

Research Article

Role of toll-like receptors and their ligands in adipocyte secretion

A Mishra^{2*}, AV Shestopalov^{1,2}, AM Gaponov², IA Alexandrov² and SA Roumiantsev^{1,2}

¹Peoples' Friendship University of Russia, Moscow, Russia

²Center for Molecular Health, Moscow, Russia

Abstract

Background: Adipose tissue is one of the main sites of energy homeostasis that regulates whole body metabolism with the help of adipokines. Disruption in its proper functioning results in adipose tissue remodeling (primarily hypertrophy and hyperplasia) which directly influences the secretion of said adipokines. Obesity characterized as chronic low-grade inflammation of the adipose tissue is one such condition that has far reaching effects on whole body metabolism. Inflammation in turn results in immune cells infiltrating into the tissue and further promoting adipocyte dysfunction.

Purpose: In our study we explored this adipose tissue-innate immunity axis by differentiating adipose tissue derived stem cells (ADSCs) into white and beige adipocytes. We further stimulated our cultures with lipopolysaccharide (LPS), flagellin, or meteorin-like, glial cell differentiation regulator (METRNL) to trigger an inflammatory response. We then evaluated Toll-like receptor (TLR) mRNA expression and secretion of interleukin (IL-6), interleukin-8 (IL-8), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) in these cultures.

Results: We found that TLR2 is the highest expressed receptor in adipocytes. Further, LPS and METRNL are strong activators of TLR2 in white and beige^{BMP7(-)} adipocytes. TLR4 was not significantly expressed in any of our cultures despite LPS stimulation. TLR9 expression is upregulated in ADSCs upon LPS and METRNL stimulation. IL-6 and IL-8 secretion is increased upon LPS stimulation in white adipocytes. METRNL activates both IL-6 and IL-8 expression in adipocyte cultures. Lastly, BDNF and NGF is secreted by all adipocyte cultures with beige^{BMP7(-)} and beige^{BMP7(+)} secreting slightly higher amounts in comparison to white adipocytes.

Conclusion: ADSCs and adipocytes alike are capable of expressing TLRs, but white adipocytes remain the highest expressing in both control and stimulated cultures. TLR2 is highly expressed in white and beige adipocytes whereas TLR4 showed no significant expression. LPS and METRNL trigger IL-6 and IL-8 secretion in adipocytes. Products of white adipocyte "browning" are capable of secreting higher amounts of BDNF and NGF in comparison to white adipocytes.

Introduction

In the recent past a renewed interest in adipocyte biology has been sparked, with particular focus on its secretory activity and by extension endocrine function. The discovery of leptin and adiponectin as the first adipokines (hormones of the adipose tissue) fundamentally changed our understanding of the adipose tissue and its impact on energy homeostasis. In addition to regulating metabolism and insulin sensitivity, the adipose tissue (via adipokines) has been speculated to play a role in neuroendocrine regulation and the immune system [1].

As one of the main sites of energy homeostasis, the adipose tissue upholds the delicate balance between lipogenesis

(triglyceride accumulation) and lipolysis (releasing energy substrates). This tightly monitored process is made possible by constant communication between various tissues – pancreas, brain, adipose tissue, skeletal muscles, liver, etc. [2]. Disruption in these metabolic processes can arise when the adipose tissue comes in contact with pathogens or pathogen-associated molecular patterns (PAMPs).

Toll-like Receptors (TLR) are a group of transmembrane proteins that recognize pathogen-associated (PAMP) and damage-associated molecular patterns (DAMP). Human adipocytes have been shown to express TLR1-10; TLR1, 2, 4, 5, and 6 are located on the cell membrane whereas TLR3, 7, 8, and 9 are located in the cytoplasm. Exogenous or

More Information

***Address for Correspondence:** A Mishra, Center for Molecular Health, Moscow, Russia, Tel: +79857750383; Email: mishra@phystech.edu

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ORCID: orcid.org/0000-0001-5594-3805

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endogenous ligands acting upon these receptors initiate a pro or anti-inflammatory cascade which can affect glucose energy homeostasis particularly in adipocytes.

Currently, pathogenesis of obesity is categorized as not only as a form of metabolic dysfunction, but also as chronic low-grade inflammation. This prolonged state of inflammation causes what is known as adipose tissue remodeling leading to an abnormal increase in size (hypertrophy) and number (hyperplasia) of adipocytes in the tissue. Hypertrophy and hyperplasia in turn negatively affect adipose tissue endocrine function by altering expression and secretion of adipokines.

Most of our understanding of adipokines and their effect on the human metabolism comes from analyzing blood samples. Although, the regulatory mechanisms involved in their synthesis remain elusive. Despite this, the existence of regulatory axes has been described – a microbiota and adipose tissue axis [3] and an adipose tissue and innate immunity axis [4]. In an attempt to better understand this relationship and possibly find a link between the three systems, we decided to conduct our research on Adipose tissue derived stem cells (ADSCs) and the products of their adipogenic differentiation.

To simulate inflammation in our cultures, we picked two known TLR agonists of bacterial origin – lipopolysaccharide and flagellin to stimulate our cultures. We also chose a myokine which has recently been found to promote synthesis of antimicrobial peptides in the gut. This myokine - meteorin-like, glial cell differentiation regulator (METRNL) also known as subfatin - is named so because of its similarity a cytokine named meteorin found circulating in the central nervous system. Though originally thought to be a myokine only secreted by contracting skeletal muscles, in recent years it has been shown to be an adipokine as well [5]. It acts on the adipose tissue via eosinophils present in their matrix and activates a pro-inflammatory response

In this paper we studied the influence of TLR agonists – PAMPs (LPS and flagellin) and METRNL on the secretory activity of ADSC cell cultures subjected to adipogenic differentiation and “beige” transdifferentiation. We then compared the levels of IL-6, IL-8, brain-derived neurotrophic factor (BDNF), and neural growth factor (NGF) secretion and TLR expression.

Materials and methods

ADSC isolation and adipogenic differentiation

ADSCs isolated from lipoaspirates (subcutaneous adipose tissue) of 10 healthy female donors (ages 24-44) were used in this experiment. The study was approved by the Local Ethics Committee of the Pirogov Russian National Research Medical University; permit number 186, granted on 26th June 2019. All donors gave their consent for participating in this study.

ADSCs from adipose tissue were isolated using 0.2%

type I collagenase (Paneco, Russia) for 45 minutes at 37°C on a shaker. The subsequent stromal vascular fraction was separated and seeded on to T-75 flasks (Corning, USA) and brought to a confluency of 80% - 85% in basal media – DMEM/F12 (Paneco, Russia) with 2 mM L-glutamine, 10% FBS and 40 µg/mL gentamycin. Once the ADSCs had been passaged a minimum of 6 times, the protocol for adipogenic differentiation was commenced.

White adipocyte cultures were cultivated by incubating ADSCs in MesenCult Adipogenic Differentiation Medium (StemCell Technologies, Canada) for 21 days.

Beige adipogenic differentiation was carried out by following two different protocols depending on the absence or presence of a “browning” factor - bone morphogenic protein (BMP7). Beige adipogenic transdifferentiation devoid of BMP7 (beige adipocytes^{BMP7(-)}) was undertaken in successive phases of induction (4 days) and maintenance (3 days) over a span of 21 days.

Adipocyte cultures incubated with 125 ng/mL of BMP7 (PeproTech, UK) for three days prior to commencing adipogenic differentiation were termed as beige adipocytes^{BMP7(+)}. During the inductive phase, adipocytes were grown in MesenCult Adipogenic Differentiation Medium with the addition of 1nM T₃ (Sigma, USA) and 1µM rosiglitazone (Sigma, USA). The cells were held in basal media for MSCs during the maintenance phase.

At the end of 21 days, the cells were treated with one of the following stimulants - 1µg/mL lipopolysaccharide (LPS) (Sigma, #L2630) 10 ng/mL flagellin (Sigma, #SRP8029), or 5 µM meteorin-like, glial cell differentiation regulator (METRNL) (Aviscera Bioscience, #00478-01-100) for a period of 16 hours.

Multiplex ELISA

The supernatants of ADSCs and adipocyte cultures were quantitatively analyzed for IL-6, IL-8, BDNF, and NGF with the help of multiplex ELISA. The kits used were Milliplex map Human Adipokine Magnetic Bead Panel 2 – Endocrine Multiplex Assay (HADK2MAG-61K – Merck, USA) and Milliplex map Human Myokine Magnetic Bead Panel (HMYOMAG-56K). The tests were performed on MAGPIX (Luminex, USA) as per the manufacturer’s instructions.

RT-qPCR

RNA was extracted with the help of ExtractRNA kit (Evrogen, Russia) and SatelliteRed (Evrogen, Russia) using manufacturer’s instructions. cDNA was synthesized using MMLV revertase and poly-T primers following manufacturer’s protocol of the kit MMLV RT (Evrogen, Russia). RT-qPCR was carried out in the amplificator CFX 1000 (BioRad laboratories, USA). The primers used for the housekeeping gene and TLRs

are listed in table 1. β -glucuronidase (GUSB) was used as the housekeeping gene in our experiments. The reaction was carried out in the following conditions: pre-heating at 95 °C for 3 minutes for 40 cycles, following by 15 seconds of denaturation at 61 °C, and finally elongation at 72 °C for 30 seconds. PCR melting curves were obtained to ensure assay specificity by analyzing the level of fluorescence between 65 °C to 95 °C at 0.5 °C intervals.

Statistical analysis was performed using parametric and nonparametric methods implementing Student's t-test and Wilcoxon signed-rank test using the program OriginLab.

Results

Expression of Toll-like Receptors in ADSCs and the products of their adipogenic differentiation.

TLR2 mRNA expression

Change in mRNA expression of TLR2 was the most pronounced out of all the receptors that we analyzed (Table 2). Overall, stimulated ADSC and adipocyte cultures expressed a change in gene expression but only the changes in white and beige^{BMP7(-)} adipocyte cultures were significant. LPS increased the expression of TLR2 by 63 and 21 times in white and beige^{BMP7(-)} adipocytes respectively. In white adipocytes METRNL stimulation increased TLR2 expression by 36 times.

Expression of Toll-like receptor (TLR) 2, 4, and 9 in Adipose-derived stem cells (ADSCs) and adipocytes. The relative expression was measured when compared with their respective control cultures. The letter p indicates *p* - value for that data set; beige^{BMP7(-)} – beige differentiation without BMP7 induction; beige^{BMP7(+)} – beige differentiation with BMP7 induction.

Table 1: Primers used to detect expression of Toll-like receptors in ADSCs and adipocytes.

Gene	Forward Primer	Reverse Primer
GUSB	CAGAGCGAGTATGGAGCAGAA	CAAATGAGCTCTCCAACCACGT
TLR2	ATAGTGACTCCCAGGAGCTC	GACCCACACCATCCACAAG
TLR4	TCTCCTGCGTGAGACCAGAAA	TTAGGCTCTGATATGCCCATCT
TLR9	ATCCTTCCCTGTAGCTGCTGT	AGCATGATGGCCTGCACCA

Table 2: Relative expression of TLRs in ADSCs and adipocytes.

		ADSC	White adipocytes	Beige ^{BMP7(-)} adipocytes	Beige ^{BMP7(+)} adipocytes
TLR2	LPS	3.43	63.14 <i>p</i> < 0.05	21.86 <i>p</i> < 0.05	0.44
	Flagellin	2.96	2.1 <i>p</i> < 0.05	1.62 <i>p</i> < 0.05	1.24
	Metnrl	7.29	36.7 <i>p</i> < 0.05	1.617	-0.24
TLR4	LPS	-1.23	1.3	5.51	1.37
	Flagellin	-1.84	1.92	1.57	-1.14
	Metnrl	-0.1	3	1.16	1.24
TLR9	LPS	5.27	2.2	16.13 <i>p</i> < 0.05	-1.2
	Flagellin	3.26 <i>p</i> < 0.05	1.52	-3.08	-1.28
	Metnrl	2.02 <i>p</i> < 0.05	4.31	0.33	-0.01

TLR4 mRNA expression

None of our cultures seemed to show a difference in TLR4 expression upon stimulation. Though it is a well-known fact that adipocytes express TLR4 on their membranes. Though LPS is a potent TLR4 agonist, our study reported no significant change from basal expression levels.

TLR9 mRNA expression

In our adipose derived ADSC cultures we observed a 3-fold and 2-fold increase in TLR9 expression upon flagellin and METRNL stimulation respectively. Additionally, beige^{BMP7(-)} adipocytes showed a 16-fold increase upon LPS stimulation. When stimulated, ADSCs and white adipocytes showed a tendency of higher expression though not always statistically significant.

Secretion of metabolites by ADSCs and products of their adipogenic differentiation.

Interleukin-6 (IL-6)

Cultures of ADSCs and the products of their white and beige adipogenic differentiation were all capable of secreting IL-6. Although, the difference in secretion between the primary ADSC culture and subsequent adipocyte cultures remained statistically insignificant. Control cultures of beige^{BMP7(+)} adipocytes secreted 3 times the amount of IL-6 in comparison to white adipocytes. This is in agreement with reported results showing that beige adipocytes tend to secrete more IL-6 than white adipocytes.

LPS drastically increased the secretion of IL-6 in white adipocytes by 11 times but did not seem to cause a change in either of the beige adipocyte cultures (Table 3). Flagellin did not cause a change in the secretion of IL-6 in any of our cell cultures. METRNL had the most profound effect on IL-6 secretion in all the adipocyte cultures. Secretion in white and beige^{BMP7(+)} adipocytes increased by 12 times and by 8 times in beige^{BMP7(-)} adipocytes.

Interleukin-8 (IL-8)

Adipocyte cultures tend to secrete higher levels of IL-8 in comparison to ADSCs, but our results did not show a significant difference. ADSCs were not affected by LPS, flagellin, or METRNL stimulation. LPS in white adipocytes caused a nearly 10-fold increase in secretion when compared to control cultures. METRNL raised IL-8 secretion in beige^{BMP7(-)} and beige^{BMP7(+)} adipocytes by 6 times and by 10 times in white adipocytes.

Brain-derived Neurotrophic Factor (BDNF)

Secreted BDNF levels remained unchanged in ADSCs despite LPS or METRNL stimulation (Table 4). Flagellin increased secretion in ADSCs by 37%. Beige^{BMP7(-)} adipocytes under basal conditions increased their BDNF secretion by 26% when compared to white adipocytes. Similarly, beige^{BMP7(+)}



Table 3: Effect on the secretion of IL-6 and IL-8 in ADSCs and adipocytes.

		IL-6				IL-8			
		Control	LPS	Flagellin	Metnrl	Control	LPS	Flagellin	Metnrl
ADSC	M	1063.57	19571.53	1845.93	20772.92	1182.87	12841.3	2382.7	23142.59
	Q1	576.35	5871.93	796.165	9533.165	240.5	11249.9	812	15050.45
	Q3	9719.29	20772.92	40436.96	65854.27	73514.09	70618.54	21073.99	123357
White	M	524.77	5777.34 <i>p1 < 0.01</i>	975.51	6627.03 <i>p1 < 0.01</i>	2218.91	21533.84 <i>p1 < 0.01</i>	6951.53	23356.45 <i>p1 < 0.01</i>
	Q1	380.985	3518.445	477.745	2651.84	918.47	16930.72	1264.985	16165.61
	Q3	844.3	20772.92	1702.645	15589.21	3279.495	26806.4	8454.705	39843.35
Beige BMP7(-)	M	2553.38	20772.92	2025.23	20772.92 <i>p1 < 0.01</i>	7395.52	23008.34	6355.29	50841.06 <i>p1 < 0.01</i>
	Q1	1365.98	11355.06	834.25	15203.46	3608.935	13614.36	3698.725	28818.17
	Q3	12045.27	20772.92	6805.24	20772.92	16063.19	29553.65	16387.48	123357
Beige BMP7(+)	M	1659.12 <i>p2 < 0.01</i>	13231.06	1720.08	20772.92 <i>p1 < 0.01</i>	6660.96	39352.4	5702.01	41624.66 <i>p1 < 0.01</i>
	Q1	1232.485	7631.015	1203.025	5342.825	3055.55	19532.48	3448.065	35181.68
	Q3	3773.59	20772.92	4984.34	20772.92	12108.15	123357	10413.3	123357

Table 4: Effect on the secretion of BDNF and NGF in ADSCs and adipocytes.

		BDNF				NGF			
		Control	LPS	Flagellin	Metnrl	Control	LPS	Flagellin	Metnrl
ADSC	M	14.64	14.64	20.06 <i>p1 < 0.01</i>	14.64	10.12	10.12	11.75	16.83
	Q1	10.62	10.62	14.325	11.46	8.05	12.12	9.495	13.525
	Q3	23.0425	23.0425	28.04	40.8575	17.6	26.86	16.39	20.135
White	M	11.6	18.47	14.59	21.18	7.22	15.1 <i>p1 < 0.01</i>	10.2	6.81 <i>p1 < 0.01</i>
	Q1	7.42	12.365	11.875	19.4775	4.29	13.12	5.055	4.505
	Q3	13.505	30.2425	15.6175	28.49	10	17.695	14.08	9.115
Beige BMP7(-)	M	14.64 <i>p2 < 0.01</i>	25.74	25.8	26.99	22.92 <i>p2 < 0.01</i>	40.2	22.52	17.05
	Q1	11.8575	14.4675	21.915	20.7225	16.65	19.95	14.375	16.205
	Q3	55.1375	28.035	39.925	45.035	46.675	58.435	32.575	17.895
Beige BMP7(+)	M	21.6 <i>p2 < 0.01</i>	24.1	23.6	36.87 <i>p1 < 0.01</i>	22.43 <i>p2 < 0.01</i>	39.31	15.88	8.39 <i>p1 < 0.01</i>
	Q1	15.9075	20.2075	16.88	25.8	13.815	17.54	12.42	8.13
	Q3	54.92	85.57	41.04	88.5775	30.735	73.365	36.945	8.65

adipocytes also facilitated secretion of the metabolite though the difference was more profound at 86% or nearly twice the amount. Lastly, METRNL increased BDNF secretion by 1.7 times in beige^{BMP7(+)} adipocytes.

Nerve Growth Factor (NGF)

NGF secretion levels remain unchanged in ADSCs under basal and stimulated conditions alike. Compared to white adipocytes, both beige^{BMP7(-)} and beige^{BMP7(+)} adipocytes secreted 3 times the amount of BDNF. LPS was able to double the secretion in white adipocyte cultures. METRNL reduced BDNF secretion in white adipocytes by a mere 5.8% and by a factor of 2.6 in beige^{BMP7(-)} adipocytes.

The table lists the secretion of Interleukin-6 (IL-6) and Interleukin-8 (IL-8) in pg/mL. The values displayed are medians along with the first (Q1) and third (Q3) quartiles. ADSCs – adipose tissue derived stem cells; White – white adipocytes; beige^{BMP7(-)} – beige differentiation without BMP7 induction; beige^{BMP7(+)} – beige differentiation with BMP7 induction. p1 is the comparison of cell cultures to their respective control cultures. p2 is the comparison of beige or brown control cultures with white adipocyte control cultures.

The table lists the secretion of Interleukin-6 (IL-6) and Interleukin-8 (IL-8) Brain-derived Neurotrophic Factor (BDNF), and Neural Growth Factor (NGF) in pg/mL. The values displayed are medians along with the first (Q1) and third (Q3) quartiles. ADSCs – adipose tissue derived stem cells; White – white adipocytes; beige^{BMP7(-)} – beige differentiation without BMP7 induction; beige^{BMP7(+)} – beige differentiation with BMP7 induction. p1 is the comparison of cell cultures to their respective control cultures. p2 is the comparison of beige or brown control cultures with white adipocyte control cultures.

Discussion

Over a decade ago, the link between lipid accumulation and inflammation observed during obesity began to be questioned. Adipose tissue remodeling as a result of chronic low-grade inflammation is described by hypertrophy, hyperplasia, increase in blood vessels, macrophage recruitment, and increased secretion of proinflammatory cytokines and interleukins [6]. Distress caused by PAMPs or DAMPs is recognized by cells that express TLRs. Following the activation of a proinflammatory cascade, cells of the immune system are recruited to tissues through cytokine and chemokine signaling.



A wide host of microbial components originating from bacteria, fungi, parasites, and viruses are recognized by TLR2. TLR2 and TLR4 have been implicated in promoting pro-inflammatory processes, regulating glucose uptake in adipocytes, and decreasing adipocyte differentiation [4,8]. These processes point to an evolutionary link between innate immunity and energy homeostasis. Due to its ability to recognize a variety of bacterial components, it is possible that TLR2 mRNA expression drastically increased in our white and beige adipocytes upon LPS and flagellin stimulation.

LPS is the most potent ligand associated with TLR4, though endogenous ligands such as free fatty acids (FFAs) released by adipocytes during lipolysis are also of great significance. Binding of LPS with TLR4 triggers the Toll/interleukin-1 receptor (TIR) cascade leading to the activation of NF κ B and Mitogen-activated protein kinase (MAPK) [9]. In our results, we did not observe a statistically significant change in TLR4 expression in any of our cultures including those stimulated with LPS. Adipocytes in culture have shown to begin secreting cytokines within 4 hours of LPS stimulation [10]. Prolonged LPS stimulation (16 hours in our study), possibly decreased TLR4 mRNA levels or simply degraded it due to a negative feedback loop. FFAs are a known product of lipolysis, which in turn is a common effect observed upon LPS stimulation in adipocytes. FFAs also bear resemblance to other TLR4 ligands and can bind to the receptor in an autocrine fashion. This could possibly cause TLR4 mRNA degradation [11] resulting in a statistically insignificant change in TLR4 expression upon LPS stimulation in all our cell cultures. It must be noted that though an increased expression was not observed, it is not the absolute indication of constitutive TLR4 expression or the presence of the receptor on the cell membrane. In other words, the basal levels of TLR4 mRNA in adipocytes might be high enough to begin with. In future studies, it would be worth considering reducing the concentration and incubation time of LPS in cell cultures so as to hopefully observe a higher TLR4 expression.

It has repeatedly been suggested that in the adipose tissue non-fat cells are the major contributors of TLR expression and not the adipocytes themselves. In murine 3T3-L1 cells TLR2 is expressed throughout adipogenic differentiation whereas TLR4 more so in mature adipocytes. One can argue that adipocyte cultures that are obtained via differentiation of ADSCs are not fully mature. Hence, their transcriptome and secretory profile resemble that of preadipocytes and not of mature adipocytes or primary adipocyte cultures. Interestingly, beige^{BMP7(+)} adipocytes in our study failed to change TLR4 expression upon stimulation with LPS, flagellin, or METRNL. A study reported the expression of TLR2 and TLR4 in brown adipocytes and observed the upregulation of only TLR2 during brown adipocyte differentiation. Additionally, TLR2 and TLR4 activation caused a decrease in basal UCP-1 expression and suppression of mitochondrial biogenesis [12]. Brown adipose tissue in the body remains mostly dormant

unless acted upon by ligands of β -adrenergic receptors (β AR). During the 16-hour incubation with our stimulants (PAMPs and METRNL), we held beige^{BMP7(+)} adipocyte cultures without their β AR agonists (T_3 and rosiglitazone). Since lipolysis is a direct consequence of brown adipose tissue activation (active UCP-1 expression), one can imagine the deleterious effects it might have if acted upon by TLR2 and TLR4 agonists. The inactivity of brown adipocytes could thus hint at an overall protective role against conditions which could potentially lead to serious conditions such as cachexia.

Unlike TLR2 and TLR4, TLR9 is found in the cytoplasm and is known to recognize PAMPs and DAMPs (prokaryotic and eukaryotic alike) [13]. A known consequence of prolonged inflammation - specifically in the case of obesity - is hyperplasia which is the increase in number of cells. Sensing stress and/or damage markers, ADSCs could release growth factors and cytokines that lead to their proliferation and differentiation into preadipocytes via TLR9.

METRNL also known as subfatin or IL-39 is a pro-inflammatory cytokine that was previously described as a myokine secreted by skeletal muscles during resistance training [14]. An extensive review on METRNL expression levels in various human tissues reported that the colon is the primary site of METRNL production [15]. Its abundance in barrier tissues (intestinal epithelium, skin, and lungs) was shown to play a role in the synthesis of antimicrobial peptides. In our study METRNL increased TLR2 expression in white adipocytes by 36 times. This could point to a link between gut microbiota and the adipose tissue. Inflammation (perhaps even as a result of obesity) has been known to cause an increase in circulating levels of LPS [16]. Perhaps this low-grade chronic inflammation also releases flagellin and METRNL from the gut promoting direct or indirect changes in adipose tissue functioning.

IL-6 is increasingly being viewed not only as a proinflammatory cytokine, but also as a modulator of whole-body metabolism. Of particular interest is its role as a myokine (cytokine secreted by myocytes) acting on several tissues like the liver, pancreas, brain, and the adipose tissue. On the other hand, IL-8 is a chemokine primarily produced by macrophages and is often used as a predictor for coronary heart disease. The adipocyte response to inflammation has been characterized as an increase in the secretion of certain cytokines and chemokines - TNF α , IL-6, IL-1 β , IL-8, and Monocyte chemoattractant and activating protein-1 (MCP-1) [17]. IL-8 levels in blood are increased during obesity which indicates its possible involvement in glucose homeostasis [18]. It has been reported that beige and brown adipocytes in culture secrete higher levels of IL-6 in comparison to white adipocytes. So much so that prolonged blocking of IL6 receptors in beige adipocytes seems to inhibit "browning" [19]. Our data seems to agree with these observations and although not much is known about IL-8 secretion in beige and brown

adipocytes, we observed it to be similar to IL-6. LPS is known to trigger a proinflammatory response in cells expressing TLR4. TLR4 activation in these cells leads to the secretion of multiple cytokines including IL6 and TNF α via the NF κ B pathway [20]. Fascinatingly the effects of LPS, flagellin, and METRNL stimulation in our study followed the same trend for both IL-6 and IL-8 secretion. This suggests that upon sensing a pro-inflammatory signal, adipocytes release cytokines (eg. IL-6) as well as chemokines (eg. IL-8) to recruit immune cells into the adipose tissue. Interestingly, METRNL was the only stimulator in our study which significantly increased IL-6 secretion in all three of our adipocyte cultures. In adipocytes, overall stimulation of “browning” can be enhanced by the autocrine function of IL-6 [21].

BDNF and NGF belong to a class of factors called neurotrophins that regulate the proper growth and functioning of neural tissue, both of which are also secreted by the adipose tissue [22]. It is interesting to note that just like the adipose tissue is capable of secreting neurotrophins, the brain is known to secrete adipokines (leptin and adiponectin). Knowing that via leptin (the satiety hormone) the adipose tissue and brain together are able to regulate appetite, it may be assumed that neurotrophins in turn are also able to influence energy homeostasis [23]. Our results showed a slight but statistically significant increase in the secretion of BDNF and NGF in beige^{BMP7(-)} and beige^{BMP7(+)} adipocytes when compare to white adipocytes. Overexpression of BDNF has been speculated to cause “browning” and upregulation of UCP-1 expression in white adipocytes. Additionally, thermogenesis due to environmental factors or exercise which induces “browning” (via the sympathetic nervous system or skeletal muscles) may be due to the endogenous effect of BDNF on white adipocytes [24]. NGF along with FGF2 secreted by brown adipocytes directly act upon the sympathetic nervous system causing an increase in brown preadipocyte recruitment required for thermogenesis [25].

In conclusion, we stimulated our cell cultures with LPS, flagellin, or METRNL in an attempt to better understand the interactions between the adipose tissue and innate immunity. We observed that TLR4 expression does not change in adipocyte cultures and ADSCs upon stimulation with TLR agonists. Although TLR2 expression in white and beige^{BMP7(-)} adipocytes was significantly increased upon PAMP stimulation. Flagellin and METRNL increased TLR9 expression in ADSCs. IL-6 and IL-8 secretion show identical tendencies with METRNL acting as the strongest activator in all adipocyte cultures. Lastly, ADSCs and adipocyte cultures are capable of secreting neurotrophic factors (BDNF and NGF). Where beige^{BMP7(-)} and beige^{BMP7(+)} adipocytes secrete higher levels when compared to white adipocytes.

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