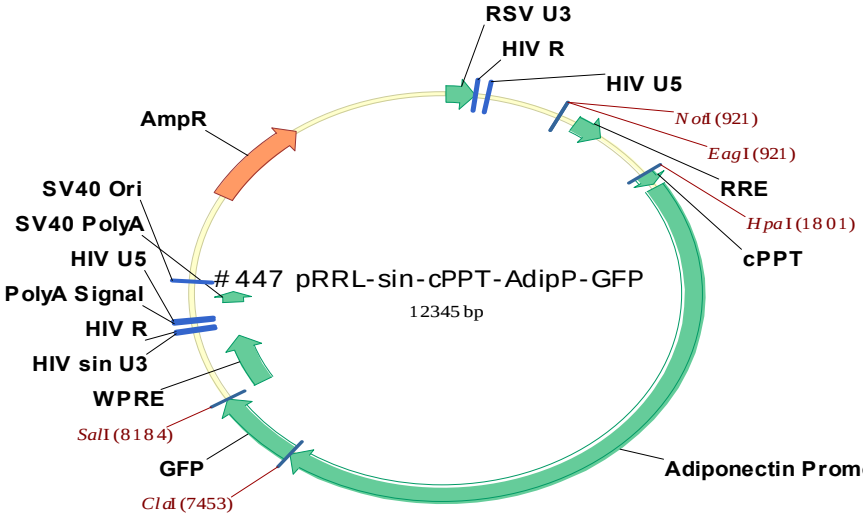


Figure 4. A schematic presentation of the map of the AdipoQ-GFP lenti-virus used.

xiii. Microscope

Cells were viewed under Observer.D1 microscope (ZEISS), images were captured using AXIO-CAM HRc (ZEISS) camera and processed with AxioVision Rel 4.6 software.

xiv. Statistical analysis

Prism was used for statistical analysis. Mann Whitney non-parametric test was used for comparisons between two groups while a two-way ANOVA was employed where multiple group comparisons were required. Values were expressed as mean ± SEM. p value <0.05 was considered significant.

E. Results

Exposure of 3T3-L1 preadipocytes to SP does not alter the mRNA expression and protein levels of adhesion molecules.

Determination of appropriate FBS concentration during treatment of 3T3-L1 preadipocytes with SP. In preliminary experiments performed to help us standardize our treatment protocols, 3T3-L1s were kept overnight in various concentrations of FBS in order to determine the effects of serum starvation on basal expression of inflammatory molecules. RNA was collected, and the expression levels of CXCL1 were examined. Although not statistically significant (p~0.54, n=6, between 0 and 10% FBS), CXCL1 expression increased as the concentration of FBS in the medium decreased. Thus, we decided to use 1% FBS medium prior to SP exposure.

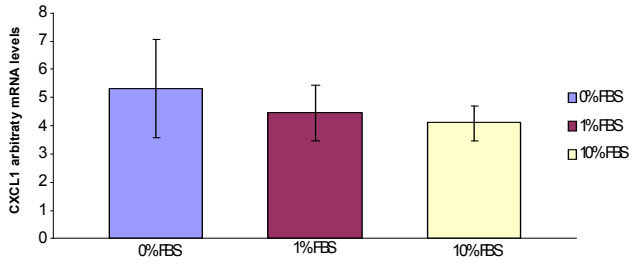


Figure 5. Effects of serum strarvation on the mRNA expression of CXCL1. Addition of varying concentrations of FBS in the medium of 3T3-L1 preadipocytes does not alter the basal expression levels of CXCL1 mRNA.

Effects of SP treatment on the expression of adhesion molecules in 3T3-L1 preadipocytes. 3T3-L1 cells were exposed to SP (10^{-8} and 10^{-7}M) for 4 or 8 hours and mRNA levels of I-CAM1 and V-CAM1 were measured. Regardless of SP treatment, I-CAM1 expression levels remained steady after SP exposure for different time points, although there was a tendency for decreased expression after 4hours exposure, with the maximum effect at 10^{-8}M SP. However, this effect is not statistically significant (Figure 6A, p value 0.18). V-CAM1 expression did not significantly altered after SP stimulation, except from a decrease at 12h, using 10^{-7}M SP (Figure 6B, p value=0.0011). TNFa was used as a positive control of ICAM1 and VCAM1 expression (last bars, values of ICAM1 with TNFa

exposure were increased by 131.32, 148.69, and 149.91 fold compared to the basal levels of ICAM

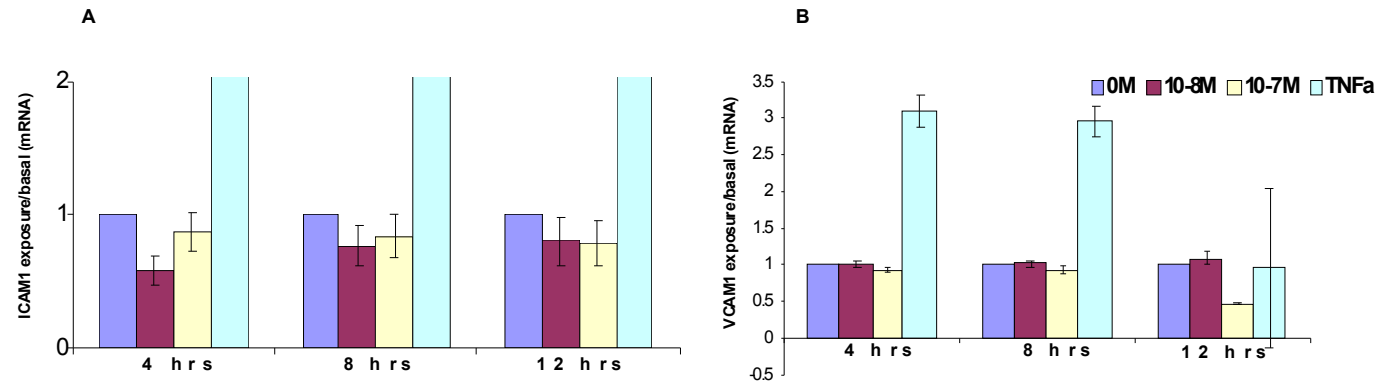


Figure 6. Exposure of 3T3-L1 preadipocytes to SP does not affect I-CAM1 and V-CAM1 mRNA expression levels. (A) Ratios of arbitrary ICAM1 mRNA expression units show no significant effects of SP on this adhesion molecule in 3T3-L1 cells in contrast to TNFα treated positive controls. **(B)** 3T3-L1 V-CAM1 expression is decreased by SP stimulation after 12hours of exposure (10^{-7}M, p value=0.00057).

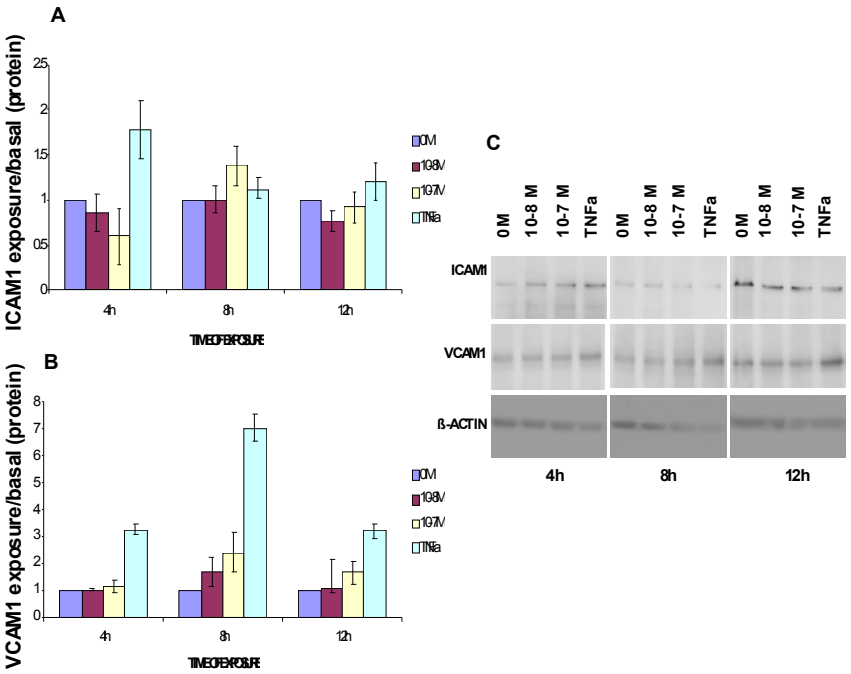


Figure 7. Effects of SP treatment on adhesion molecule protein levels in 3T3-L1 cells. SP treatment does not significantly alter the levels of **(A**) ICAM1 and **(B)** VCAM1 protein expression. All values are presented as a ratio of protein units after SP exposure to basal levels of expression. **(C)** Western blot image of the data described in panels A and B. No statistically significant differences were

expression at 4, 8 and 12 hours respectively).

To verify the previous observations on ICAM1 and VCAM1 mRNA expression, the effects of SP on the protein levels of the same adhesion molecules were examined in 3T3-L1 preadipocytes. No difference in the protein levels of I-CAM1 or V-CAM1 was identified (Figure 7B, p value=0.58 for V-CAM1 at 8hours with10^{-7}M SP). TNFa, used as a positive control, significantly increased ICAM 1 and VCAM1 protein expression (ICAM1 and VCAM1 n=5, and for TNFa n=3).

Effects of SP stimulation on Rat Mesenteric Preadipocytes.

In light of our previous studies showing increases in inflammatory pathway stimulation of primary human preadipocytes in response to SP, rat mesenteric preadipocytes were stimulated with 10 $^{-7}$M of SP for 6 hours and its effects on the expression of proinflammatory cytokines was examined. Our results show a statistically significant increase of TACR2 mRNA levels (Figure 4, p value= 0.030, n=3) and trends towards increase of IL-6, BDNF, TNF, CXCL-1 and CCL-2 cytokine-mRNA levels after stimulation. Although non-significant, these changes show a tendency for SP to induce proinflammatory responses (see discussion for analysis). Perhaps, increases in the number of samples tested may increase the significance in our findings.

TACR-1 and IL-1β transcription levels were also tested, but

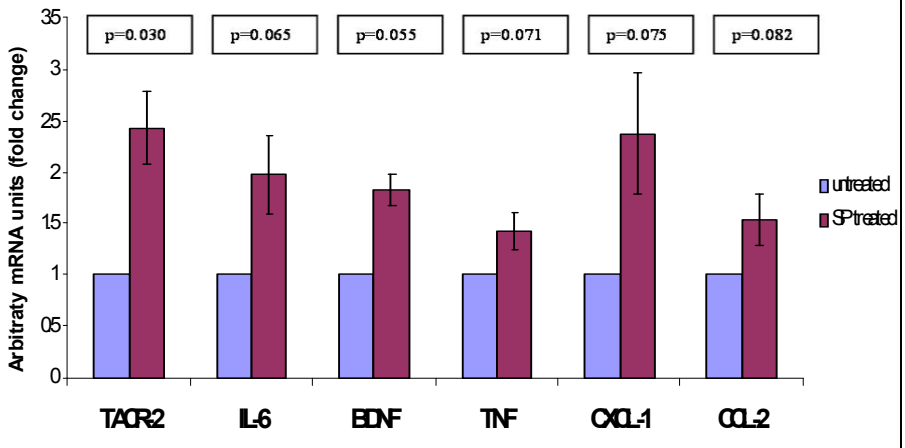


Figure 8. Effects of SP treatment on primary rat mesenteric preadipocytes. SP increases the mRNA expression levels of Tacr2 in ray mesenteric preadipocytes (first columns). Although there is also a strong trend for increased mRNA expression for several proinflammatory molecules tested, these changes were not statistically significant. The mRNA expression levels after treatment are presented as fold difference from the control (untreated).

were undetectable (Figure 8, n=3)

SP Treatment induces disease and site-dependent responses on Human Mesenteric Preadipocytes.

Human mesenteric preadipocytes from controls and IBD patients (from both involved and uninvolved parts of the intestine) were exposed to 10^{-7} M SP for 8 hours and the total RNA was collected. The basal expression levels of NK1R were examined between mesenteric pre-adipocytes of control and IBD patients. Our group had previously demonstrated NK1R expression in human mesenteric pre-adipocytes of obese patients (84).

In this study, we used samples from 4 control, 2 Ulcerative Colitis (UC) uninvolved samples, 4 UC involved samples, 1 Crohn's Disease (CD) uninvolved sample and 1 CD involved sample.

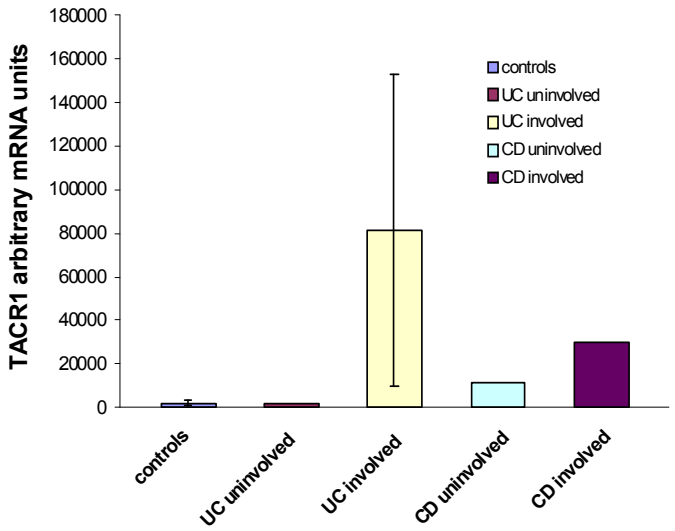


Figure 9. Effects of SP treatment in Human mesenteric preadipocytes isolated from inflamed or non-inflammed adipose tissue during IBD. SP-dependent increases in NK1R mRNA in adipocytes from UC patients (close to the inflamed section) are not statistically significant (middle bar, p= 0.3). Conclusions cannot be drawn for the rest of the conditions due to inadequate sample size.

We compared basal NK1R (*Tacr1*) expression between controls and samples from UC patients close to the involved intestine. Although no statistical significance was observed between the two

groups (Figure 9, p=0.3, n=4), this may be due to the large variability created by a single outlier value in our analysis. This will be re-evaluated as more patient samples included in this study will be analyzed (we have already obtained and isolated cells from 14 UC patients). Despite the trends that start to develop when CD patient samples are plotted it is difficult to discern any valid conclusions due to the inadequate number of samples included in this study.

SP increases the expression of IL-8 and IL-1β mRNA in primary human mesenteric preadipocytes. Previous studies from our group have demonstrated increased IL-8 mRNA expression as well as protein release from human mesenteric preadipocytes in response to SP stimulation (59). Treatment of human mesenteric preadipocytes isolated from UC patients from areas proximal to the inflamed intestine increased the mRNA expression of the proinflammatory cytokines IL-8 and IL-1β (Figure 10A and C, p<0.01 and 0.001, for IL-8 and IL-1β, respectively, n=4). Although not statistically significant, SP appear to induce a trend towards increase of these cytokines in preadipocytes isolated from control patients (Figure 10A and C, first block, p=0.06 and 0.0502, for IL-8 and IL-1β,

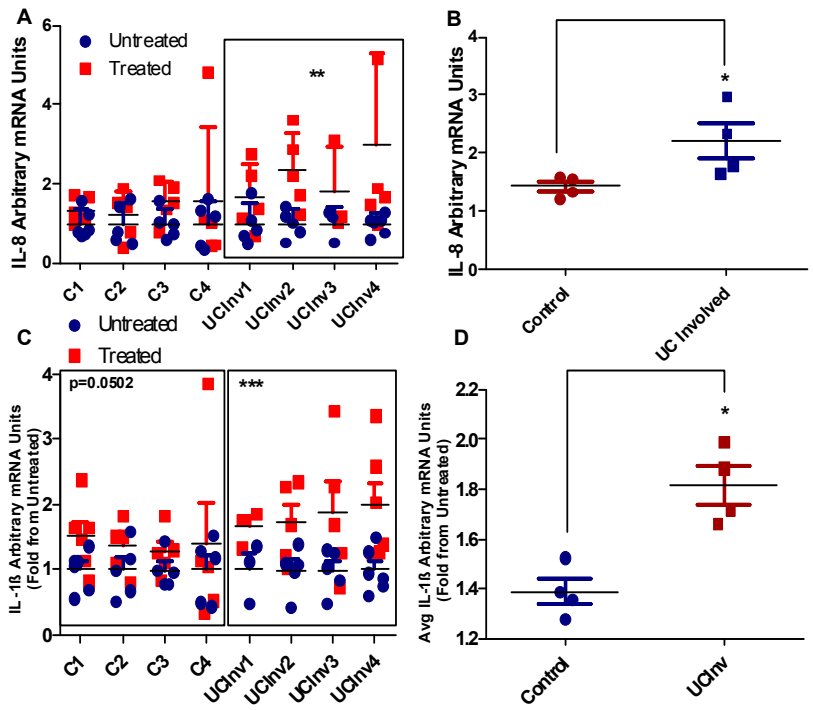


Figure 10. UC augments SP-induced IL-8 and IL-1β expression from human mesenteric preadipocytes. Treatment with SP increases IL-8 (A) and IL-1β (C) mRNA expression in primary human mesenteric preadipocytes. This induction is significantly more robust for both IL-8 (B) and IL-1β (D) in preadipocytes isolated from UC patients compared to "healthy" controls.

respectively). Analysis of additional patient samples undergoing in our laboratory may result in a statistically significant increased expression of these proinflammatory molecules. However, comparing the fold change in mRNA expression of IL-8 and IL-1β in response to SP between preadipocytes from control and UC patients (1.42 vs 2.22 and 1.39 vs 1.81, respectively) we observed that the latter responded more intensely in both cases (Figure 10B and D, $p< 0.05$ for both IL-8 and IL-1β, n=4). Thus, UC seems to be priming cells of the adipose tissue for more robust responses to proinflammatory stimuli, such as SP.

We also studied mRNA expression of NK2R and NK3R in these cells. We found NK2R mRNA being in all of the samples tested (1 control,1 UC-both involved and uninvolved), while NK3R was undetectable (1 UC-both involved and uninvolved). Also, I-CAM1 (1 control, 1 UC uninvolved, 2 UC involved), V-CAM1 (1 control, 1 UC-involved and uninvolved), IL-6 (1 control, 1 UC involved), BDNF mRNA was detected in one control sample with no significant differences either among the samples or the exposed and non –exposed (although more samples need to be examined). Finally, on the 1 control sample IL-2, IL-10, IL-12b and Adiponectin were examined but found undetectable. Again, those genes should be tested on more samples of multiple patients.

IL-8 secretion was also examined with ELISA in one of the control samples, and no statistically significant difference was observed after 6 hours of exposure. However, the measurement must be repeated with samples and for different exposure time points.

Effects of SP treatment on *in vitro* differentiated Human Mesenteric Differentiated Adipocytes*Effects of differentiation on NK1R mRNA expression in human mesenteric preadipocytes.* First we investigated whether differentiation and the accumulation of lipid in adipocytes affected the levels of NK1R mRNA in human mesenteric preadipocytes and adipocytes isolated fro control and

IBD patients. Although we detected a steep drop (undetectable for most differentiated cell samples, Figure 11) in receptor levels with differentiation both in UC and CD samples, more samples are needed to verify our data in the case of UC samples (Figure 7, 2 out of 3 differentiated samples had no detectable NK-1R mRNA) while no concrete analysis can be made for the rest of the groups until more samples are added to their analysis.

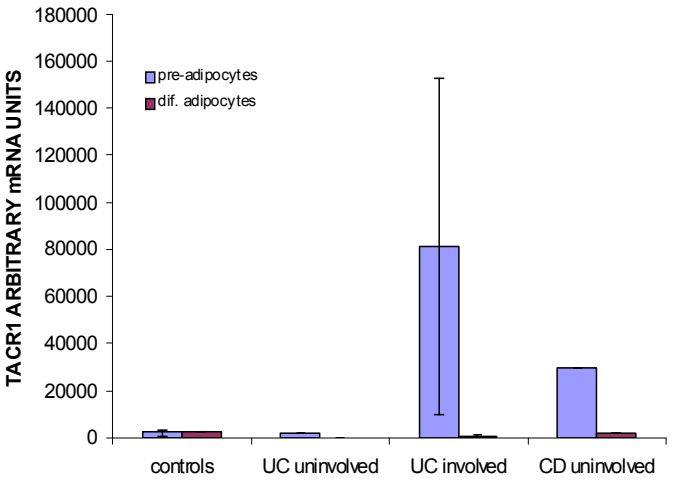


Figure 11. Differentiation affects NK1R levels during UC. Human mesenteric preadipocytes were induced to differentiate and NK1R mRNA levels were assessed in comparison to those of undifferentiated preadipocytes. NK1R mRNA was undetected for the majority (2/3) of differentiated adipocytes tested while it was detected in all preadipocyte lysates. More samples are required for all groups for better evaluation of this study though.

Effects of SP treatment on cytokine expression of in vitro differentiated human mesenteric preadipocytes. We have also performed several experiments where IL-8 and IL-1β mRNA levels were measured in human mesenteric preadipocytes in response to SP treatment. As also mentioned above more samples will need to be evaluated in order to be able to present any valid data on this study (between 1 and 3 from controls, UC involved, UC uninvolved and CD involved and uninvolved patient samples have been analyzed thus far).

Effects of SP on mediators of Human Mesenteric Preadipocyte differentiation.

In order to examine the possible role of SP in adipocyte differentiation, pre-adipocytes were exposed to SP at different time points during differentiation and the protein expression levels of intracellular mediators (transcription factors) that regulate this process (see introduction) were measured. Total protein was collected at 4 different stages (before the initiation of differentiation and

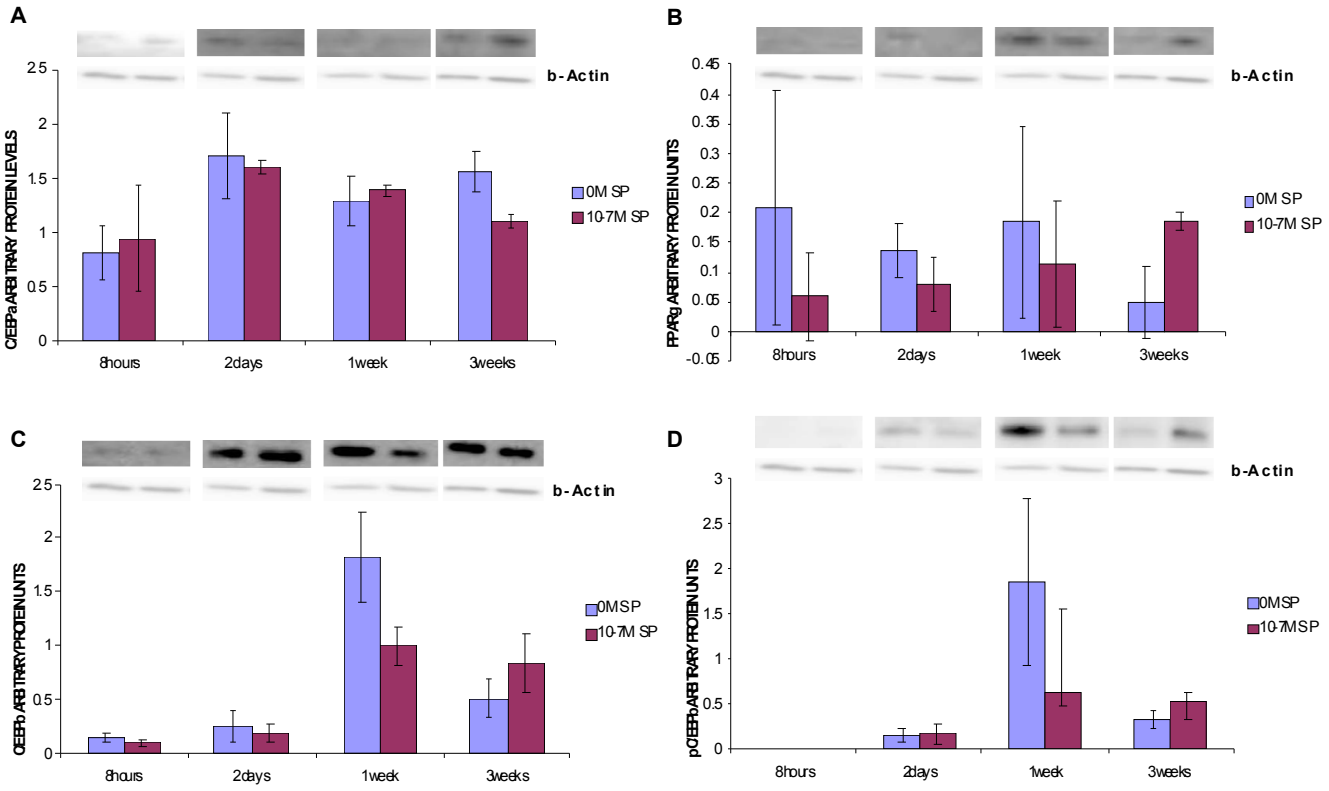


Figure 12. Effects of SP on preadipocyte differentiation-inducing transcription factors. Protein levels of (A) C/EBPα, (B) PPARγ, (C) C/EBPβ and (D) p-C/EBPβ were estimated in pre-adipocytes exposed to 10^{-7}M SP during differentiation. Although the levels of these transcription factors change during differentiation (as expected), their levels remain largely unaffected by SP treatment.

after an 8hr treatment, and at 2/7/21 days after the induction of differentiation) and the levels of C\EBPa, C\EBPb (both phosphorylated and un-phosphorylated forms) and PPARg were measured.

C\EBPa was not detectable before the initiation of differentiation, but it appeared after two days of differentiation (highest levels) and was largely unaffected by SP treatment (although there is a trend towards a decrease at 3 weeks, Figure 12A, p value=0.085, n=4). PPARγ is also unaffected by SP during differentiation (Figure 12B, p=0.117, n=4). C\EBPβ seems to be expressed exist in low levels in the beginning and increased during the 1st and 3rd weeks of differentiation. However, it seems unaffected by SP exposure although a trend for decreased expression appears during week one (Figure 12C, n=4). Phospho-CEBPβ was undetectable before the initiation of differentiation, and reached its highest expression levels during the 3rd week of differentiation (Figure 12D). Phospho-C/EBPβ responses to SP treatment were almost identical to those of its unphosphorylated isoform. The employment of different antibodies against C/EBPβ, that detect its many different isoforms, is required for a better assessment of the effects of SP in this molecule. Indeed, the abundance of different isoforms as well as their ratios are very important parameters in the evaluation of the effects of this transcription factor on preadipocyte differentiation.

Expression of GFP under the control of the adiponectin promoter in Human Mesenteric Preadipocytes using the pRRL lentiviral vector.

Human mesenteric preadipocytes of a control patient were infected with the lentiviral construct and after 24 hrs the cells were either incubated in differentiation medium or regular growth medium for one week. The cells were observed under an inverted fluorescent microscope daily. GFP expression was observed only in the differentiating cells within 24 hrs of the induction of differentiation and continued until day 7 when the cells were harvested for total RNA isolation (Figure 13D). Non-

differentiating cells did not exhibit any green fluorescence demonstrating the differentiation-dependent activity of the promoter (Figure 13B). Untransduced cells were used to delineate any potential effects of cellular auto fluorescence on our observations (Figure 13A and C).

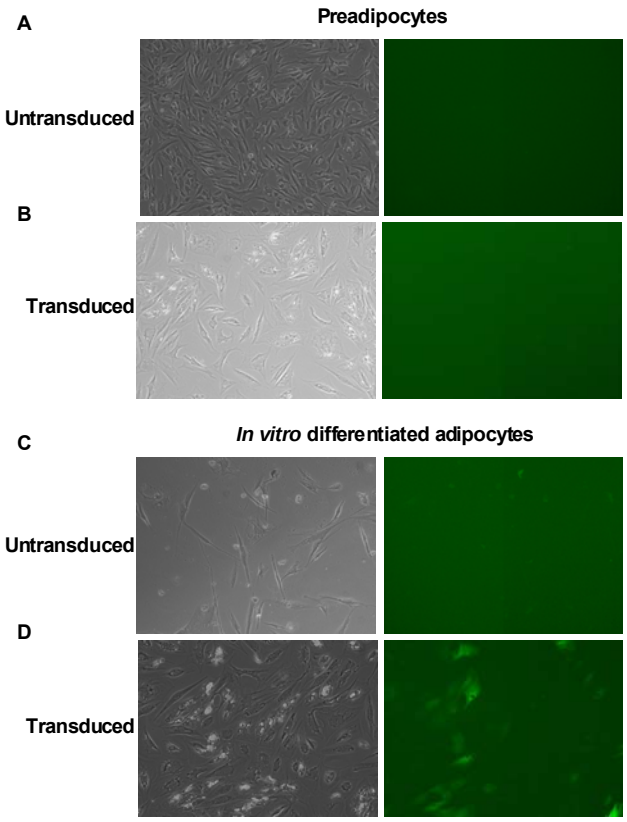


Figure 13. Adipoq Promoter driven GFP-expressing Lentivirus was successfully transduced to human mesenteric pre-adipocytest. Transduction of preadipocytes with pRRLAdipoq-Ires-GFP lenti-virus dies not induce adiponectin promoter induced GFP expression (B) an activity that was rectified 7 days after the induction of differentiation (D). Cellular auto fluorescence did not affect our observations in this study (A, C).

E. Discussion

The investigation of fat tissue function and its effects on other tissues or systems in the body is a novel and emerging field. Interesting knowledge is rapidly accumulating on the vast adipose tissue-associated effects on many diseases or conditions that predispose to diseases in humans. However, there are significant difficulties that pertain to adipose tissue research.

The only existing immortalized cell line is the mouse derived 3T3-L1, which, as of late, exhibits large batch-related variability in the ability of the cells to differentiate as well as in the responses to treatment. In addition, the isolation and culture of primary adipocytes is strenuous and provides cells with a finite division potential which in turn limits our ability to propagate large quantities for treatment. Isolation of preadipocytes from mice requires sacrifice of multiple animals for a low cellular yield, due to the small size of their fat depots. Isolation of rat cells is more feasible, due to the larger size of their depots. However, although they are responsive to SP treatment, they do not seem to express abundant NK1R, contrast to NK-2R which is more readily expressed, thus differing from human pre-adipocytes in this respect. This interesting observation will require further investigation utilizing more animals and preadipocytes isolated from animal models of colitis.

Apart from those difficulties, mature adipocytes can not be cultured, so must either be used immediately after removal from the animal and for short term experiments. Alternatively pre-adipocytes must be differentiated *in vitro* and then treated as mature adipocytes. Differentiation is time-consuming (depending on the cell origin can take up to 21days), and thus poses a limit to the amount of experiments to be conducted.

All the previously described difficulties and disadvantages in the use of animal mesenteric pre-adipocytes apply to human mesenteric pre-adipocytes as well. Additionally, the abundance of human samples depends on the rate that the affiliated hospitals and collaborators provide samples, and of

course, human mesenteric pre-adipocytes present enormous patient-related diversity since they derive from individuals with diverse genetic background. On the other hand, research on human mesenteric preadipocytes presents one very important advantage: they are more relevant to the human condition and thus results deriving from these studies can be translated more precisely as to their relation to disease pathophysiology and the potential identification of treatment approaches on patients.

To overcome the difficulties of using pre-adipocytes of human origin, we intent to use an immortalized cell line (83, 85) recently created, infect it with a lenti-virus in order to over-express NK1R and test the intracellular signaling pathways after SP treatment. Also, to more effectively utilize the rat pre-adipocytes that we harvest, we intent to try and identify molecules that can potentially increase naturally NK1R expression in rats in order to intensify the potential effects of SP in these cells. Use of preadipocytes isolated from either SP or NK1R knock out animals and the inducible fat-specific transgenic animals that are currently being designed are also potential tools available to us for the investigation of the effects of this neuropeptide on adipose tissue function.

Our data suggest that human mesenteric pre-adipocytes from samples of UC and CD patients may have increased levels of NK1R mRNA (Figure 9). However, the uninvolved samples of UC patients seem to have similar levels to the controls. This (pending the analysis and characterization of more samples) may indicate a role of SP-NK1R signaling for the adipose tissue in the processes within the inflamed intestine during IBD, and these effects may differ between disease stages (suggested by the difference of involved and uninvolved tissues). Differentiated adipocytes seem to produce an independent and unpredictable pattern of NK1R expression compared to preadipocytes. Many of the differentiated adipocytes used in this study demonstrate significantly lower NK1R when matched and compared to their preadipocyte precursors isolated from the same patient. In addition,

while most groups show a similar pattern of NK1R expression between preadipocytes and adipocytes (after *in vitro* differentiation), in cells isolated from UC patients close to the involved intestine, the receptor mRNA decreases substantially in the differentiated cells.

Additionally, we identified changes in the ability of IBD patients to respond to SP compared to "healthy" controls. Figure 6 depicts the ability of SP to induce IL-8 and IL-1β mRNA expression from both control and UC patient-derived preadipocytes. However, this response is more prominent for both cytokines in cells from UC patients suggesting that these cells have altered, UC-induced, intracellular conditions that exacerbate the proinflammatory effects of SP on preadipocytes. A comparison can be drawn here between these studies and previous observations from members of our group where preadipocytes from obese patients demonstrated a higher than 2-fold increased in IL-8 mRNA expression compared to untreated controls (59), a response that resembles the one of UC patients. This may be due to the underlying proinflammatory environment of the organism during these conditions (UC and obesity) that may predispose these cells to more robust responses to other proinflammatory stimuli. Furthermore, early studies with differentiated adipocytes showed decreased cytokine mRNA expression in response to SP treatment. However, analysis of cells from additional patients needs to be performed in order to draw safer conclusions on this subject.

The effects of SP on adipose tissue physiology may also be exerted beyond the level of the induction of proinflammatory mediator production to effects on adipose tissue mass via expansion or even differentiation. The potential effects of SP on adipose tissue expansion via preadipocyte replication have already been demonstrated in previous studies. Gross et. al. have shown that SP increases preadipocyte replication and decreases apoptosis in human mesenteric preadipocytes via the induction of intracellular signaling cascades (40). Interestingly, some of these effects may not be regulated via SP-NK1R interactions.

In our studies we examined whether SP has the capacity to affect preadipocyte differentiation. Early results show increased PPARγ activity in response to SP after the end of the differentiation protocol (3 weeks). PPARγ is a central regulator of preadipocyte and an important contributor to insulin sensitization in mature adipocytes (86, 87)**.** In addition, a SP-induced shift in C/EBPβ and phosphor-C/EBPβ expression seems to occur between weeks 1 and 3 although not statistically significant. Additional experiments may solidify these data which at least in part suggest a role for SP in preadipocyte differentiation with a potential effect in the function of the resulting adipocytes. To prove this point, future experiments should focus on the effects of SP on functions associated with differentiated state (e.g. response to insulin stimulation). In addition to these studies, more in depth analysis of the expression of different non-phosphorylated and phosphorylated isoforms of these molecules should be performed using the available antibodies for their detection. Indeed, studies have demonstrated differential effects of these molecules after phosphorylation (C/EBPα, PPARγ, C/EBPβ) or relative to the abundance of their different isoforms (C/EBPβ) (88, 89)**.**

Creation of an adipose-specific expression model

Several animal models of altered adiposity exist and they may be both genetic (e.g. *ob/ob (90)*) or diet-induced (DIO (91)). These models provided valuable data on the potential effects of increased or reduced fat tissue levels in the development of several diseases (92, 93). However, the assessment of the contribution of adipocyte-derived molecules (necessary for the identification of potential therapeutic targets) on these diseases has been stalled by the lack of availability of adequate fat tissue-specific expression animal models. Previous attempts that have created such models employ the *cre-lox* system to drive the expression of molecules under the control of fatty acid-binding protein promoters (e.g aP2 promoter (94)). These attempts, although somewhat successful, have produced “leaky” models with expression of the desired product in cells other that adipocytes

(macrophages in the case of aP2). We now possess an adipocyte-specific promoter construct (83) and have already demonstrated its ability to drive GFP expression under the control of the adiponectin promoter (Figure 13). In addition to its demonstrated tissue specificity in mice as we describe above (83), the system that we are in the process of creating is also inducible under the addition of doxycycline allowing us to control its function at the desired time during the lifespan of the animal. Thus we can avoid unwanted expression or silencing of the genes of interest and concentrate on the effects during particular time points of the experiment.

Conclusion

The emergence of adipose tissue as an important regulator of human pathophysiology coupled with the limited research on its function identifies it as a promising field for the identification of targets that may affect the development and progression of human disease. Furthermore adipose tissue plasticity, both in size and function, during human disease strengthens the belief that interventions to alter the expression of fat-derived mediators may provide promising future therapeutic approaches for pathological conditions known to be affected by changes in adiposity. Ours and previous studies provide insight in a field where investigatory tools (cell lines, animal models) are limited. We demonstrate our ability to isolate and use several primary adipocytic cell lines from different species including humans. Furthermore, we have initiated experiments for the creation of the most reliable adipose-specific expression animal model with the additional advantage of inducible induction of the message of interest in order to avoid unwanted early effects that may affect animal pathophysiology which is often the case with transgenic animals.

We also demonstrate differential effects of the neuropeptide SP on preadipocytes from control and IBD patients suggesting that these cells may exist in different pre-activation states under different positions and insinuating a role for adipose tissue-derived proinflammatory molecules in the

development of the disease. Finally, we provide early evidence for the potential effects of SP on pradipocyte differentiation via effects on the expression of transcription factors that are central to this process. Such effects, if further verified, may provide insight on potential effects of SP on the development of a central identifying feature of Crohn's disease, namely of "creeping" fat, via the promotion of preadipocyte differentiation around the inflamed intestine.

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