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Abstract: Brown adipose tissue (BAT) plays essential role in metabolic- and thermoregulation and displays morphological and functional plasticity in response to environmental and metabolic challenges. BAT is a heterogeneous tissue containing adipocytes and various immune-related cells, however, their interaction in regulation of BAT function is not fully elucidated. Fractalkine is a chemokine synthesized by adipocytes, which recruits fractalkine receptor (CX3CR1)-expressing leukocytes into the adipose tissue. Using transgenic mice, in which the fractalkine receptor, Cx3cr1 gene was replaced by Gfp, we evaluated whether deficiency in fractalkine signaling affects BAT remodeling and function in high-fat-diet - induced obesity. Homo- and heterozygote male CX3CR1-GFP mice were fed with normal or fat enriched (FatED) diet for 10 weeks. Interscapular BAT was collected for histological and qPCR analysis. Heterozygous animals in which fractalkine signaling remains intact, gain more weight during FatED than CX3CR1 deficient gfp/gfp homozygotes. FatED in controls resulted in macrophage recruitment in the BAT with increased expression of proinflammatory mediators (Il1a,b, Tnfa and Ccl2). Local BAT inflammation was accompanied by increased expression of lipogenic enzymes and resulted in BAT "whitening". By contrast, fractalkine receptor deficiency prevented accumulation of tissue macrophages, selectively attenuated the expression of Tnfa, Il1a and Ccl2, increased BAT expression of lipolytic enzymes (Atgl, Hsl and Mgtl) and upregulated genes involved thermo-metabolism (Ucp1, Pparg Pgc1a) in response to FatED. These results highlight the importance of fractalkine-CX3CR1 interaction in recruitment of macrophages into the BAT of obese mice which might contribute to local tissue inflammation, adipose tissue remodeling and regulation of metabolic-related genes.

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March, 07, 2016

Dear Editors,

Enclosed please find our manuscript entitled: Brown Adipose Tissue in Obesity: Fractalkine-receptor Dependent Immune Cell Recruitment and Metabolic-related Gene Expression" to be published in BBA Molecular and Cell Biology of Lipids.

In this study, we aimed to identify the chemokine mechanism which is responsible for recruitment of macrophages in the brown adipose tissue in response to high fat diet. We have found that fat-enriched diet results in "whitening" the BAT of obese animals, upregulation of local production of proinflammatory cytokines, chemokines, impaired induction of lipolytic enzymes and theromogenic genes. Among the chemokines upregulated in obese mice BAT, fractalkine might play a crucial role in diet-induced morphological and functional rearrangements, because mice with targeted deletion of fractalkine receptor gain less weight and have reduced proinflammatory cytokine expression in the BAT. Mice with deficient fractalkine signaling were able to mount significant upregulation of thermometabolic and lipolytic enzymes's genes in the BAT when exposed to fat enriched diet.

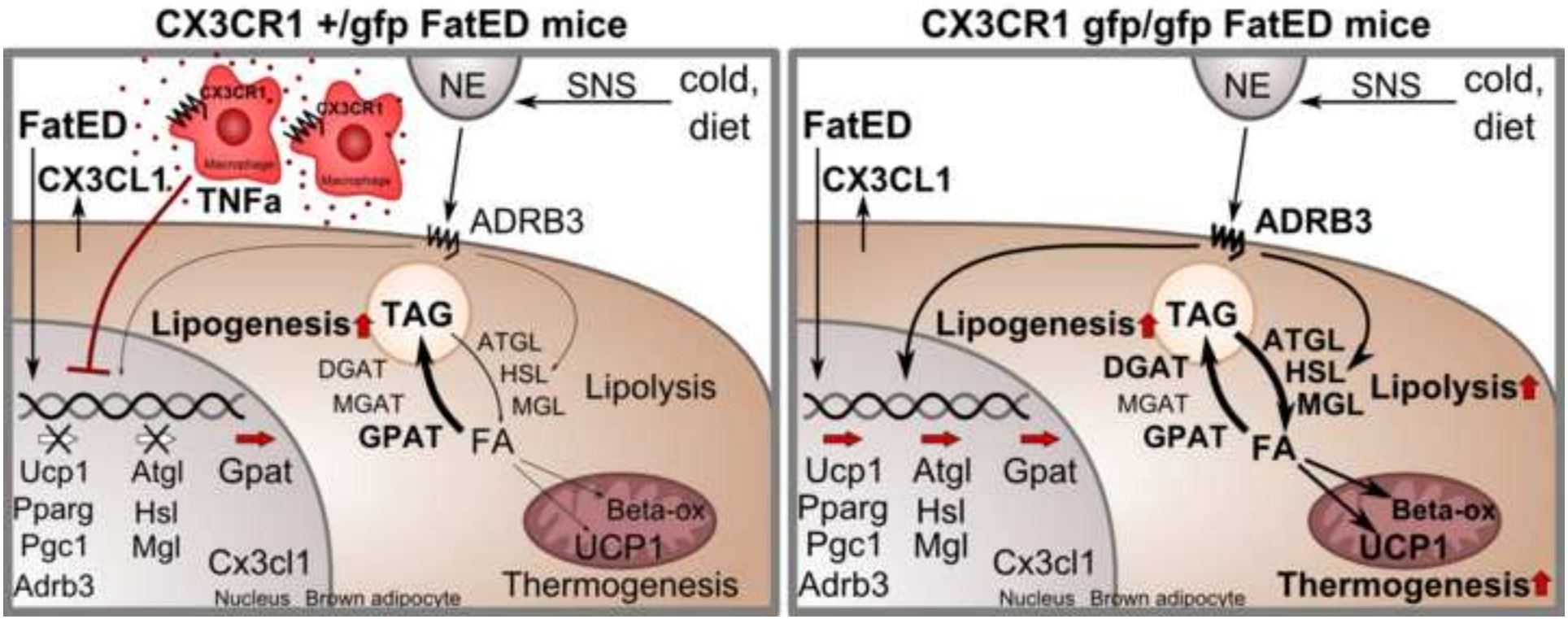
These results are novel and important, because the role of fractalkine in BAT function has not been addressed before. On the basis of our present and previous findings, fractalkine signaling might be a potential target to fight obesity and metabolic inflammation.

This study is a continuation of our previously published paper (Polyák et al. BBI 38:25-35,2014) in which we studied the role of fractalkine in the hypothalamus, ***WAT*** and liver. Here we confirm that this present manuscript describes our original findings on ***BAT*** and has not been and will not be submitted for publication elsewhere.

Sincerely,

Krisztina J. Kovács, PhD

Graphical Abstract (for review)



Highlights (for review)

- Fat enriched diet results in accumulation of leukocytes into the BAT and local inflammation.
- BAT accumulation of macrophages depends on fractalkine signaling.
- Thermogenic and lipolytic genes are induced in FatED mice with impaired fractalkine signaling.
- Fractalkine receptor deficient mice are protected from FatED-induced obesity.

Brown Adipose Tissue in Obesity: Fractalkine-receptor Dependent Immune Cell Recruitment Affects Metabolic-related Gene Expression

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Abstract

Brown adipose tissue (BAT) plays essential role in metabolic- and thermoregulation and displays morphological and functional plasticity in response to environmental and metabolic challenges. BAT is a heterogeneous tissue containing adipocytes and various immune-related cells, however, their interaction in regulation of BAT function is not fully elucidated. Fractalkine is a chemokine synthesized by adipocytes, which recruits fractalkine receptor (CX3CR1)-expressing leukocytes into the adipose tissue. Using transgenic mice, in which the fractalkine receptor, *Cx3cr1* gene was replaced by *Gfp,* we evaluated whether deficiency in fractalkine signaling affects BAT remodeling and function in high-fat-diet - induced obesity. Homo- and heterozygote male CX3CR1-GFP mice were fed with normal or fat enriched (FatED) diet for 10 weeks. Interscapular BAT was collected for histological and qPCR analysis. Heterozygous animals in which fractalkine signaling remains intact, gain more weight during FatED than CX3CR1 deficient gfp/gfp homozygotes. FatED in controls resulted in macrophage recruitment in the BAT with increased expression of proinflammatory mediators *(Il1a,b, Tnfa and Ccl2*). Local BAT inflammation was accompanied by increased expression of lipogenic enzymes and resulted in BAT "whitening". By contrast, fractalkine receptor deficiency prevented accumulation of tissue macrophages, selectively attenuated the expression of *Tnfa, Il1a and Ccl2,* increased BAT expression of lipolytic enzymes *(Atgl, Hsl and Mgtl)* and upregulated genes involved thermo-metabolism (*Ucp1, Pparg Pgc1a*) in response to FatED. These results highlight the importance of fractalkine-CX3CR1 interaction in recruitment of macrophages into the BAT of obese mice which might contribute to local tissue inflammation, adipose tissue remodeling and regulation of metabolic-related genes.

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Keywords: fractalkine, macrophage, inflammation, triglyceride metabolism, thermogenesis, obesity, BAT

Abbreviations: BAT – brown adipose tissue, WAT – white adipose tissue, CX3CL1 – fractalkine, CX3CR1 – fractalkine receptor, GFP – green fluorescent protein, HFD – high fat diet, FatED – fat enriched diet, ND – normal diet, PND – postnatal day, qPCR – quantitative real time polymerase chain reaction, IL1A – interleukin 1 alpha, IL1B – interleukin 1 beta, IL6 – interleukin 6, TNFa – tumor necrosis factor alpha, CCL2 (MCP1) - chemokine (C-C motif) ligand 2, UCP1 – uncoupling protein 1, PPARG - peroxisome proliferator-activated receptor gamma, PGC1A (PPARGC1A) - peroxisome proliferator-activated receptor gamma, coactivator 1 alpha, TH - tyrosine hydroxylase, ADRB3 - adrenoceptor beta 3, DIO2 - Type 2 Iodothyronine Deiodinase, GLUT4 - Glucose transporter type 4, DGAT1 - diacylglycerol O-acyltransferase 1, MGAT - mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase, GPAT - glycerol-3-phosphate acyltransferase, ATGL (PNPLA2) - adipose triglyceride lipase, HSL (LIPE) - lipase, hormone-sensitive, MGL - monoglyceride lipase, FA – fatty acid.

1. Introduction

Obesity and diabetes are worldwide epidemics driven by the disruption in energy balance [1]. Brown adipose tissue (BAT) is the major site for cold- and diet-induced thermogenesis with which BAT significantly affects systemic glucose and lipid metabolism [2-4]. In 2007 Nedergaard et al. published that adult humans possess active BAT [5]. The amount of BAT is inversely correlated with body-mass index, especially in older people [6]. Metabolically active BAT seems to be particularly low in patients with obesity or diabetes [7]. These results suggest a significant role of brown adipose tissue in adult human metabolism and opens new opportunities to develop therapeutic interventions to treat obesity.

Brown adipocytes and inducible brown-in-white (brite, beige) adipocytes are multilocular and contain significantly higher number of mitochondria than other adipocytes in the body [8]. These cells are specialized to dissipate energy in the form of heat by uncoupled thermogenesis, mediated by the dissociation of mitochondrial respiratory chain electron transport from ATP synthesis via the action of uncoupling protein UCP1. In addition to adipocytes, adipose tissues contains various immune-related cells including resident macrophages, eosinophils, mast cells and T cells, which significantly contribute to their function via release (adipo)cytokines and transmitters in paracrine or endocrine fashion. [9-12]. Both types of adipose tissues (BAT and WAT) are sensitive to environmental (temperature) - hormonal (T3, leptin, insulin, corticosteroid) - and metabolic (high fat diet) cues and display significant cellular and functional remodeling in response to these challenges. For instance, high fat diet results in hypertrophy and hyperplasia of white adipocytes and recruitment of monocytes into the WAT [13]. Furthermore, in obese animals and humans there is a shift from alternatively (anti-inflammatory) polarized macrophages to those that produce predominantly proinflammatory mediators [14, 15]. However,

the accumulation of macrophages to BAT, the mechanisms that recruit and activate them, and their effect on thermometabolic genes has not been fully elucidated. Because these changes contribute to insulin resistance and low grade systemic metabolic inflammation which is seen in a subset of obese patients with metabolic X [16], it is important to understand the mechanisms that recruit and activate adipose tissue macrophages and the means with which local inflammation affects lipid metabolism and thermoregulation.

Fractalkine (CX3CL1) is a chemokine expressed in endothelial cells, vascular smooth muscle cells, hepatocytes, adipocytes and neurons as a transmembrane protein and involved in trafficking and capturing various leukocytes (monocytes, macrophages, microglia) expressing its'cognate receptor, CX3CR1 [17, 18]. Fractalkine -released from the cell surface by proteolytic cleavage- acts in paracrine and endocrine manner and has been identified in the WAT as a novel adipocytokine with increased expression in obese individuals [19]. It has been shown previously that lack of CX3CL-CX3CR1 signaling results in reduced macrophage accumulation into white adipose tissue and reduced body weight gain during the development of obesity [20].

The aim of the present study was to identify the role of fractalkine/CX3CR1 signaling in the recruitment of monocytes into the brown adipose tissue and to reveal the role of local inflammation in regulation of genes involved in triglyceride- and thermo-metabolism in obese mice.

2. Materials and methods

2.1. Animals and diet

Experiments were performed in male CX3CR1 +/gfp (+/gfp), and CX3CR1 gfp/gfp (gfp/gfp) mice [17]. Animals were obtained from the European Mouse Mutant Archive (EMMA $Cx3cr1^{tm1Litt}$ MGI:2670351). The background C57Bl/6J strain has been shown to be genetically vulnerable to diet-induced obesity [21]. In these mice, the *Cx3cr1* gene was replaced by a *Gfp* reporter gene such that heterozygote CX3CR1 +/gfp mice express GFP in cells of the myeloid linage and retain receptor function, whereas monocytes in homozygote CX3CR1 gfp/gfp mice are labeled with GFP and lack functional CX3CR1. Genotype of the animals has been verified by PCR using combination of three different primers as described by Jung et al [17].

Animals were housed in groups of 4-5/cage at the minimal disease (MD) level of the Medical Gene Technology Unit of our Institute, had free access to food and water and were maintained under controlled conditions: temperature, 21 °C ± 1 °C; humidity, 65%; light-dark cycle, 12-h light/12-h dark cycle, lights on at 07:00. At 35 days of age, both CX3CR1 +/gfp (n=25) and CX3CR1 gfp/gfp (n=25) mice were randomly distributed into two groups. The first group, normal diet (ND), received standard chow (VRF1 (P), Special Diets Services (SDS), Witham, Essex, UK.). The second group received fat-enriched diet (FatED), by providing a 2:1 mixture of standard chow and lard (Spar Budget, Budapest, Hungary). The energy content and macronutrient compositions of the two diets are given in Table 1. All procedures were conducted in accordance with the guidelines set by the European Communities Council Directive (86/609 EEC) and approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine (permit number: 22.1/3347/003/2007).

Table 1. Energy content and macronutrient composition of diets

	ND - standard chow		**FatED - mixed chow**	
	g%	kcal%	g%	kcal%
Protein	19,1	22,5	12,7	9,7
Carbohydrate	55,3	65,0	36,9	28,0
Fat	4,8	12,6	36,5	62,3
kcal/g	3,40		5,27	

2.2. Experimental design

Mice were fed with normal diet (ND) or fat enriched diet (FatED) starting at age of 35 days. Mice were decapitated ten weeks later, interscapular brown adipose tissues were collected, sampled and stored at -70°C for qPCR, tissue samples were also obtained for histology. A set of animals underwent cold tolerance test.

2.3. Histology

BAT tissue samples were immersion fixed in 4% w/v paraformaldehyde in 0.1 mol l^{-1} phosphate buffer, pH 7.4 (PB) for 3 days and stored in 1% w/v paraformaldehyde in 0.1 mol l^{-1} PB at 4°C then were embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E). Microscopic slides were digitalized with Pannoramic Digital Slide Scanner (3DHISTECH Kft., Hungary). Lipid droplet areas of brown adipose cells were counted under 40x magnification in one field of view with ImageJ software (NIH, USA).

2.4. Core body temperature measurement and cold challenge

Rectal temperature was measured with Multithermo thermometer (Seiwa Me Laboratories Inc., Tokyo, Japan). To assess cold tolerance, set of animals (n = 30) from both genotypes were fasted for 5 hours, then placed into new individual cages with minimal bedding and transferred to cold room (4^{o}C). Rectal temperature was measured before and 60, 120, 180 and 240 min after cold exposure.

2.5. Gene expression analysis by quantitative real-time PCR

Total RNA was isolated from brown adipose tissue samples with QIAGEN RNeasyMiniKit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination, DNase I (Fermentas) treatment was used. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific). Amplification was not detected in the RT-minus controls. cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The designed primers (Invitrogen) were used in real-time PCR reaction with Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on ABI StepOnePlus instrument. The gene

159 expression was analyzed by ABI StepOne 2.3 program. The amplicon was tested by Melt Curve
160 Analysis. Measurements were normalized to ribosomal protein S18 (*Rps18*) expression [22].
161
162 *2.6. Primer design*
163 Primers used for the comparative CT (threshold cycle) experiments were designed by the Primer
164 Express 3.0 program. Primer sequences are shown in Table 2.
165
166 ***Table 2. Mouse specific primer sequences used for rtPCR***
167

Gene	Forward sequence	Reverse sequence
Adrb3	ACCGCTCAACAGGTTTGA	GGGGCAACCAGTCAAGAAGAT
Atgl	GCCATGATGGTGCCCTATACT	TCTTGGCCCTCATCACCAGAT
Ccl2 (Mcp1)	CCAGCACCAGCACCAGCCAA	TGGATGCTCCAGCCGGCAAC
Cx3cl1	CCGCGTTCTTCCATTTGTGT	GGTCATCTTGTCGCACATGATT
Dgat1	GTTTCCGTCCAGGGTGGTAGT	CGCACCTCGTCCTCTTCTAC
Dio2	ACAAACAGGTTAAACTGGGTGAAG	CGTGCACCACACTGGAATTG
Gfp	GGACGACGGCAACTACAAGA	AAGTCGATGCCCTTCAGCTC
Glut4	AGGAACTGGAGGGTGTGCAA	GGATGAAGTGCAAAGGGTGAG
Gpat	AGTGAGGACTGGGTTGACTG	GCCTCTTCCGGCTCATAAGG
Hsl	AGCCTCATGGACCCTCTTCTA	TCTGCCTCTGTCCCTGAATAG
Il1a	CCATAACCCATGATCTGGAAGAG	GCTTCATCAGTTTGTATCTCAAATCAC
Il1b	CTCGTGGTGTCGGACCCATATGA	TGAGGCCCAAGGCCACAGGT
Il6	CTCTGCAAGAGACTTCCATCC	AGTCTCCTCTCCGGACTTGT

Mgat	TGGTTCTGTTTCCCGTTGTTC	GAAACCGGCCCGTTACTCAT
Mgl	CTTGCTGCCAAACTGCTCAA	GGTCAACCTCCGACTTGTTCC
Pgc1a (Ppargc1a)	ATGTGCAGCCAAGACTCTGT	TTCCGATTGGTCGCTACACC
Pparg2	CTCCTGTTGACCCAGAGCAT	TGGTAATTTCTTGTGAAGTGCTCA
Rps18	TCCAGCACATTTTGCGAGTA	TTGGTGAGGTCGATGTCTGC
Th	TCTCAGAGCAGGATACCAAGCA	GCATCCTCGATGAGACTCTGC
Tnfa	CAGCCGATGGGTTGTACCTT	GGCAGCCTTGTGCCTTGA
Ucp1	GGTCAAGATCTTCTCAGCCG	AGGCAGACCGCTGTACAGTT

2.7. Statistical analysis

Statistical analysis was performed by factorial ANOVA with Newman–Keuls post-hoc test in Statistica 11 (StatSoft Inc.). The results are shown as means ± SEM. In all cases $p< 0.05$ was considered significant.

3. Results

3.1. *Fractalkine receptor deficiency prevents FatED-induced obesity*

In agreement with our previous findings [20], 10 weeks on FatED increased body weight of mice (diet effect: $F(1,14) = 20.84$, $p < 0.001$), but the weights were significantly lower in fractalkine receptor deficient, gfp/gfp FatED group (genotype*diet effect: $F(1,14) = 6.59$, $p < 0.05$) (Fig. 1A).

Although the daily food consumption of all FatED mice was lower, the daily energy intake was comparable to those on normal diet. Because we did not detect significant genotype effect in cumulative food- and energy intake and fecal output, these factors may not be responsible for the differences seen in body weight gain (Supplementary Table 1).

However, significant diet- and genotype effects have been revealed in the relative BAT weight. There was an increase in response to FatED (diet effect: $F(1,14) = 11.5$, $p < 0.01$; genotype effect: $F(1,14) = 4.63$, $p < 0.05$), but it was significantly lower in gfp/gfp group (Fig.1B).

3.2. Fractalkine deficient mice activate thermogenesis in response to acute cold.

The core temperature of *ad libitum* fed mice was not different (Fig. 1C). When fasted mice were placed to cold, the rectal temperature of all mice gradually decreased. However, after 2 hours in cold, the temperature of homozygous animals started to increase back to the normal and the increase in FatED mice was significantly higher than that seen in heterozygous animals fed by control- or FatED (Fig. 1D-E).

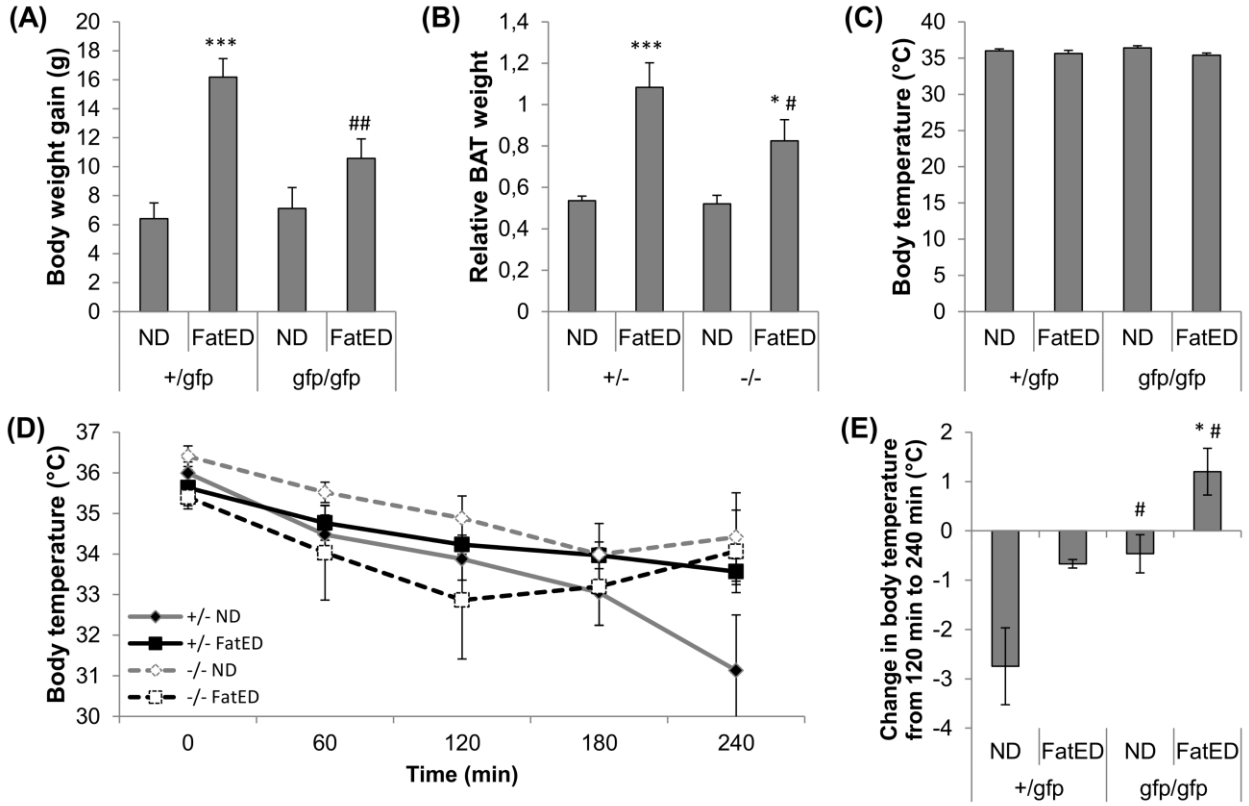

Figure 1. Body weight gain, BAT weight and cold tolerance test.

*Mean±SEM values of body weight gain (A) and relative BAT weight (B) of mice kept on normal (ND) or fat enriched diet (FatED) for 10 weeks. C) Core body temperature at room temperature. D-E) Changes in body temperature during cold tolerance test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ND, # $p < 0.05$, ## $p < 0.01$ vs. +/gfp (Newman–Keuls post hoc comparison). FatED – fat enriched diet, ND – normal diet.*

3.3. Lack of fractalkine receptor attenuates diet-induced accumulation of macrophages into the BAT.

To reveal leukocyte recruitment into the BAT, we relied on GFP transgene expression, which occurs in myeloid cells of CX3CR1+/gfp and gfp/gfp mice. Increased number of leukocytes were observed in the BAT sections of FatED mice. Furthermore, "crown like structures" (CLS) - similar to those found in WAT of obese animals [23] - were observed in BAT: enlarged adipocytes filled with single lipid droplet were surrounded by numerous immune cells in FatED

+/gfp mice (Fig. 2A). No similar cellular scenario has been detected in gfp/gfp FatED and all ND groups.

Because *Gfp* expression in the tissue is proportional to the number of macrophages, we calculated the normalized *Gfp* mRNA levels to compare the number of macrophages within the BAT of our experimental groups. In CX3CR1 +/gfp mice, FatED resulted in an increase of *Gfp* expression, however, in Cx3cr1 homozygotes the relative quantity of *Gfp* did not change in response to FatED, suggesting that lack of fractalkine receptor prevents the accumulation of CX3CR1+ monocytes into BAT (Fig.2).

CCL2 (MCP-1) and CX3CL1, among others, are monocyte attracting chemokines, which serve as signals for monocytes to accumulate to the sites of inflammation. CCL2 also contributes to the local proliferation of tissue macrophages. To reveal the importance of these chemokines in accumulation of GFP-positive immune cells to the BAT, we have compared their relative expression in mice exposed to ND or FatED.

As shown in Figure 2 fractalkine (*Cx3cl1*) mRNA level was elevated in response to FatED in both genotypes, however, diet-induced expression of *Ccl2* was detected only in CX3CR1 +/gfp mice (*Gfp*: diet effect: $F(1,11) = 8.68$, $p < 0.05$; genotype effect: $F(1,11) = 38.97$, $p < 0.001$. *Cx3cl1*: diet effect: $F(1,11) = 33.13$, $p<0.001$. *Ccl2*: diet effect: $F(1,11) = 9.08$, $p<0.05$; genotype effect: $F(1,11) = 7.99$, $p< 0.05$; genotype * diet: $F(1,11) = 7.87$, $p< 0.05$).

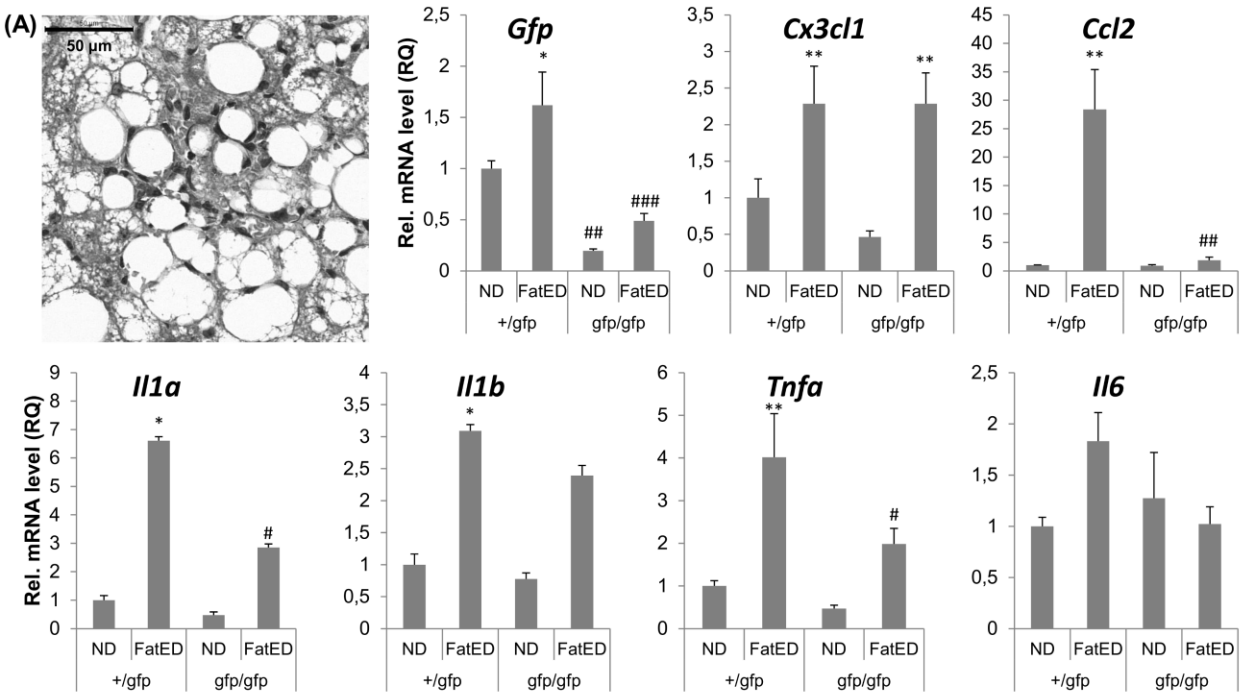

Figure 2. Macrophage accumulation to BAT and expression of proinflammatory cytokines *A) Representative image of CX3CR1 +/gfp FatED BAT. Adipocytes with enlarged lipid droplets in the BAT of CX3CR1 +/gfp mice are surrounded by leukocytes. Scale bar = 50 µm. Mean ± SEM values for relative mRNA levels in BAT: Gfp, chemokines: Cx3cl1, Ccl2, pro-inflammatory cytokines: Il1a, Il1b, Tnfa, Il6. * p < 0.05, ** p < 0.01vs. ND, # p < 0.05, ## p < 0.01 vs. +/gfp (Newman–Keuls post hoc comparison). FatED – fat enriched diet, ND – normal diet.*

3.4. Recruitment of macrophages in the BAT is accompanied by increased expression of proinflammatory cytokines

As we have detected significant differences in the number of *Gfp*-positive profiles in the BAT of mice exposed to FatED, next, we compared the mRNA levels of pro-inflammatory cytokines produced by macrophages in the BAT. *Il1a, Il1b* and *Tnfa* were higher in +/gfp FatED group (*Il1a*: diet effect: $F(1,11) = 11.93$, $p < 0.01$; *Il1b*: diet effect: $F(1,11) = 19.09$, $p < 0.01$; *Tnfa*: diet effect: $F(1,11) = 23.75$, $p < 0.001$; genotype effect: $F(1,11) = 7.61$, $p < 0.05$), but in gfp/gfp FatED mice only *Il1b* expression was elevated, although the increase was not significant (Fig. 2).

3.5. *Fat-enriched diet results in "whitening" of BAT*

As shown in Fig. 3A, fat enriched diet resulted in "whitening" of interscapular brown adipose tissue. Histological analysis of BAT revealed enlarged brown adipose cells with few large lipid droplets in +/gfp FatED mice, reminiscent of white adipocytes filled with a single lipid droplet were also present. In gfp/gfp mice kept on FatED, multilocular brown adipocytes were more abundant than in +/gfp FatED mice and comparable to those BAT cells in ND mice. Frequency distribution analysis of lipid droplet areas in BAT revealed that FatED shifted the droplet areas to larger sizes, less droplets were under 15 μm^2 and more over 135 μm^2 ($F(1,14) = 8.62$, $p < 0.05$; $F(1,14) = 16.76$, $p < 0.01$, respectively). In gfp/gfp FatED mice significantly more small lipid droplets were present than in +gfp heterozygotes (Fig. 3B).

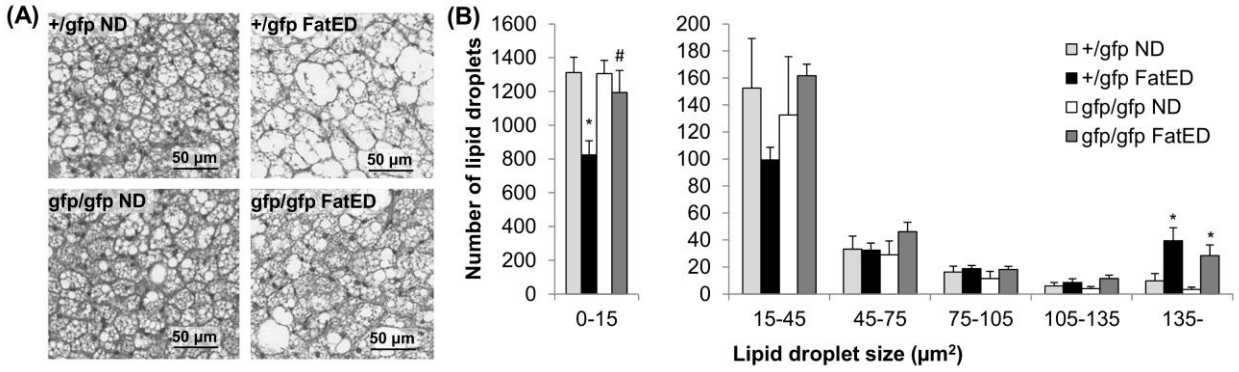

Figure 3. Quantitative histological analysis of BAT

*A) Representative histological images of hematoxylin-eosin stained BAT sections. FatED fed CX3CR1 +/gfp mice have larger lipid droplets. Scale bars = 50 µm. B) Frequency distribution of lipid droplet areas in one field of view. * $p < 0.05$ vs. ND, ** $p < 0.01$ vs. ND, # $p < 0.05$ vs. +/gfp, ## $p < 0.01$ vs. +/gfp (Newman–Keuls post hoc comparison). FatED – fat enriched diet, ND – normal diet.*

3.6. *Fat storage and lipolytic/lipogenic enzymes in BAT*

To analyze whether differences in lipid metabolism contribute to diet-induced phenotypic- and morphological changes in BAT of +/gfp and gfp/gfp animals, we have measured expression of enzymes involved in lipid synthesis and lipolysis in the BAT.

FatED upregulated lipogenic enzymes, *Dgat1* and *Gpat* mRNA expression in both genotypes. Lipolytic enzyme expression did not change in response to FatED in +/gfp mice. In gfp/gfp ND fed mice express lower levels of *Atgl* and *Mgl*, but FatED upregulated all lipolytic enzymes' mRNA expression (Fig. 4) (Lipogenic enzymes: *Dgat1* (diet effect: $F(1,13) = 76.94$, $p < 0.001$; diet * genotype: $F(1,13) = 6.63$, $p < 0.05$); *Mgat* (diet effect: $F(1,13) = 9.79$, $p < 0.01$); *Gpat* (diet effect: $F(1,13) = 129.54$, $p < 0.001$; genotype effect: $F(1,13) = 9.36$, $p < 0.01$; diet * genotype: $F(1,13) = 12.44$, $p < 0.01$). Lipolytic enzymes: *Atgl* (diet effect: $F(1,13) = 22.12$, $p < 0.001$); *Hsl* (diet effect: $F(1,13) = 18.02$, $p < 0.001$); *Mgl* (diet effect: $F(1,13) = 32.10$, $p < 0.001$)).

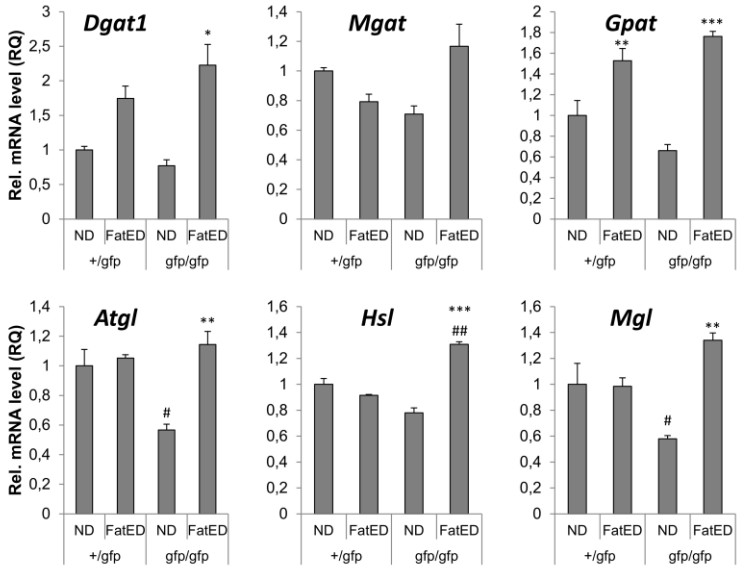

Figure 4. Gene expression of lipogenic and lipolytic enzymes in the BAT. *Mean ± SEM values for relative mRNA levels in BAT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. ND, # $p < 0.05$, ##$p < 0.01$ vs. +/gfp (Newman–Keuls post hoc comparison). FatED – fat enriched diet, ND – normal diet.*

3.7. *Fractalkine receptor deficiency affects the expression of BAT thermogenic and metabolic-related markers*

Because BAT significantly contributes to energy expenditure via non-shivering thermogenesis by using fatty acids and glucose as fuels, next we investigated the diet-induced expression of thermogenic related markers in the BAT of mice with or without fractalkine signaling.

In +/gfp mice, fat enriched diet did not affect expression of *Ucp1, Pparg and Pgc1a,* however, *Dio2* and *Adrb3* mRNA levels were elevated. Gfp/gfp mice express less *Ucp1, Pparg2 and Pgc1a,* than +/gfp mice during normal dieting, exposure to FatED resulted in significantly elevated expression of these mRNAs in the BAT. *Pparg2 and Adrb3* mRNA levels in gfp/gfp FatED mice was higher than in +/gfp FatED mice (Fig. 5) (*Ucp1*: diet effect: $F(1,11) = 23.68$, $p < 0.001$; genotype * diet: $F(1,11) = 13.74$, $p < 0.01$; *Pparg2:* diet effect: $F(1,11) = 26.88$, $p < 0.001$, genotype * diet: $F(1,11) = 43.91$, $p < 0.001$; *Pgc1a:* diet effect: $F(1,11) = 15.74$, $p < 0.01$; genotype * diet: $F(1,11) = 7.75$, $p < 0.05$. *Dio2:* diet effect: $F(1,11) = 24.70$, $p < 0.001$; genotype effect: $F(1,11) = 17.35$, $p < 0.01$. *Adrb3*: diet effect: $F(1,11) = 89.78$, $p < 0.001$; genotype effect: $F(1,11) = 7.56$, $p < 0.05$; genotype * diet: $F(1,11) = 28.23$, $p < 0.001$).

Because adipose tissue macrophages synthesize and release catecholamines locally in response to cold [24], we have been interested how *Th,* the key enzyme in catecholamine synthesis, varies in the BAT in response to diet. Neither the genotype nor the diet affected *Th* expression in the BAT.

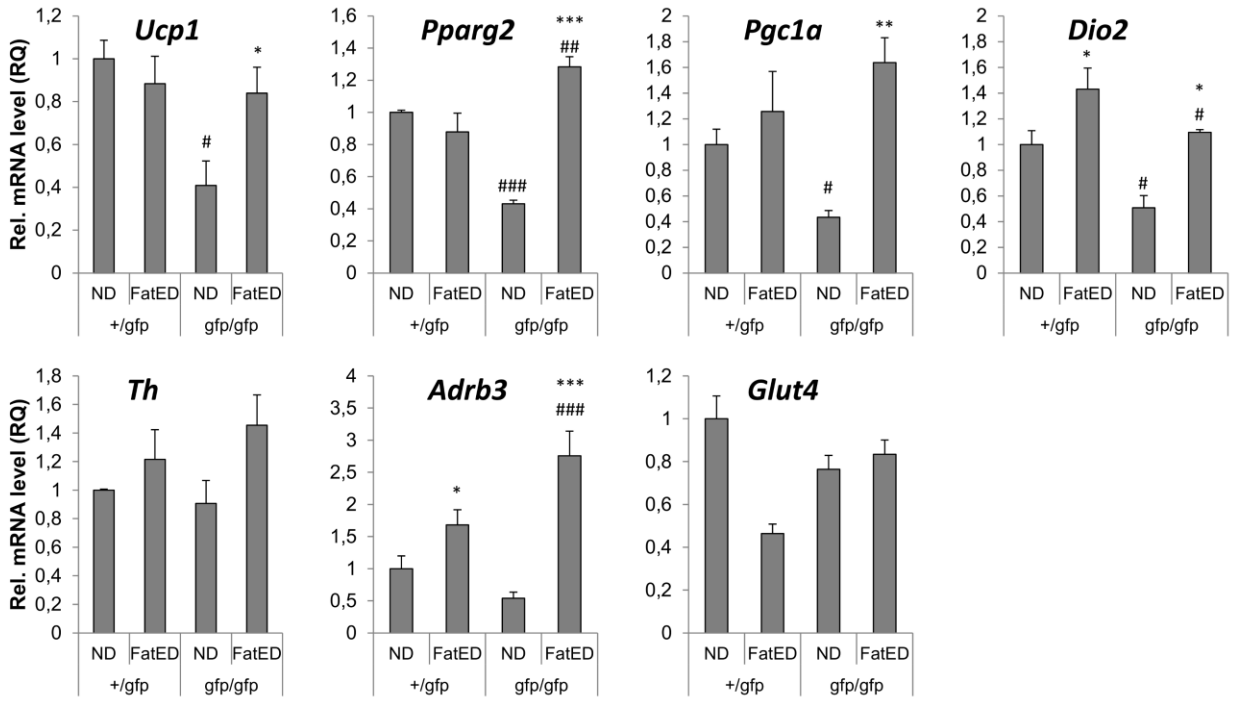

Figure 5. Gene expression of BAT thermogenic and metabolic-related markers. Mean ± SEM values for relative mRNA levels in BAT * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. ND; # $p < 0.05$, ## $p < 0.01$ ###, $p < 0.001$ vs. +/gfp (Newman–Keuls post hoc comparison). FatED – fat enriched diet, ND – normal diet.

4. Discussion

Present results reveal significant morpho-functional immune- and metabolic rearrangements in the brown adipose tissue of mice kept on fat enriched diet. We have found "whitening" of BAT in FatED fed mice with increased accumulation and recruitment, of mononuclear cells in the BAT, differential overexpression of pro-inflammatory mediators that results in local metabolic inflammation. Fractalkine/fractalkine receptor system is involved in the recruitment of mononuclear cells into the BAT and regulation of adipose inflammation, since mice lacking functional fractalkine receptor display reduced expression of proinflammatory cytokines and improved profile of diet-induced genes involved in lipid metabolism and thermogenesis. These changes in the BAT might contribute to the obesity-resistant phenotype of mice lacking fractalkine signaling.

Both type of adipose tissue displays significant morphological and functional plasticity driven by metabolic-, environmental- and hormonal cues [25]. “Browning” of the white adipose tissue is well recognized. For instance, clusters of UCP1 expressing cells referred to as “brite” (brown in white or beige) adipocytes appear in the white adipose tissue in response to cold, while there is a downregulation of *Ucp1* mRNA levels together with phenotypic appearance of white adipocytes in the BAT at thermoneutral conditions [26]. Here we have shown „whitening” of BAT in response to fat-enriched diet in mice, which is due to coalescence of lipid droplets. Similar, distorted lipid droplet architecture has also been reported in mice kept on high fat diet for 13 weeks [27, 28]. Increase of the size of lipid droplets might indicate an imbalance between lipid synthesis and lipolysis. Indeed, our present results show that obese CX3CR1 +/gfp mice were unable to induce lipolytic enzyme expression in the BAT, which might be responsible for fat deposition in BAT of these animals.

In addition to morphological changes of adipocytes we found recruitment/accumulation of mononuclear cells into the BAT of +/gfp mice kept on fat-enriched diet. These data are consistent with previous results showing that genetic and diet-induced obesity results in chronic inflammation in the BAT [29-31]. By contrast, Fitzgibbons et al. found very low level of immune cell enriched transcripts in the BAT from C57BL6/J mice fed a high-fat diet for 13 weeks [27]. Thus the extent of BAT inflammation is largely depends on the strain and conditions used. Our estimation of leukocyte accumulation is based on the normalized expression of *Gfp* mRNA in CX3CR1-gfp transgenic mice [20] in which monocytes (except eosinophils and neutrophils), a special subset of NK cells and dendritic cells express *Gfp* as described previously [17]. Recruitment of leukocytes into the white adipose tissue and their role in metabolic inflammation is well recognized both in genetic- and diet-induced rodent models as well as in human obesity [32]. Feeding a high fat diet to C57Bl6 mice has been shown to promote large increases of

various leukocytes, among those T cells and neutrophils are the first on the scene, followed by monocytes by 8-10 weeks on diet [33]. Adipose tissue macrophages have been mechanistically implicated in low grade, long lasting, metabolic inflammation and glucose intolerance seen in diet-induced obesity [34, 35]. It has been hypothesized that dying adipocytes initiate macrophage recruitment to the adipose tissue, however, recent findings emphasize the role of various chemokines originating from adipocytes and/or from the stromal vascular fraction. In this respect the monocyte attractant protein, CCL2 (MCP1) and its receptor CCR2 have been the most intensively studied [36]. Although *Mcp1* mRNA level in the adipose tissue is elevated within 7 days and plasma MCP1 concentration increased 4 weeks after starting high fat diet, genetic disruption of MCP1 signaling did not confer resistance to diet-induced obesity in mice or reduce adipose tissue macrophage infiltration in the WAT [36], indicating involvement of additional monocyte attractants.

Fractalkine has been recently identified as an adipo-chemokine, which is elevated in obese people and patients with type2 diabetes [19]. CX3CL1 is implicated in recruitment of leukocytes in clinical syndromes of adipose tissue inflammation and atherosclerosis. Indeed, we have recently shown increased expression of fractalkine in the epididymal white fat pad of obese mice to be accompanied with increased number of tissue macrophages and upregulated expression of proinflammatory cytokines [20]. Here we report, for the first time, that fat enriched diet induces fractalkine expression in brown adipose tissue as well. Based on the facts that expression of fractalkine (CX3CL1) has been significantly elevated in all groups fed with FatED, while mice lacking the fractalkine receptor accumulated significantly less GFP+ cells into the BAT and display less severe local tissue inflammation than controls, we propose a role of fractalkine/fractalkine receptor system in recruitment of macrophages into the BAT.

By contrast, data from Morris et al. indicate that CX3CR1 is not required for trafficking of macrophages to- and their retention in, the epididymal WAT in mice with diet-induced obesity [37].

Here we report significant increase of mRNA expression of proinflammatory cytokines *Il1a*, *Il1b* and *Tnfa* in the BAT of FatED mice, the pattern and rate of increase of cytokines were comparable with those observed in WAT. It remains unknown however, if these cytokines originate in resident or recruited population of immune cells within the BAT. When compared to heterozygote controls, we have found reduced expression of proinflammatory mediators in the BAT of CX3CR1 gfp/gfp mice, indicating the role of fractalkine signaling in activation of monocytes in the BAT as well. Accumulation of proinflammatory adipose tissue macrophages in obese BAT shows similarities to foam cell formation in atherosclerotic plaques, which is also dependent on the presence of CX3CR1 [38]. It should be recognized, however, that different subsets of monocytes use different chemokine patterns with which to accumulate in various inflammatory targets [39].

One interesting finding of the present study is the lack of diet-induced elevation of CCL2 (MCP1) in fractalkine receptor deficient mice, suggesting some mechanistic relationship between these chemokines.

Among the cytokines induced by FatED in the BAT, TNFa might play a prominent role in morphofunctional rearrangements. For instance, TNFa decreased the expression of functionally active ADRB3 receptors in brown adipocytes and consequently attenuated the thermogenic and lipolytic actions of SNS activity [40]. Studies on 3T3-L1 adipocytes revealed that TNFa decreases the expression of lipolytic enzymes *Atgl* and *Hsl* [41] Conversely, TNFa deficiency in genetically obese (ob/ob) mice resulted in less severe obesity, decrease in brown adipocyte apoptosis, and increased expression of *Adrb3* and *Ucp1* with significant improvement in

thermogenetic capacity [40]. Fractalkine receptor deficient mice (gfp/gfp), in which FatED- did not induce local *Tnfa* expression, are protected from excessive weight gain, display improved glucose tolerance and induction of *Adrb3, Atgl* and *Hsl* mRNA in the BAT.

The next obvious question was how FatED-induced proinflammatory environment affects energy expenditure/cold tolerance and BAT expression of metabolic-related and thermogenic genes. CX3CR1 +/gfp mice were unable to increase BAT expression of *Ucp1, Pparg2,* and *Pgc1a* in response to FatED, which might explain their obesity prone phenotype and impaired cold tolerance during fat enriched diet. Indeed, it has been recently shown that macrophage derived proinflammatory cytokines in general-, and TNFa in particular, suppress the induction of *Ucp1* promoter activity and mRNA expression [42, 43]. Nevertheless, the interaction between adipose tissue macrophages, proinflammatory cytokines and adipocytes is quite complex and occurs at several functionally distinct loci of obesity.

These results clearly suggest that diet-induced recruitment of macrophages in the BAT of +/gfp mice through the release of proinflammatory cytokines like TNFa, results in local inflammation and may attenuate the sympathetic nervous system (SNS) induced thermogenesis and lipolysis in BAT, leading to fat accumulation, driving a vicious circle. However, impaired fractalkine signaling (in gfp/gfp mice) breaks this circle by attenuating the accumulation of brown adipose tissue macrophages and their cytokine production, which results in diet-induced upregulation of *Atgl*, *Hsl* and *Mgl* lipogenic enzymes and *Ucp1* mRNA in the BAT, which changes are likely to contribute to the improved thermoadaptive response and the leaner phenotype seen in fractalkine receptor deficient mice.

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Supplementary Material (for online publication)

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Food intake (g)

		Weeks									
Genotype	Diet	1	2	3	4	5	6	7	8	9	10
+/gfp	ND	3,41 ± 0,58	4,63 ± 0,28	3,97 ± 0,37	4,52 ± 0,14	4,45 ± 0,15	3,69 ± 0,15	7,33 ± 0,37	3,99 ± 0,34	4,9 ± 0,26	3,31 ± 0,36
	FatED	2,88 ± 0,11	3,38 ± 0,11	2,58 ± 0,1	2,83 ± 0,08	2,84 ± 0,18	3,24 ± 0,16	3,17 ± 0,15	3,03 ± 0,1	3,02 ± 0,16	3,53 ± 0,24
gfp/gfp	ND	3,53 ± 0,53	5,75 ± 0,79	4,14 ± 0,15	4,69 ± 0,36	4,44 ± 0,27	4,23 ± 0,35	6,62 ± 0,11	4,38 ± 0,8	5,15 ± 0,16	4,1 ± 0,26
	FatED	2,7 ± 0,09	3,25 ± 0,19	2,68 ± 0,19	2,88 ± 0,22	2,74 ± 0,08	3,32 ± 0,23	3,07 ± 0,19	2,9 ± 0,13	2,94 ± 0,16	3,37 ± 0,14

Energy intake (kcal)

		Weeks									
Genotype	Diet	1	2	3	4	5	6	7	8	9	10
+/gfp	ND	11,59 ± 1,99	15,74 ± 0,96	13,5 ± 1,26	15,4 ± 0,5	15,13 ± 0,53	12,55 ± 0,51	24,93 ± 1,26	13,59 ± 1,15	16,66 ± 0,89	11,27 ± 1,25
	FatED	15,14 ± 0,6	17,82 ± 0,62	13,6 ± 0,53	14,92 ± 0,46	14,95 ± 0,98	17,07 ± 0,85	16,69 ± 0,83***	15,95 ± 0,53	15,92 ± 0,86	18,56 ± 1,27**
gfp/gfp	ND	12,01 ± 1,82	19,56 ± 2,68	14,08 ± 0,53	15,95 ± 1,23	15,09 ± 0,94	14,38 ± 1,22	22,5 ± 0,37	14,91 ± 2,74	17,52 ± 0,55	13,94 ± 0,89
	FatED	14,2 ± 0,52	17,13 ± 1	14,09 ± 1,05	15,14 ± 1,16	14,45 ± 0,43	17,5 ± 1,21	16,17 ± 1,03**	15,28 ± 0,7	15,5 ± 0,88	17,73 ± 0,77

Faeces (g)

		Weeks					
Genotype	Diet	1	3	4	6	7	8
+/gfp	ND	0,93 ± 0,04	0,93 ± 0,04	0,85 ± 0,04		0,89 ± 0,02	0,99 ± 0,07
	FatED	0,52 ± 0,04***	0,54 ± 0,02***	0,47 ± 0,03***	0,51 ± 0,03	0,42 ± 0,01***	0,44 ± 0,02***
gfp/gfp	ND	0,95 ± 0,03	0,91 ± 0,08	0,81 ± 0,03		0,96 ± 0,16###	1,02 ± 0,03
	FatED	0,51 ± 0,01***	0,55 ± 0,02***	0,49 ± 0,02***	0,51 ± 0,02	0,43 ± 0,01***	0,48 ± 0,01***

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