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## **Evaluation of macrophage plasticity in brown and white adipose tissue**

M. Teresa Ortega, Linglin Xie, Silvia Mora, Stephen K. Chapes

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Corresponding Author: Dr. Stephen Keith Chapes, Ph.D.

Corresponding Author's Institution: Kansas State University

First Author: M. Teresa Ortega, Ph.D.

Order of Authors: M. Teresa Ortega, Ph.D.; Linglin Xie, Ph.D.; Silvia Mora, Ph.D.; Stephen Keith Chapes, Ph.D.

**Abstract:** There are still questions about whether macrophage differentiation is predetermined or is induced in response to tissue microenvironments. C2D macrophage cells reside early in the macrophage lineage in vitro, but differentiate to a more mature phenotype after adoptive transfer to the peritoneal cavity (PEC-C2D). Since C2D macrophage cells also traffic to adipose tissue after adoptive transfer, we explored the impact of white adipose tissue (WAT), brown adipose tissue (BAT) and in vitro cultured adipocytes on C2D macrophage cells.

When PEC-C2D macrophage cells were cultured with preadipocytes the cells stretched out and CD11b and Mac-2 expression was lower compared to PEC-C2D macrophage cells placed in vitro alone. In contrast, PEC-C2D cells co-cultured with adipocytes maintained smaller, round morphology and more cells expressed Mac-2 compared to PEC-C2D co-cultured with preadipocytes. After intraperitoneal injection, C2D macrophage cells migrated into both WAT and BAT. A higher percentage of C2D macrophage cells isolated from WAT (WAT-C2D) expressed Ly-6C (33%), CD11b (11%), Mac-2 (11%) and F4/80 (29%) compared to C2D macrophage cells isolated from BAT (BAT-C2D). Overall, BAT-C2D macrophage cells had reduced expression of many cytokine, chemokine and receptor gene transcripts when compared to in vitro grown C2D macrophages, while WAT-C2D macrophage cells and PEC-C2D up-regulated many of these gene transcripts. These data suggest that the C2D macrophage phenotype can change rapidly and distinct phenotypes are induced by different microenvironments.

1 **Evaluation of macrophage plasticity in brown and white adipose tissue**

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4 M. Teresa Ortega<sup>1†</sup>, Linglin Xie<sup>1†</sup>, Silvia Mora<sup>2</sup>, and Stephen K. Chapes<sup>1\*</sup>

5 <sup>1</sup>Division of Biology, Kansas State University, Manhattan, KS, 66506 and <sup>2</sup> Department of

6 Cellular and Molecular Physiology, Institute of Translational Medicine, The University of

7 Liverpool, Crown Street, Liverpool L69 3BX, United Kingdom

8 †Co-first authors

9

10 **Running Title: Macrophage-adipose interactions**

11

12

13 \*Address correspondence to:

14 Stephen K. Chapes

15 116 Ackert Hall

16 Kansas State University

17 Manhattan, KS 66506-4901

18 E mail:[skcbiol@ksu.edu](mailto:skcbiol@ksu.edu)

19 Voice: 785-532-6795

20 Fax: 785-532-6653

21

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23

24 **ABSTRACT**

25           There are still questions about whether macrophage differentiation is predetermined or is  
26 induced in response to tissue microenvironments. C2D macrophage cells reside early in the  
27 macrophage lineage *in vitro*, but differentiate to a more mature phenotype after adoptive transfer  
28 to the peritoneal cavity (PEC-C2D). Since C2D macrophage cells also traffic to adipose tissue  
29 after adoptive transfer, we explored the impact of white adipose tissue (WAT), brown adipose  
30 tissue (BAT) and *in vitro* cultured adipocytes on C2D macrophage cells.

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32 out and CD11b and Mac-2 expression was lower compared to PEC-C2D macrophage cells  
33 placed *in vitro* alone. In contrast, PEC-C2D cells co-cultured with adipocytes maintained  
34 smaller, round morphology and more cells expressed Mac-2 compared to PEC-C2D co-cultured  
35 with preadipocytes. After intraperitoneal injection, C2D macrophage cells migrated into both  
36 WAT and BAT. A higher percentage of C2D macrophage cells isolated from WAT (WAT-  
37 C2D) expressed Ly-6C (33%), CD11b (11%), Mac-2 (11%) and F4/80 (29%) compared to C2D  
38 macrophage cells isolated from BAT (BAT-C2D). Overall, BAT-C2D macrophage cells had  
39 reduced expression of many cytokine, chemokine and receptor gene transcripts when compared  
40 to *in vitro* grown C2D macrophages, while WAT-C2D macrophage cells and PEC-C2D up-  
41 regulated many of these gene transcripts. These data suggest that the C2D macrophage  
42 phenotype can change rapidly and distinct phenotypes are induced by different  
43 microenvironments.

44

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46 **Key words: macrophage, plasticity, adipocyte, adipose tissue, trafficking**

47

48 **1. Introduction**

49           Macrophages are found throughout the body and serve as initiators and effectors of the  
50 innate immune system [1-6]. Macrophages differentiate from bone marrow hematopoietic stem  
51 cells through various stages including, macrophage-colony forming cells to monoblasts,  
52 promonocytes and finally into monocytes [7,8]. Monocytes enter the bloodstream, where they  
53 circulate before migrating into tissues. There they differentiate into tissue-specific macrophages  
54 [9]. Macrophages are a heterogeneous group of cells which have different functions,  
55 morphologies and phenotypic properties [7,9]. Heterogeneity is commonly associated with  
56 macrophages as a consequence of the functions, organ sites and immune status of the host [9,10].  
57 However, there is controversy about macrophage adaptation to microenvironmental signals *in*  
58 *vivo* [10-13]. Some think that since subpopulations of macrophages have either proinflammatory  
59 (M1) or anti-inflammatory (M2) properties, there are predetermined fates for monocytes and  
60 macrophages as opposed to the microenvironmental signaling leading to the macrophage  
61 plasticity [10,14].

62           C2D macrophage cells reside early in the macrophage lineage *in vitro*, but differentiate to  
63 a more mature, phenotype after adoptive transfer to the peritoneal cavity (PEC-C2D) [15]. These  
64 macrophage cells differentiate and traffic like primary macrophages and can provide insight into  
65 macrophage function [16]. In particular, they can provide evidence about macrophage plasticity  
66 in response to different microenvironments.

67           White adipose tissue (WAT) and brown adipose tissue (BAT) have distinct physiological  
68 functions. WAT is an energy storage and endocrine organ [17,18]. In contrast, BAT functions  
69 as an energy-dissipating organ through adaptive-thermogenesis [19]. These adipocyte depots  
70 display different morphology, cellular characteristics, body localizations and function [19-24].

71 Previous studies have suggested that macrophage function varies considerably in  
72 different fat depots [25]. Some have also suggested that macrophage plasticity is an artifact of *in*  
73 *vitro* manipulations [10]. Given the controversy about macrophage adaptation to  
74 microenvironmental signals *in vivo* [10-13] and the fact that little is known about BAT-  
75 macrophage interactions, we investigated whether macrophage phenotype is predetermined or is  
76 adaptable.

77

## 78 **2. Materials and methods**

### 79 *2.1 Mouse strains*

80 C57BL/6J (B6) mice were originally obtained from the Jackson Laboratory (Bar Harbor,  
81 ME). Male and female, 8-16 week-old mice were bred in the rodent facility of the Division of  
82 Biology at Kansas State University and used in these experiments. Mice were fed a normal  
83 mouse chow diet (5001, PMI International, St. Louis, MO) and were allowed to feed *Ad libitum*.  
84 All animal experiments were approved by the Institutional Animal Care and Use Committee.

85

### 86 *2.2 Antibodies and Reagents*

87 Collagenase (Type II), insulin from bovine pancreas, 3-Isobutyl-1-methylxanthine  
88 (IBMX) and dexamethasone were obtained from Sigma-Aldrich Co. (St. Louis, MO).  
89 Carboxyfluorescein diacetate, succinimidyl (CFDA-SE) ester was purchased from Molecular  
90 probes (Eugene, OR). APC conjugated anti-CD11c, APC conjugated anti-F4/80, APC  
91 conjugated anti-CD11b, ALEXA Fluor 647 conjugated anti-Mac2, and their isotype control  
92 antibodies were purchased from eBioscience (San Diego, CA). Biotin conjugated anti-Ly-6C

93 (ER-MP20) and its isotype control antibody were from BD Pharmingen (San Jose, CA). APC  
94 conjugated Streptavidin was purchased from eBioscience (San Diego, CA).

95

### 96 *2.3 Cell lines and cell culture*

97         The C2D macrophage cell line was created as described by our group [26]. These cells  
98 were derived from C2D mouse bone marrow and selected in the presence of macrophage colony  
99 stimulating factor (M-CSF). These cells have the *MHCII*<sup>-/-</sup> and *Tlr4*<sup>Lps-n</sup> genotype and are  
100 histocompatible with mice of the H-2<sup>b</sup> haplotype. C2D cells were grown in Dulbecco's  
101 Modified Eagle's Medium with 4% fetal bovine serum (DMEM<sub>4</sub>) supplemented with 0.3%  
102 Glutamax and 10% Opti-MEM in 150-mm tissue culture plates.

103         3T3L1 adipocytes were obtained from the American Type Culture Collection (Manassas,  
104 VA). Adipocytes were cultured and differentiated as described previously [27]. Briefly, 3T3L1  
105 cell differentiation was induced by culturing cells in DMEM containing 10% FBS (DMEM<sub>10</sub>), 1  
106 μM dexamethasone, 1.7 μM insulin and 0.5 mM IBMX for 4 days. On the fourth day, the  
107 3T3L1 cells were cultured in DMEM<sub>10</sub> with 1.7 μM insulin. On day 8, 3T3L1 cells were  
108 maintained in DMEM<sub>10</sub>. Undifferentiated preadipocytes and adipocytes differentiated for 6-8  
109 days were used in the experiments. 3T3L1 cells (1×10<sup>6</sup> cells) were directly co-cultured with  
110 1×10<sup>6</sup> C2D cells grown exclusively *in vitro* or 1×10<sup>6</sup> cells adoptively transferred C2D  
111 macrophage cells isolated from the peritoneal cavity (PEC-C2D).

112         Bone marrow derived macrophages (BM-Mo) were differentiated from B6 mouse bone  
113 marrow cells isolated from the femora, tibiae, and humeri. Briefly, the bones were recovered and  
114 cleaned of all non-osseous tissue. The marrow cavity was flushed with a sterile PBS solution.  
115 The red blood cells were lysed by incubating in ammonium chloride lysis buffer (0.15 M NH<sub>4</sub>Cl,

116 10 mM KHCO<sub>3</sub>, and 0.1 mM Na<sub>2</sub>EDTA, pH 7.3) for 5 min in ice. Cells were centrifuged (300 x  
117 g, 5 min) and washed two times with DMEM<sub>2</sub>. Bone marrow cells were seeded and incubated in  
118 M-CSF medium (DMEM<sub>10</sub>, OPTI-MEM, 0.01 M HEPES, 50 ng/ml gentamycin, 1.5 ng/ml  
119 rMCSF-1) for 7 days at 37 °C, 8 % CO<sub>2</sub>. BM-Mo were indirectly co-cultured with collagenase-  
120 digested white adipose tissue (WAT) gonadal fat pads or collagenase- digested BAT perispleen  
121 or interscapular fat pads as described below.

122

#### 123 *2.4 Adoptive transfer of labeled cells*

124 C2D cells were suspended in sterile, pre-warmed (37°C) phosphate buffered saline (PBS;  
125 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) at a concentration of 1.5 x 10<sup>6</sup> cells per  
126 ml, further stained with CFDA-SE according to the manufacturer's protocol. Briefly, C2D cells  
127 were incubated with 22 µM of CFDA-SE solution at 37 °C for 15 minutes. After centrifugation  
128 at 370 x g for 10 minutes, cell pellets were suspended in pre-warmed PBS and incubated in 37°C  
129 for an additional 20 minutes. Cells were then washed twice in PBS, and suspended at a  
130 concentration of 4 x 10<sup>7</sup> cells per ml in PBS. One and one-half ml of the cell suspension of  
131 CFDA-SE labeled C2D or normal C2D cells was injected intraperitoneally (*i.p.*) per mouse.

132

#### 133 *2.5 Peritoneal cell extraction and fat tissue isolation*

134 PEC-C2D macrophage cells were obtained from B6 mice by peritoneal lavage 36 hours  
135 after intraperitoneal injection of 4 x 10<sup>7</sup> of C2D macrophage cells labeled with CFDA-SE. The  
136 peritoneal exudate red blood cells were lysed as described in section 2.3. One-half of the cells  
137 were treated with 1 mg/ml collagenase type II at 37°C with shaking (60 rpm) for 40 minutes.



138 Control or collagenase-treated cells were washed three times with PBS and  $3 \times 10^6$  cells were  
139 plated into 150-mm cell culture plates and incubated in DMEM<sub>4</sub> for 16 hours.

140 Isolation of adipocytes and CFDA-SE labeled C2D macrophage cells was performed as  
141 previously described [15,16]. Adipocytes were isolated from both mouse gonadal fat pads  
142 (depots connected to the uterus and ovaries in females and the epididymis and testes in males)  
143 and perispleen adipose tissues by collagenase digestion [28,29]. We confirmed BAT origin by  
144 quantitating the mRNA of PRDM16 by qRT-PCR [30] and/or UCP-1 [31] in tissues collected  
145 from perispleen and interscapular isolates (data not shown). Gonadal fat pads weighed an  
146 average of 268 mg while perispleen fat averaged 98 mg. Interscapular fat pads weighed an  
147 average of 61 mg. The fat pads were minced and incubated for 10 min in pre-warmed (37°C)  
148 Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM  
149 MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mM Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 2.5 mM D-glucose, and 2 %  
150 bovine serum albumin, pH 7.4), thereafter the samples were incubated with Type II collagenase  
151 (1mg/ml) for 40 min at 37°C with constant shaking at 60 rpm. The WAT or BAT cells were  
152 passed through a 100 µm cell strainer; cells were then centrifuged at 370 x g for 1 minute and  
153 washed with Krebs Ringer buffer twice. Additionally, the adipocytes isolated from the paired  
154 gonadal fat pads were separated into 2 major fractions. The floating upper layer was primarily  
155 white adipocytes and the pelleted fraction was a mixture of stromal-vascular fraction (SVF) cells  
156 containing macrophages. Both cell fractions were collected and washed twice with KRP buffer.

157 WAT and BAT cells were scored for numbers and viability on a hemacytometer using  
158 trypan blue exclusion. Viability was 91±0%, 81±3% and 87±0% for PEC, BAT and SVF cells,  
159 respectively, after isolation and collagenase treatment. In collagenase-digested samples, we  
160 isolated an average of  $1.3 \times 10^5$  C2D macrophage cells per mouse from gonadal WAT and  $8.9 \times$

161  $10^4$  C2D macrophage cells from perispleen BAT or  $3.5 \times 10^4$  C2D macrophage cells from  
162 interscapular BAT.  $1 \times 10^5$  cells were pelleted onto a cytopsin slide for differential staining. A  
163 mixture of white adipocytes (upper layer) and SVF cells was co-incubated at  $37^\circ\text{C}$  in DMEM<sub>10</sub>,  
164 for 16 hours at a concentration of  $1 \times 10^5$  cells/ml in a 150-mm culture dish. The adipocytes  
165 remained dispersed in the medium and the SVF cells attached to the 150-mm culture plate. The  
166 adipocytes isolated from perispleen adipose were collected from the cell pellets and washed  
167 twice with KRP buffer. Cells isolated from perispleen BAT ( $3 \times 10^6$ ) were cultured at  $37^\circ\text{C}$  in  
168 DMEM<sub>10</sub> in a 150-mm culture plate for 16 hours.

169

## 170 *2.6 Flow cytometry analysis of C2D macrophage cells*

171 Cell sorting was based on C2D macrophage cell CFDA-SE fluorescence, with the lowest  
172 10 % of the positive cells not selected. Briefly, cell sorting was performed with either a  
173 FACSVantage SE cell sorter (Becton Dickson, Rockville, MD) or a MoFlo XDP Sterile Cell  
174 Sorter (Beckman Coulter), using specimen optimization and calibration techniques according to  
175 the manufacturer's recommendations. Cells were sorted at a rate of 15,000 cells per second and  
176 approximately  $1 \times 10^6$  viable (trypan blue exclusion), positive cells per group were collected on  
177 ice and centrifuged at  $350 \times g$  for 5 min at  $4^\circ\text{C}$  for PCR Array or qRT-PCR analysis.

178 We found a loss of cell surface markers following collagenase treatment. For example,  
179 Mac-2 was down regulated over 50% after a 40 minute collagenase treatment based on control  
180 PEC-C2D (data not shown). However, we were also concerned that this incubation would also  
181 influence the cells. Therefore, we also evaluated the changes in TNF gene expression over time  
182 after the PEC-C2D cells were cultured *in vitro*. We found C2D macrophage gene expression  
183 was reduced some but was still positive for at least 24 hrs (data not shown). Therefore, we felt a

184 reasonable approach to phenotype the cell surface molecules of the recovered C2D macrophage  
185 cells from PEC, BAT and WAT would be to allow the cells to recover *in vitro* for 16 hours at  
186 37°C in medium. Therefore, control or collagenase-treated C2D macrophage cells isolated from  
187 the peritoneal cavity (PEC-C2D), WAT and BAT were resuspended in DMEM<sub>4</sub> and incubated  
188 for 16 hours prior to labeling cell surface proteins and assessment by flow cytometry.

189 Cells were transferred to wells of 96-well, round-bottom plates and they were blocked  
190 with PBS-goat serum (50:50; 50 µl) at 4 °C for 0.5 hour. Subsequently, macrophage cell surface  
191 proteins were identified by direct labeling. Briefly, blocked cells were incubated with the isotype  
192 or specific antibody diluted in Hank's Buffered Salt Solution (HBSS; 0.137 M NaCl, 5.4 mM  
193 KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>)  
194 for 1 hour in the dark at 4°C. After two washes with HBSS, cells were fixed in 1% formalin.  
195 Labeled cell surface proteins were assessed by flow cytometry. We gated on live, CFDA-SE-  
196 positive or CFDA-SE negative cells, subsequently we assayed for the presence or absence of the  
197 selected cell surface markers.

198

### 199 *2.7 Real time quantitative RT-PCR analysis*

200 RNA was obtained by suspending the pelleted cells in 1 ml of TriReagent (Molecular  
201 Research Center). The solution was transferred to 2.0 ml Heavy Phase Lock Gel tubes (5  
202 Prime). 200 µl of chloroform was added and the mixture was shaken for 15 seconds. The  
203 samples were then centrifuged at 12,000 x g for 10 minutes at 4°C and the aqueous phase was  
204 transferred to clean 1.5 ml tubes. 500 µl of isopropanol was added and RNA was precipitated at  
205 -20°C for 24 hours. Samples were subsequently centrifuged at 12,000 x g for 10 minutes. The  
206 RNA pellet was washed with 1 ml of 70% ethanol and samples were centrifuged at 7.4 x g for 5

207 minutes. The 70% ethanol was decanted from the pellet; the pellet was allowed to slightly air  
208 dry and was resuspended in 50 µl of nuclease-free water. RNA samples were purified and  
209 DNase treated with EZRNA total RNA kit (Omega Bio-Tek, Inc.). One step qRT-PCR was  
210 performed using the SuperScript III Platinum SYBR Green kit (Invitrogen; Carlsbad, CA)  
211 according to the manufacturer's protocol. Primers were designed with the PrimerQuest software  
212 (*IDT; http://www.idtdna.com*) using sequence data from NCBI sequence database as following:  
213 *TNF-α*(NM\_013693) forward 5'-tctcatgcaccaccatcaaggact and reverse 5'-  
214 tgaccactctccctttgcagaact; *IL-6* (NM\_031168.1) forward 5'-tctcatgcaccaccatcaaggact and reverse  
215 5'-tgaccactctccctttgcagaact; *ILI-β*(NM\_008361) forward 5'-aagggtgcttccaaacctttgac and reverse  
216 5'-atactgctgctgaagctcttgt; *Arg-1*(NM\_007482) forward 5'-tggetttaaccttggttcttctg and reverse  
217 5'-catgtggcgcattcacagtcact; *Ym-1* (M94584) forward 5'-caccatggccaagctcattcttgt and reverse 5'-  
218 tattggcctgtccttagcceaact; *Fizz-1*(NM\_020509.3) forward 5'-actgcctgtgcttactcgttgact and reverse  
219 5'-aaagctgggttctccaccttca; *Prdm16* (BC059838) forward 5'-tcatcccaggagagctgcatcaaa and  
220 reverse 5'-atcacaggaacacgctacacggat; *Ucp-1* (NM009463.3) forward 5'-ttgagctgctccacagcgcc  
221 and reverse 5'-gttgcctgatgcgggcacga;  $\beta$ -*actin* (NM\_007393) forward 5'-  
222 tgtgatgtgggaatgggtcagaa and reverse 5'-tgtggtgccagatcttctccatgt. The qRT-PCRs were  
223 performed in a Cepheid SmartCycler System (Sunnyvale, CA). Fold increase in transcript  
224 expression was calculated:  $E(\text{gene of interest})^{\Delta\text{ct target}}/E(\text{housekeeping})^{\Delta\text{ct housekeeping}}$  where E  
225 (efficiency)= $10^{(-1/\text{slope})}$  as was previously described [32].

## 226 2.8 PCR array analysis

227 Expression analysis of 84 cytokines, chemokines and corresponding receptor genes  
228 involved in inflammatory responses was performed with the mouse inflammatory cytokines and  
229 receptors RT<sup>2</sup> profiler<sup>TM</sup> PCR array system (SuperArray Bioscience Corporation, Frederick,

230 MD). 1.2 µg of total RNA was obtained from CFDA SE labeled C2D cells sorted from the PEC,  
231 WAT, and BAT of C57BL/6J mice (n= 2 pooled RNA samples, 4 mice per pooled sample).  
232 Genomic DNA was digested with RNase-free DNAase, followed by first strand cDNA synthesis  
233 and then quantitative mRNA analysis according to manufacturer's protocol. The quantitative  
234 real-time PCR array was done on a BioRad iCycler (BioRad Laboratory, Hercules, CA)  
235 performed with the RT<sup>2</sup> SYBR Green/Fluorescein qPCR Master Mix (SuperArray Bioscience  
236 Corporation, Frederick, MD). Expression of mRNA for each gene was normalized to the  
237 expression of β-actin and compared to the data obtained with the negative control (RNA from  
238 cultured C2D cells) according to the ΔΔCt method [32].

239

#### 240 *2.9 Immunofluorescence and image analysis*

241 Gonadal fat pads and perispleen adipose tissue were washed in Krebs-Ringer phosphate  
242 (KRP) buffer fixed in 10% formalin/PBS and were cut into 50-µm-thick slices using a TC-2  
243 tissue sectioner (Sorvall Instruments). Tissue slices were mounted onto glass slides, and  
244 differential contrast interference (DIC) images of tissue and CFDA SE-labeled C2D macrophage  
245 cells were observed on a model LSM 5 Pascal Zeiss laser scanning confocal microscope. Tissues  
246 were visualized with 20X/0.5 and 40X/0.75 Plan Neofluor objectives with DIC. CFDA SE-  
247 labeled C2D macrophage cells were visualized using the 488-nm line of an argon ion gas laser  
248 (excitation of CFDA SE), an FT 488 primary dichroic beam splitter, a FT 545 secondary dichroic  
249 beam splitter, a 505-nm to 530-nm-bandpass filter, a photomultiplier tube, and LSM5 Pa software,  
250 version 3.2 SP2. The number of CFDA SE-labeled macrophages per square micrometer of area  
251 of adipose tissue was determined using ImageJ v1.37 (NIH). Images were then imported to  
252 Adobe Photoshop (Adobe Systems, Inc.) for figure processing.

253

254 *2.10 Statistical analysis.*

255 Flow cytometry data, cell distribution data and qRT-PCR data were presented as the  
256 mean  $\pm$  standard error of mean (SEM) of independent experiments (n=3 samples, 3 mice per  
257 sample unless stated otherwise in the Figure legend). Differences in mean were determined  
258 using Student's *t* test (paired, two-tailed) or were determined using the Mann-Whitney rank-sum  
259 test. Differences in cell distribution were assessed using the Chi-Square ( $\chi^2$ ) test. All tests were  
260 calculated using the StatMost statistical package (Data XIOM, Los Angeles, CA). Differences  
261 were considered significantly different when  $P < 0.05$ . To assess differences in the samples  
262 assayed in the PCR Arrays the mean Ct values between macrophage isolates were compared  
263 using minimum significant difference (MSD) [33]. Any difference between means greater than  
264 or equal to the MSD was considered to be a statistically different, while differences less than the  
265 minimum significant difference were considered to be non-significant. An MSD was calculated  
266 using the following equation:  $MSD = 2 \times (s_{\text{pool}}) \times (\sqrt{(1/n_1 + 1/n_2)})$ , where *s* pool is the standard  
267 deviation pooled across all genes and all groups, *n*<sub>1</sub> and *n*<sub>2</sub> are the numbers of replicates for the  
268 two treatments. In this study, *n*<sub>1</sub>=*n*<sub>2</sub>=2, and the pooled standard deviation was equal to 1.61 and  
269 the MSD was equal to 3.22.

270

271 **3. Results**

272 *3.1 Morphological and phenotypic changes of C2D macrophage cells in response to adipocytes*  
273 *in vitro*

274 To determine how different tissue environments impact macrophage phenotypes, we  
275 investigated macrophage responses to preadipocytes and adipocytes. In our experiments we used

276 the C2D macrophage cell line and 3T3L1 cells before or after, differentiation into adipocytes.  
277 We previously established the specific macrophage phenotype expressed by C2D cells after they  
278 were injected *i.p.* (PEC-C2D) or before they respond to other microenvironments [15,16,26]. In  
279 order to visualize and identify the C2D macrophages, C2D cells were labeled with CFDA-SE  
280 prior to their injection into the animals or their co-culture with (3T3L1) preadipocyte/adipocyte  
281 cells *in vitro* for two days. Subsequently, these cells were recovered and analyzed by  
282 fluorescence-activated cell sorting. Cells from peritoneal lavages, BAT and SVF cells from  
283 WAT were sorted by FACS analysis as described in Figure 1. C2D cells grown *in vitro* (Figure  
284 1A, region 1) and C2D CFDA-SE cells (Figure 1B, region 2) were used as negative and positive  
285 controls for gating, respectively, and for sorting C2D CFDA-SE positive cells from mixed cell  
286 samples such as C2D CFDA-SE macrophages co-cultured with 3T3L1 adipocytes (Figure 1C).

287 In comparison to the larger stretched morphology of C2D macrophage cells grown *in*  
288 *vitro* (Figure 2A, panels a and d), the PEC-C2D macrophage cells were round after cell isolation  
289 (Figure 2B, panels a and d). When C2D or PEC-C2D macrophage cells were cultured with  
290 preadipocytes, the cells stretched out (Figure 2A and 2B, panels b and e). In contrast, when C2D  
291 macrophage cells or PEC-C2D cells were co-cultured with differentiated 3T3L1 adipocytes, we  
292 found that the cells maintained a mostly smaller, round morphology (Figure 2A and 2B, panels c  
293 and f), suggesting that differentiated 3T3L1 adipocytes inhibit normal adherence and stretching  
294 of the C2D macrophage cells.

295 The morphological differences in C2D and PEC-C2D macrophage cells co-cultured with  
296 adipocytes were accompanied by changes in cell phenotype defined by cell surface molecules  
297 detected using flow cytometry. *In vitro*, 15% of the C2D macrophage cells expressed Mac-2 but  
298 not CD11b; indicative of an immature macrophage phenotype [15]. C2D macrophage cells

309 acquire a more differentiated phenotype after adoptive transfer *in vivo* with high levels of  
300 macrophage-specific molecules CD11b, Mac2, F4/80, cfms and low levels of CD11c, and Gr-1  
301 (Ly6G) [15,16]. We observed no change in the numbers of cells that expressed either of the cell  
302 surface markers on C2D macrophage cells co-cultured with pre-adipocytes compared to C2D  
303 macrophage cells cultured alone (Figure 3A). However, we observed a statistically significant  
304 ( $P<0.05$ ) increase in the number of C2D macrophages that expressed CD11b when co-cultured  
305 with 3T3L1 adipocytes (Figure 3A). For PEC-C2D macrophage cells, their maturation in the  
306 peritoneal cavity is accompanied by an increase in the number of cells that express CD11b  
307 [15,16]. Significantly more PEC-C2D macrophage cells co-cultured *in vitro* with adipocytes  
308 expressed Mac-2 than PEC-C2D macrophage cells cultured alone or with preadipocytes (Figure  
309 3B,  $P<0.05$ ). We observed a significant decrease ( $P<0.05$ ) in the number of PEC-C2D  
310 macrophage cells expressing CD11b and Mac-2 when incubated with preadipocytes (Figure 3B).

311

### 312 *3.2 Assessment of C2D macrophage cells after trafficking into WAT and BAT*

313 We previously found that C2D macrophage cells could be isolated from gonadal WAT  
314 [15]. However, macrophage trafficking to BAT has not been well characterized. We used  
315 confocal microscopy to visualize and count C2D macrophages in WAT and BAT. We counted  
316 an average of 97 C2D macrophages/mm<sup>2</sup> in BAT compared to 146 C2D macrophages/mm<sup>2</sup> in  
317 WAT ( $P>0.05$ , T test,  $n\geq 9$  fields scored per tissue; Figure 2C). We also assessed if C2D  
318 macrophage immigration to WAT and BAT induced inflammation. We did differential staining  
319 of white blood cells isolated from the adipose tissue 36 hrs after *i.p.* injection of C2D  
320 macrophage cells. This survey revealed distinct cell distributions. The white cell distribution in  
321 WAT was 8±1% PMN, 60±2% macrophage/monocyte and 33±1% lymphocytes compared to



322 3±2% PMN, 79±2% macrophage/monocytes and 18±1% lymphocytes in BAT ( $P<0.01$ ;  $\chi^2$  test).  
323 We also assessed C2D macrophage cell localization within WAT and BAT. WAT-C2D  
324 appeared between adipocytes and some appeared to spread around the adipose cells (Figure 2C,  
325 panels a, c, e). In contrast, BAT-C2D appeared only between adipocytes and generally had a  
326 round appearance (Figure 2C, panels b, d, f).

327 To determine if the C2D macrophage cells isolated from BAT and WAT maintained the  
328 same phenotype they expressed in the peritoneum or if they responded to the different tissue  
329 environments, we compared BAT-C2D and WAT-C2D for the expression of Ly-6C, Mac-2,  
330 CD11b and F4/80. Cells were labeled with CFDA and the CFDA-SE positive cells were  
331 assessed (Figure 4A). We observed that 33% of the WAT-C2D expressed Ly-6C, while there  
332 were very few Ly6C-positive (<1%) BAT-C2D. A significantly higher number of WAT-C2D  
333 macrophage cells expressed ( $P<0.05$ ) Mac-2 compared to BAT-C2D macrophage cells. We  
334 observed over 30% of the WAT-C2D macrophage cells expressed F4/80, but almost no BAT-  
335 C2D expressed F4/80 (Figure 4A). Significantly more WAT-C2D cells expressed CD11b than  
336 BAT-C2D (Figure 4A). When we compared C2D macrophage phenotype to the phenotype of  
337 the recipients' macrophages, we observed that a higher percentage of WAT-C2D cells expressed  
338 F4/80 compared to the recipient's macrophages (CFDA negative cells; Figure 4B). We detected  
339 no significant differences in the % positive cells that expressed Ly-6C, Mac-2 or CD11b when  
340 we compared WAT-C2D and recipient macrophages (Figure 4B).

341 To further characterize the impact of the adipose microenvironment on recently migrated  
342 macrophages, we measured transcript levels in C2D macrophages that were isolated and sorted  
343 from the peritoneal cavity, WAT or BAT. We measured the expression of an array of  
344 inflammatory chemokines and cytokines and their receptor genes by quantitative PCR. As

345 shown in Figure 5, the overall expression of chemokine, cytokine and receptor genes was  
346 dramatically down-regulated in BAT-C2D cells relative to the gene expression of C2D cells  
347 maintained *in vitro*, compared to those of PEC-C2D and/or sorted WAT-C2D cells. When  
348 WAT-C2D cells were compared to PEC-C2D macrophage cells, transcript levels for several  
349 chemokines were lower (Figure 5A). These included MIP-3b/CCL19 (3 vs. 9 fold), NAP-  
350 3/CXCL1 (-0.5 vs. 5 fold), CCL11 (1 vs. 6 fold), CXCL5 (2 vs. 4 fold), CXCL9 (3 vs. 7) and  
351 CXCL12 (4 vs. 12 fold). MIP-1a/CCL3 and MCP-5/CCL12 were also down-regulated in PEC-  
352 C2D compared to C2D macrophages grown *in vitro*.

353 Interestingly we found that two cytokines receptor genes had higher transcript levels in  
354 PEC-C2D macrophage cells compared to WAT-C2D macrophage cells; CD121a/IL 1r1 (8 vs. 2  
355 fold) and CD130/IL6st (6 vs. 3 fold). Additionally, C3 (5 vs.1 fold) had higher expression in  
356 PEC-C2D cells compared to WAT C2D cells (Figure 5E).

### 357 *3.3 Macrophages gene expression in response to WAT and BAT in vitro*

358 C2D macrophage cells exhibited distinct phenotypes in response to WAT or BAT  
359 adipose environments. Therefore, to confirm that C2D macrophage behavior reflected that of  
360 primary macrophages, we indirectly co-cultured BM-Mo in transwell plates (top) with  
361 collagenase-digested WAT and BAT (bottom). RNA from the BM-Mo was isolated and the  
362 transcript levels of *TNF- $\alpha$* , *IL-6*, *IL-1 $\beta$* , *Arg-1*, *Ym-1* and *Fizz-1* were assessed using qRT-PCR  
363 (Table 1). WAT incubated BM-Mo macrophages had higher *TNF- $\alpha$*  and *IL-6* transcript levels  
364 than BAT-BM Mo (Table 1). This would be consistent with the observations seen with C2D  
365 macrophages. In contrast, there were no differences in *IL-1 $\beta$*  or the anti inflammatory genes  
366 *Arg-1*, *Ym-1* and *Fizz-1* between BM-Mo inoculated in WAT or BAT (Table 1).

367

368 **4. Discussion**

369 Adipose tissue contains a heterogeneous array of cells including preadipocytes and  
370 adipocytes along with resident and inflammatory macrophages constituting up to 40 percent of  
371 the cell population [34]. Additionally, the trafficking of C2D macrophage cells to both WAT  
372 and BAT provided a unique opportunity to determine the impact of these distinct adipose  
373 environments on recently immigrating macrophages and how different microenvironments affect  
374 macrophage plasticity. We used the C2D macrophage cell line to investigate this question. This  
375 is a powerful model because the cells are phenotypically defined both *in vitro* and *in vivo* and the  
376 phenotypic change of the C2D macrophages in response to WAT or BAT paralleled the *in vitro*  
377 response of primary macrophages {Table 1; also see reference [35]}. Therefore, by knowing the  
378 characteristics of the C2D macrophages before and after exposure to the different adipose tissues  
379 we know exactly what changes are due to their immediate exposure to different  
380 microenvironments. Indeed, the finding that preadipocytes allowed C2D macrophage cells to  
381 spread regardless of their differentiation state, while white adipocytes inhibited macrophage  
382 spreading supports the hypothesis that macrophage phenotype is dependent on the adipose tissue  
383 microenvironment.

384 WAT has been well characterized. There are differences in CD68<sup>+</sup> macrophages between  
385 visceral and subcutaneous WAT [36]. “Obese” WAT has increased proinflammatory cytokine  
386 transcripts [37,38] and secreted cytokines such as TNF [39], angiotensinogen, PAI-1,  
387 PGAR/FIAF, IL-6, leptin, and resistin [40-42]. In particular, isolated adipocytes secrete TNF-  
388 alpha, IL-6, IL-8, IL-1Ra, IL-10, leptin, adiponectin, resistin [41] and visfatin [40,43] and  
389 various populations of CD14<sup>+</sup> CD31<sup>+</sup> adipose tissue macrophages (ATMs) [40] or MGL1<sup>+</sup>  
390 ATMs have increased *IL10*, *Arg1*, and *Pgc1b* transcript levels [44] or secrete MCP-1, MIP-1 $\alpha$

391 and IL-8 [43]. *Nos2* and *IL1b* transcripts also go up in MGL1<sup>-</sup> CCR2<sup>+</sup> macrophage populations  
392 around necrotic adipocytes [44]. One explanation for the differences may be the origins of the  
393 tissues. Brown adipose cells may be more closely related to muscle cells than white adipose  
394 cells [45,46]. Macrophages in normal muscle are angiogenic or anti-inflammatory [47]. In  
395 addition, BAT and WAT express and secrete different autocrine, paracrine and endocrine  
396 signals. WAT has been recognized as an endocrine organ. It produces and secretes a plethoric  
397 collection of adipokines [48-50]. Among them for example, adipisin, leptin and adiponectin are  
398 highly expressed in WAT, whereas their production in BAT is associated only with  
399 thermogenically inactive BAT cells [51]. In contrast, BAT has been reported to express other  
400 cellular mediators, such as basic fibroblast growth factor [52] and prostaglandins E2 and F2 $\alpha$   
401 [53]. BAT cells also produce T4 thyroxine deiodinase type II [54] and nitric oxide synthase  
402 enzymes (eNOS and iNOS) [55] which enables it to produce T3 and NO, respectively.  
403 **Uncoupling protein, unique to BAT, also exhibits chloride channel properties [56]. Chloride**  
404 **channels can regulate NADPH oxidase membrane depolarization [57] and can regulate**  
405 **phagocyte cell function [58]. Therefore, it is possible that BAT can regulate the C2D**  
406 **macrophage cell phenotype because of UCP's unique ability to regulate oxidative metabolism.**  
407 While it is not clear at present what specific molecular mechanisms are responsible for the  
408 distinct phenotypic differences between WAT-C2D and BAT-C2D, we have shown that there are  
409 significant interactions between adipocytes and macrophages that is mediated by cytokines and  
410 cell-cell contact that affects the differentiation and function of both macrophages and adipocytes  
411 [35]. This current study extends the macrophage interaction to include brown adipocytes by  
412 showing that C2D macrophages traffic to the BAT and they acquire a phenotype unique to that

413 tissue. The data support the hypothesis that macrophage plasticity is dependent upon  
414 environmental signals and is not predetermined as some have suggested [10].

415

416 It is possible that the adoptive transfer technique or procedures used in recovering  
417 macrophages for our study may have impacted the results. First, adoptive transfer could have  
418 induced a peritonitis or inflammation in the adipose tissue. We do not believe this to be the case.  
419 Although, we detected some neutrophils (28% PMNs in PEC, 3% in BAT and 10% in WAT), the  
420 lack of an acute inflammatory response where one would expect a large PMN inflammation  
421 (>80% PMN) [59] and extensive macrophage activation [60] suggests that we did not induce a  
422 peritonitis or abnormal inflammation in these tissues. We found that the recipient host  
423 macrophages that were isolated from the SVF had a similar cell surface phenotype to the WAT-  
424 C2D cells. These data suggest that the C2D macrophages were acquiring a “resident  
425 macrophage phenotype” as opposed to a proinflammatory phenotype which would be expected  
426 of recently immigrating macrophages in obese mice [18,43]. This hypothesis is supported by  
427 the fact that we saw inconsistent evidence of a proinflammatory phenotype in WAT-C2D  
428 because both M1 (e.g. TNF- $\alpha$ ) and M2 (IL-10) [61,62] markers were up regulated. The  
429 differential counts of the cells in WAT and BAT also did not reflect an inflammatory milieu.  
430 The CD11b expression on WAT-C2D macrophages would also be reflective of cells which are  
431 undergoing normal cell trafficking [63].

432 Ruan *et al.* found that the isolation of adipocytes with collagenase for 2 hours led to the  
433 activation of the adipocytes when they were assayed *in vitro* [64]. Our macrophages were  
434 isolated with a 40 minute collagenase treatment and that exposure could have affected them and  
435 we cannot rule out this possibility. However, the adipocytes were separated from the

436 macrophages quickly and the expression of some of the genes of interest (e.g. TNF and TNFR)  
437 take several hours to upregulate [64]. In addition, if collagenase had a general activating action  
438 on the C2D macrophage cells [65], we probably would not have seen the general down  
439 regulation of C2D macrophage cell gene transcripts in BAT unless BAT had a suppressive  
440 environment.

441         Lastly, we were concerned that the 16 h incubation that we included before we assessed  
442 surface marker expression could have affected the macrophage phenotype [66]. We see changes  
443 in C2D macrophages when they are reintroduced to *in vitro* culture. However, two observations  
444 suggest that the cell surface expressions we report are an accurate sampling of the C2D  
445 macrophage phenotype. 1) The changes induced *in vivo* were still evident after an additional 16  
446 hours of *in vitro* culture and 2) the differential expression of surface markers such as CD11b in  
447 BAT and WAT paralleled the general changes in transcript level in those same tissues. The  
448 RNA used for those analyses was not subject to the 16 h. incubations.

449         In summary, the WAT microenvironment altered C2D macrophage cells differently than  
450 BAT. The changes in WAT were dependent upon the differentiation of both the macrophages  
451 and the adipocytes. In addition, WAT caused C2D macrophage cells to upregulate many genes  
452 and molecules compared to when they were isolated from BAT. To our knowledge, this is the  
453 first study to directly compare the macrophages that have recently trafficked to different adipose  
454 tissues in the absence of complicating chronic diseases or altered genetic states. The evidence  
455 that infiltrating macrophages begin to display unique tissue-specific phenotypes in normal mice  
456 reaffirms the adaptive nature of macrophages to their environment. Determining the properties  
457 of adipose tissue that make BAT and WAT so different may give us clues on how to regulate  
458 macrophages to prevent disease.

459

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470

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649 **Figure legends**

650

651 **Fig. 1. Gating strategies for cell sorting of CFDA-SE positive macrophage cells and effects**

652 **of *in vitro* culture and collagenase treatment on C2D macrophage phenotype.** A) C2D

653 macrophage cells were sorted based on negative expression of CFDA-SE, B) C2D CFDA-SE

654 macrophage cells were sorted based on positive CFDA-SE expression; C) Example of C2D

655 CFDA-SE<sup>+</sup> cells that were sorted from a mixed cell sample such as C2D CFDA-SE<sup>+</sup>

656 macrophages (region 2) co-cultured with 3T3L1 adipocytes (region 1); D) PEC-C2D

657 macrophages were treated with isotype control antibody (top) or anti Mac-2 antibody (middle

658 and bottom) then assessed by flow cytometry. Cells were treated with PBS (middle) or

659 collagenase (bottom) for 40 minutes before antibody probing.

660

661 **Fig. 2. Change in C2D macrophage cell morphology during co-cultured with adipocytes or**

662 **pre-adipocytes *in vitro* and after infiltration into BAT or WAT *in vivo*.** A) C2D macrophage

663 cells were labeled with CFDA-SE or B) C2D macrophage cells labeled with CFDA-SE and

664 isolated from peritoneal cavity (PEC-C2D) were cultured a) alone or co-cultured with b) 3T3L1

665 pre-adipocytes or c) adipocytes as described in the Materials and Methods. Panels a, b and c;

666 Cells viewed on the fluorescent microscope (Magnification x 200). Panels d, e and f are phase

667 contrast images of cells in a, b and c. C) WAT-C2D and BAT-C2D were collected from mice

668 two days after adoptive transfer. C2 D macrophages, WAT and BAT were processed as

669 described in Materials and Methods. Panels a and c images from the confocal microscope (x

670 100). Panels b and d are phase contrast images of the same fields.

671

672 **Fig. 3. Phenotype changes of C2D macrophage cells co-cultured with adipocytes or pre-**  
673 **adipocytes *in vitro*.** C2D or PEC-C2D cells labeled with CFDA-SE were cultured alone or co-  
674 cultured with 3T3L1 adipocytes or pre-adipocytes and the cell mixtures were immunostained for  
675 flow cytometry as described in Materials and Methods. C2D macrophage cell phenotypes were  
676 analyzed within CFDA-SE<sup>+</sup> population. A) C2D macrophage cells grown *in vitro* were cultured  
677 alone, co-cultured with 3T3L1 adipocytes or with pre-adipocytes. B) PEC-C2D macrophage  
678 cells were cultured alone, co-cultured with adipocytes or with pre-adipocytes. The data is  
679 presented as the mean  $\pm$  SEM (n= 3 independently collected samples per treatment group).  
680 Different letters indicate a significant difference between control, preadipocytes or adipocytes for  
681 CD11b (lower case) or Mac-2 (upper case) cell surface proteins. A *P* value of < 0.05 was  
682 considered significant.

683

684 **Figure 4. Phenotype changes in C2D macrophage cells isolated from WAT or BAT *in vivo***  
685 **after *i.p.* adoptive transfer.** C2D macrophage cells were isolated from WAT and BAT and  
686 immunostained to detect Ly-6C, Mac-2, CD11b and F4/80 by flow cytometry. A) Surface  
687 marker expression was assessed on CFDA-SE-positive cells isolated from WAT or BAT. B)  
688 Surface marker expression was assessed on CFDA-SE-positive cells (left) or CFDA-SE-negative  
689 cells (right) in the stromal vascular fraction isolated from WAT. The data is represented as mean  
690  $\pm$  SEM (n= 3-6 independently collected samples per adipose tissue type). Comparisons were  
691 done between samples stained for the same surface markers in panels A or B. \* indicates a  
692 significant difference with a *P* value of < 0.05.

693

694 **Fig. 5. Expression analysis of C2D macrophage cells isolated from WAT or BAT.** C2D  
695 macrophage cells were isolated from BAT and WAT by collagenase treatment as described in  
696 Materials and Methods. PEC-C2D (black bars), WAT-C2D (white bars) or BAT-C2D (grey bars)  
697 were purified by FACS and gene transcripts were quantified by qRT-PCR as described in the  
698 Materials and Methods. The data is presented as the mean  $\pm$  SEM (n=2 independent RNA  
699 samples; fat pads from 4 mice per pooled sample). Significant differences found between: \*  
700 PEC-C2D *vs.* WAT-C2D, PEC-C2D *vs.* BAT-C2D and WAT-C2D *vs.* BAT-C2D; † PEC-C2D  
701 *vs.* BAT-C2D and WAT-C2D *vs.* BAT-C2D; § PEC-C2D *vs.* WAT-C2D and PEC-C2D *vs.*  
702 BAT-C2D; ■ WAT-C2D *vs.* BAT-C2D; ○ PEC-C2D *vs.* WAT-C2D.

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1 **Evaluation of macrophage plasticity in brown and white adipose tissue**

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3

4 M. Teresa Ortega<sup>1†</sup>, Linglin Xie<sup>1†</sup>, Silvia Mora<sup>2</sup>, and Stephen K. Chapes<sup>1\*</sup>

5 <sup>1</sup>Division of Biology, Kansas State University, Manhattan, KS, 66506 and <sup>2</sup> Department of

6 Cellular and Molecular Physiology, Institute of Translational Medicine, The University of

7 Liverpool, Crown Street, Liverpool L69 3BX, United Kingdom

8 †Co-first authors

9

10 **Running Title: Macrophage-adipose interactions**

11

12

13 \*Address correspondence to:

14 Stephen K. Chapes

15 116 Ackert Hall

16 Kansas State University

17 Manhattan, KS 66506-4901

18 E mail:[skcbiol@ksu.edu](mailto:skcbiol@ksu.edu)

19 Voice: 785-532-6795

20 Fax: 785-532-6653

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24 **ABSTRACT**

25           There are still questions about whether macrophage differentiation is predetermined or is  
26 induced in response to tissue microenvironments. C2D macrophage cells reside early in the  
27 macrophage lineage *in vitro*, but differentiate to a more mature phenotype after adoptive transfer  
28 to the peritoneal cavity (PEC-C2D). Since C2D macrophage cells also traffic to adipose tissue  
29 after adoptive transfer, we explored the impact of white adipose tissue (WAT), brown adipose  
30 tissue (BAT) and *in vitro* cultured adipocytes on C2D macrophage cells.

31           When PEC-C2D macrophage cells were cultured with preadipocytes the cells stretched  
32 out and CD11b and Mac-2 expression was lower compared to PEC-C2D macrophage cells  
33 placed *in vitro* alone. In contrast, PEC-C2D cells co-cultured with adipocytes maintained  
34 smaller, round morphology and more cells expressed Mac-2 compared to PEC-C2D co-cultured  
35 with preadipocytes. After intraperitoneal injection, C2D macrophage cells migrated into both  
36 WAT and BAT. A higher percentage of C2D macrophage cells isolated from WAT (WAT-  
37 C2D) expressed Ly-6C (33%), CD11b (11%), Mac-2 (11%) and F4/80 (29%) compared to C2D  
38 macrophage cells isolated from BAT (BAT-C2D). Overall, BAT-C2D macrophage cells had  
39 reduced expression of many cytokine, chemokine and receptor gene transcripts when compared  
40 to *in vitro* grown C2D macrophages, while WAT-C2D macrophage cells and PEC-C2D up-  
41 regulated many of these gene transcripts. These data suggest that the C2D macrophage  
42 phenotype can change rapidly and distinct phenotypes are induced by different  
43 microenvironments.

44

45

46 **Key words: macrophage, plasticity, adipocyte, adipose tissue, trafficking**

47



48 **1. Introduction**

49           Macrophages are found throughout the body and serve as initiators and effectors of the  
50 innate immune system [1-6]. Macrophages differentiate from bone marrow hematopoietic stem  
51 cells through various stages including, macrophage-colony forming cells to monoblasts,  
52 promonocytes and finally into monocytes [7,8]. Monocytes enter the bloodstream, where they  
53 circulate before migrating into tissues. There they differentiate into tissue-specific macrophages  
54 [9]. Macrophages are a heterogeneous group of cells which have different functions,  
55 morphologies and phenotypic properties [7,9]. Heterogeneity is commonly associated with  
56 macrophages as a consequence of the functions, organ sites and immune status of the host [9,10].  
57 However, there is controversy about macrophage adaptation to microenvironmental signals *in*  
58 *vivo* [10-13]. Some think that since subpopulations of macrophages have either proinflammatory  
59 (M1) or anti-inflammatory (M2) properties, there are predetermined fates for monocytes and  
60 macrophages as opposed to the microenvironmental signaling leading to the macrophage  
61 plasticity [10,14].

62           C2D macrophage cells reside early in the macrophage lineage *in vitro*, but differentiate to  
63 a more mature, phenotype after adoptive transfer to the peritoneal cavity (PEC-C2D) [15]. These  
64 macrophage cells differentiate and traffic like primary macrophages and can provide insight into  
65 macrophage function [16]. In particular, they can provide evidence about macrophage plasticity  
66 in response to different microenvironments.

67           White adipose tissue (WAT) and brown adipose tissue (BAT) have distinct physiological  
68 functions. WAT is an energy storage and endocrine organ [17,18]. In contrast, BAT functions  
69 as an energy-dissipating organ through adaptive-thermogenesis [19]. These adipocyte depots  
70 display different morphology, cellular characteristics, body localizations and function [19-24].

71 Previous studies have suggested that macrophage function varies considerably in  
72 different fat depots [25]. Some have also suggested that macrophage plasticity is an artifact of *in*  
73 *vitro* manipulations [10]. Given the controversy about macrophage adaptation to  
74 microenvironmental signals *in vivo* [10-13] and the fact that little is known about BAT-  
75 macrophage interactions, we investigated whether macrophage phenotype is predetermined or is  
76 adaptable.

## 77

## 78 **2. Materials and methods**

### 79 *2.1 Mouse strains*

80 C57BL/6J (B6) mice were originally obtained from the Jackson Laboratory (Bar Harbor,  
81 ME). Male and female, 8-16 week-old mice were bred in the rodent facility of the Division of  
82 Biology at Kansas State University and used in these experiments. Mice were fed a normal  
83 mouse chow diet (5001, PMI International, St. Louis, MO) and were allowed to feed *Ad libitum*.  
84 All animal experiments were approved by the Institutional Animal Care and Use Committee.

### 85

### 86 *2.2 Antibodies and Reagents*

87 Collagenase (Type II), insulin from bovine pancreas, 3-Isobutyl-1-methylxanthine  
88 (IBMX) and dexamethasone were obtained from Sigma-Aldrich Co. (St. Louis, MO).  
89 Carboxyfluorescein diacetate, succinimidyl (CFDA-SE) ester was purchased from Molecular  
90 probes (Eugene, OR). APC conjugated anti-CD11c, APC conjugated anti-F4/80, APC  
91 conjugated anti-CD11b, ALEXA Fluor 647 conjugated anti-Mac2, and their isotype control  
92 antibodies were purchased from eBioscience (San Diego, CA). Biotin conjugated anti-Ly-6C

93 (ER-MP20) and its isotype control antibody were from BD Pharmingen (San Jose, CA). APC  
94 conjugated Streptavidin was purchased from eBioscience (San Diego, CA).

95

### 96 *2.3 Cell lines and cell culture*

97         The C2D macrophage cell line was created as described by our group [26]. These cells  
98 were derived from C2D mouse bone marrow and selected in the presence of macrophage colony  
99 stimulating factor (M-CSF). These cells have the *MHCII*<sup>-/-</sup> and *Tlr4*<sup>Lps-n</sup> genotype and are  
100 histocompatible with mice of the H-2<sup>b</sup> haplotype. C2D cells were grown in Dulbecco's  
101 Modified Eagle's Medium with 4% fetal bovine serum (DMEM<sub>4</sub>) supplemented with 0.3%  
102 Glutamax and 10% Opti-MEM in 150-mm tissue culture plates.

103         3T3L1 adipocytes were obtained from the American Type Culture Collection (Manassas,  
104 VA). Adipocytes were cultured and differentiated as described previously [27]. Briefly, 3T3L1  
105 cell differentiation was induced by culturing cells in DMEM containing 10% FBS (DMEM<sub>10</sub>), 1  
106 μM dexamethasone, 1.7 μM insulin and 0.5 mM IBMX for 4 days. On the fourth day, the  
107 3T3L1 cells were cultured in DMEM<sub>10</sub> with 1.7 μM insulin. On day 8, 3T3L1 cells were  
108 maintained in DMEM<sub>10</sub>. Undifferentiated preadipocytes and adipocytes differentiated for 6-8  
109 days were used in the experiments. 3T3L1 cells (1×10<sup>6</sup> cells) were directly co-cultured with  
110 1×10<sup>6</sup> C2D cells grown exclusively *in vitro* or 1×10<sup>6</sup> cells adoptively transferred C2D  
111 macrophage cells isolated from the peritoneal cavity (PEC-C2D).

112         Bone marrow derived macrophages (BM-Mo) were differentiated from B6 mouse bone  
113 marrow cells isolated from the femora, tibiae, and humeri. Briefly, the bones were recovered and  
114 cleaned of all non-osseous tissue. The marrow cavity was flushed with a sterile PBS solution.  
115 The red blood cells were lysed by incubating in ammonium chloride lysis buffer (0.15 M NH<sub>4</sub>Cl,

116 10 mM KHCO<sub>3</sub>, and 0.1 mM Na<sub>2</sub>EDTA, pH 7.3) for 5 min in ice. Cells were centrifuged (300 x  
117 g, 5 min) and washed two times with DMEM<sub>2</sub>. Bone marrow cells were seeded and incubated in  
118 M-CSF medium (DMEM<sub>10</sub>, OPTI-MEM, 0.01 M HEPES, 50 ng/ml gentamycin, 1.5 ng/ml  
119 rMCSF-1) for 7 days at 37 °C, 8 % CO<sub>2</sub>. BM-Mo were indirectly co-cultured with collagenase-  
120 digested white adipose tissue (WAT) gonadal fat pads or collagenase- digested BAT perispleen  
121 or interscapular fat pads as described below.

122

#### 123 *2.4 Adoptive transfer of labeled cells*

124 C2D cells were suspended in sterile, pre-warmed (37°C) phosphate buffered saline (PBS;  
125 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) at a concentration of 1.5 x 10<sup>6</sup> cells per  
126 ml, further stained with CFDA-SE according to the manufacturer's protocol. Briefly, C2D cells  
127 were incubated with 22 µM of CFDA-SE solution at 37 °C for 15 minutes. After centrifugation  
128 at 370 x g for 10 minutes, cell pellets were suspended in pre-warmed PBS and incubated in 37°C  
129 for an additional 20 minutes. Cells were then washed twice in PBS, and suspended at a  
130 concentration of 4 x 10<sup>7</sup> cells per ml in PBS. One and one-half ml of the cell suspension of  
131 CFDA-SE labeled C2D or normal C2D cells was injected intraperitoneally (*i.p.*) per mouse.

132

#### 133 *2.5 Peritoneal cell extraction and fat tissue isolation*

134 PEC-C2D macrophage cells were obtained from B6 mice by peritoneal lavage 36 hours  
135 after intraperitoneal injection of 4 x 10<sup>7</sup> of C2D macrophage cells labeled with CFDA-SE. The  
136 peritoneal exudate red blood cells were lysed as described in section 2.3. One-half of the cells  
137 were treated with 1 mg/ml collagenase type II at 37°C with shaking (60 rpm) for 40 minutes.

138 Control or collagenase-treated cells were washed three times with PBS and  $3 \times 10^6$  cells were  
139 plated into 150-mm cell culture plates and incubated in DMEM<sub>4</sub> for 16 hours.

140 Isolation of adipocytes and CFDA-SE labeled C2D macrophage cells was performed as  
141 previously described [15,16]. Adipocytes were isolated from both mouse gonadal fat pads  
142 (depots connected to the uterus and ovaries in females and the epididymis and testes in males)  
143 and perispleen adipose tissues by collagenase digestion [28,29]. We confirmed BAT origin by  
144 quantitating the mRNA of PRDM16 by qRT-PCR [30] and/or UCP-1 [31] in tissues collected  
145 from perispleen and interscapular isolates (data not shown). Gonadal fat pads weighed an  
146 average of 268 mg while perispleen fat averaged 98 mg. Interscapular fat pads weighed an  
147 average of 61 mg. The fat pads were minced and incubated for 10 min in pre-warmed (37°C)  
148 Krebs-Ringer phosphate (KRP) buffer (12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM  
149 MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 mM Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 2.5 mM D-glucose, and 2 %  
150 bovine serum albumin, pH 7.4), thereafter the samples were incubated with Type II collagenase  
151 (1mg/ml) for 40 min at 37°C with constant shaking at 60 rpm. The WAT or BAT cells were  
152 passed through a 100 µm cell strainer; cells were then centrifuged at 370 x g for 1 minute and  
153 washed with Krebs Ringer buffer twice. Additionally, the adipocytes isolated from the paired  
154 gonadal fat pads were separated into 2 major fractions. The floating upper layer was primarily  
155 white adipocytes and the pelleted fraction was a mixture of stromal-vascular fraction (SVF) cells  
156 containing macrophages. Both cell fractions were collected and washed twice with KRP buffer.

157 WAT and BAT cells were scored for numbers and viability on a hemacytometer using  
158 trypan blue exclusion. Viability was 91±0%, 81±3% and 87±0% for PEC, BAT and SVF cells,  
159 respectively, after isolation and collagenase treatment. In collagenase-digested samples, we  
160 isolated an average of  $1.3 \times 10^5$  C2D macrophage cells per mouse from gonadal WAT and  $8.9 \times$

161  $10^4$  C2D macrophage cells from perispleen BAT or  $3.5 \times 10^4$  C2D macrophage cells from  
162 interscapular BAT.  $1 \times 10^5$  cells were pelleted onto a cytopsin slide for differential staining. A  
163 mixture of white adipocytes (upper layer) and SVF cells was co-incubated at  $37^\circ\text{C}$  in DMEM<sub>10</sub>,  
164 for 16 hours at a concentration of  $1 \times 10^5$  cells/ml in a 150-mm culture dish. The adipocytes  
165 remained dispersed in the medium and the SVF cells attached to the 150-mm culture plate. The  
166 adipocytes isolated from perispleen adipose were collected from the cell pellets and washed  
167 twice with KRP buffer. Cells isolated from perispleen BAT ( $3 \times 10^6$ ) were cultured at  $37^\circ\text{C}$  in  
168 DMEM<sub>10</sub> in a 150-mm culture plate for 16 hours.

169

## 170 *2.6 Flow cytometry analysis of C2D macrophage cells*

171 Cell sorting was based on C2D macrophage cell CFDA-SE fluorescence, with the lowest  
172 10 % of the positive cells not selected. Briefly, cell sorting was performed with either a  
173 FACSVantage SE cell sorter (Becton Dickson, Rockville, MD) or a MoFlo XDP Sterile Cell  
174 Sorter (Beckman Coulter), using specimen optimization and calibration techniques according to  
175 the manufacturer's recommendations. Cells were sorted at a rate of 15,000 cells per second and  
176 approximately  $1 \times 10^6$  viable (trypan blue exclusion), positive cells per group were collected on  
177 ice and centrifuged at  $350 \times g$  for 5 min at  $4^\circ\text{C}$  for PCR Array or qRT-PCR analysis.

178 We found a loss of cell surface markers following collagenase treatment. For example,  
179 Mac-2 was down regulated over 50% after a 40 minute collagenase treatment based on control  
180 PEC-C2D (data not shown). However, we were also concerned that this incubation would also  
181 influence the cells. Therefore, we also evaluated the changes in TNF gene expression over time  
182 after the PEC-C2D cells were cultured *in vitro*. We found C2D macrophage gene expression  
183 was reduced some but was still positive for at least 24 hrs (data not shown). Therefore, we felt a

184 reasonable approach to phenotype the cell surface molecules of the recovered C2D macrophage  
185 cells from PEC, BAT and WAT would be to allow the cells to recover *in vitro* for 16 hours at  
186 37°C in medium. Therefore, control or collagenase-treated C2D macrophage cells isolated from  
187 the peritoneal cavity (PEC-C2D), WAT and BAT were resuspended in DMEM<sub>4</sub> and incubated  
188 for 16 hours prior to labeling cell surface proteins and assessment by flow cytometry.

189 Cells were transferred to wells of 96-well, round-bottom plates and they were blocked  
190 with PBS-goat serum (50:50; 50 µl) at 4 °C for 0.5 hour. Subsequently, macrophage cell surface  
191 proteins were identified by direct labeling. Briefly, blocked cells were incubated with the isotype  
192 or specific antibody diluted in Hank's Buffered Salt Solution (HBSS; 0.137 M NaCl, 5.4 mM  
193 KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>)  
194 for 1 hour in the dark at 4°C. After two washes with HBSS, cells were fixed in 1% formalin.  
195 Labeled cell surface proteins were assessed by flow cytometry. We gated on live, CFDA-SE-  
196 positive or CFDA-SE negative cells, subsequently we assayed for the presence or absence of the  
197 selected cell surface markers.

198

### 199 *2.7 Real time quantitative RT-PCR analysis*

200 RNA was obtained by suspending the pelleted cells in 1 ml of TriReagent (Molecular  
201 Research Center). The solution was transferred to 2.0 ml Heavy Phase Lock Gel tubes (5  
202 Prime). 200 µl of chloroform was added and the mixture was shaken for 15 seconds. The  
203 samples were then centrifuged at 12,000 x g for 10 minutes at 4°C and the aqueous phase was  
204 transferred to clean 1.5 ml tubes. 500 µl of isopropanol was added and RNA was precipitated at  
205 -20°C for 24 hours. Samples were subsequently centrifuged at 12,000 x g for 10 minutes. The  
206 RNA pellet was washed with 1 ml of 70% ethanol and samples were centrifuged at 7.4 x g for 5

207 minutes. The 70% ethanol was decanted from the pellet; the pellet was allowed to slightly air  
208 dry and was resuspended in 50 µl of nuclease-free water. RNA samples were purified and  
209 DNase treated with EZRNA total RNA kit (Omega Bio-Tek, Inc.). One step qRT-PCR was  
210 performed using the SuperScript III Platinum SYBR Green kit (Invitrogen; Carlsbad, CA)  
211 according to the manufacturer's protocol. Primers were designed with the PrimerQuest software  
212 (IDT; <http://www.idtdna.com>) using sequence data from NCBI sequence database as following:  
213 *TNF-α*(NM\_013693) forward 5'-tctcatgcaccaccatcaaggact and reverse 5'-  
214 tgaccactctccctttgcagaact; *IL-6* (NM\_031168.1) forward 5'-tctcatgcaccaccatcaaggact and reverse  
215 5'-tgaccactctccctttgcagaact; *ILI-β*(NM\_008361) forward 5'-aagggtgcttccaaacctttgac and reverse  
216 5'-atactgctgctgaagctcttgt; *Arg-1*(NM\_007482) forward 5'-tggetttaaccttggttcttctg and reverse  
217 5'-catgtggcgcattcacagtcact; *Ym-1* (M94584) forward 5'-caccatggccaagctcattcttgt and reverse 5'-  
218 tattggcctgtccttagcceaact; *Fizz-1*(NM\_020509.3) forward 5'-actgcctgtgcttactcgttgact and reverse  
219 5'-aaagctgggttctccaccttca; *Prdm16* (BC059838) forward 5'-tcatcccaggagagctgcatcaaa and  
220 reverse 5'-atcacaggaacacgctacacggat; *Ucp-1* (NM009463.3) forward 5'-ttgagctgctccacagcgcc  
221 and reverse 5'-gttgcctgatcggggcacga;  $\beta$ -*actin* (NM\_007393) forward 5'-  
222 tgtgatggtgggaatgggtcagaa and reverse 5'-tgtggtgccagatcttctccatgt. The qRT-PCRs were  
223 performed in a Cepheid SmartCycler System (Sunnyvale, CA). Fold increase in transcript  
224 expression was calculated:  $E(\text{gene of interest})^{\Delta\text{ct target}}/E(\text{housekeeping})^{\Delta\text{ct housekeeping}}$  where E  
225 (efficiency)= $10^{(-1/\text{slope})}$  as was previously described [32].

## 226 2.8 PCR array analysis

227 Expression analysis of 84 cytokines, chemokines and corresponding receptor genes  
228 involved in inflammatory responses was performed with the mouse inflammatory cytokines and  
229 receptors RT<sup>2</sup> profiler<sup>TM</sup> PCR array system (SuperArray Bioscience Corporation, Frederick,



230 MD). 1.2  $\mu$ g of total RNA was obtained from CFDA SE labeled C2D cells sorted from the PEC,  
231 WAT, and BAT of C57BL/6J mice (n= 2 pooled RNA samples, 4 mice per pooled sample).  
232 Genomic DNA was digested with RNase-free DNAase, followed by first strand cDNA synthesis  
233 and then quantitative mRNA analysis according to manufacturer's protocol. The quantitative  
234 real-time PCR array was done on a BioRad iCycler (BioRad Laboratory, Hercules, CA)  
235 performed with the RT<sup>2</sup> SYBR Green/Fluorescein qPCR Master Mix (SuperArray Bioscience  
236 Corporation, Frederick, MD). Expression of mRNA for each gene was normalized to the  
237 expression of  $\beta$ -actin and compared to the data obtained with the negative control (RNA from  
238 cultured C2D cells) according to the  $\Delta\Delta$ Ct method [32].

239

#### 240 *2.9 Immunofluorescence and image analysis*

241 Gonadal fat pads and perispleen adipose tissue were washed in Krebs-Ringer phosphate  
242 (KRP) buffer fixed in 10% formalin/PBS and were cut into 50- $\mu$ m-thick slices using a TC-2  
243 tissue sectioner (Sorvall Instruments). Tissue slices were mounted onto glass slides, and  
244 differential contrast interference (DIC) images of tissue and CFDA SE-labeled C2D macrophage  
245 cells were observed on a model LSM 5 Pascal Zeiss laser scanning confocal microscope. Tissues  
246 were visualized with 20X/0.5 and 40X/0.75 Plan Neofluor objectives with DIC. CFDA SE-  
247 labeled C2D macrophage cells were visualized using the 488-nm line of an argon ion gas laser  
248 (excitation of CFDA SE), an FT 488 primary dichroic beam splitter, a FT 545 secondary dichroic  
249 beam splitter, a 505-nm to 530-nm-bandpass filter, a photomultiplier tube, and LSM5 Pa software,  
250 version 3.2 SP2. The number of CFDA SE-labeled macrophages per square micrometer of area  
251 of adipose tissue was determined using ImageJ v1.37 (NIH). Images were then imported to  
252 Adobe Photoshop (Adobe Systems, Inc.) for figure processing.

253

254 *2.10 Statistical analysis.*

255 Flow cytometry data, cell distribution data and qRT-PCR data were presented as the  
256 mean  $\pm$  standard error of mean (SEM) of independent experiments (n=3 samples, 3 mice per  
257 sample unless stated otherwise in the Figure legend). Differences in mean were determined  
258 using Student's *t* test (paired, two-tailed) or were determined using the Mann-Whitney rank-sum  
259 test. Differences in cell distribution were assessed using the Chi-Square ( $\chi^2$ ) test. All tests were  
260 calculated using the StatMost statistical package (Data XIOM, Los Angeles, CA). Differences  
261 were considered significantly different when  $P < 0.05$ . To assess differences in the samples  
262 assayed in the PCR Arrays the mean Ct values between macrophage isolates were compared  
263 using minimum significant difference (MSD) [33]. Any difference between means greater than  
264 or equal to the MSD was considered to be a statistically different, while differences less than the  
265 minimum significant difference were considered to be non-significant. An MSD was calculated  
266 using the following equation:  $MSD = 2 \times (s_{\text{pool}}) \times (\sqrt{(1/n_1 + 1/n_2)})$ , where *s* pool is the standard  
267 deviation pooled across all genes and all groups, *n*<sub>1</sub> and *n*<sub>2</sub> are the numbers of replicates for the  
268 two treatments. In this study, *n*<sub>1</sub>=*n*<sub>2</sub>=2, and the pooled standard deviation was equal to 1.61 and  
269 the MSD was equal to 3.22.

270

271 **3. Results**

272 *3.1 Morphological and phenotypic changes of C2D macrophage cells in response to adipocytes*  
273 *in vitro*

274 To determine how different tissue environments impact macrophage phenotypes, we  
275 investigated macrophage responses to preadipocytes and adipocytes. In our experiments we used

276 the C2D macrophage cell line and 3T3L1 cells before or after, differentiation into adipocytes.  
277 We previously established the specific macrophage phenotype expressed by C2D cells after they  
278 were injected *i.p.* (PEC-C2D) or before they respond to other microenvironments [15,16,26]. In  
279 order to visualize and identify the C2D macrophages, C2D cells were labeled with CFDA-SE  
280 prior to their injection into the animals or their co-culture with (3T3L1) preadipocyte/adipocyte  
281 cells *in vitro* for two days. Subsequently, these cells were recovered and analyzed by  
282 fluorescence-activated cell sorting. Cells from peritoneal lavages, BAT and SVF cells from  
283 WAT were sorted by FACS analysis as described in Figure 1. C2D cells grown *in vitro* (Figure  
284 1A, region 1) and C2D CFDA-SE cells (Figure 1B, region 2) were used as negative and positive  
285 controls for gating, respectively, and for sorting C2D CFDA-SE positive cells from mixed cell  
286 samples such as C2D CFDA-SE macrophages co-cultured with 3T3L1 adipocytes (Figure 1C).

287 In comparison to the larger stretched morphology of C2D macrophage cells grown *in*  
288 *vitro* (Figure 2A, panels a and d), the PEC-C2D macrophage cells were round after cell isolation  
289 (Figure 2B, panels a and d). When C2D or PEC-C2D macrophage cells were cultured with  
290 preadipocytes, the cells stretched out (Figure 2A and 2B, panels b and e). In contrast, when C2D  
291 macrophage cells or PEC-C2D cells were co-cultured with differentiated 3T3L1 adipocytes, we  
292 found that the cells maintained a mostly smaller, round morphology (Figure 2A and 2B, panels c  
293 and f), suggesting that differentiated 3T3L1 adipocytes inhibit normal adherence and stretching  
294 of the C2D macrophage cells.

295 The morphological differences in C2D and PEC-C2D macrophage cells co-cultured with  
296 adipocytes were accompanied by changes in cell phenotype defined by cell surface molecules  
297 detected using flow cytometry. *In vitro*, 15% of the C2D macrophage cells expressed Mac-2 but  
298 not CD11b; indicative of an immature macrophage phenotype [15]. C2D macrophage cells

309 acquire a more differentiated phenotype after adoptive transfer *in vivo* with high levels of  
300 macrophage-specific molecules CD11b, Mac2, F4/80, cfms and low levels of CD11c, and Gr-1  
301 (Ly6G) [15,16]. We observed no change in the numbers of cells that expressed either of the cell  
302 surface markers on C2D macrophage cells co-cultured with pre-adipocytes compared to C2D  
303 macrophage cells cultured alone (Figure 3A). However, we observed a statistically significant  
304 ( $P<0.05$ ) increase in the number of C2D macrophages that expressed CD11b when co-cultured  
305 with 3T3L1 adipocytes (Figure 3A). For PEC-C2D macrophage cells, their maturation in the  
306 peritoneal cavity is accompanied by an increase in the number of cells that express CD11b  
307 [15,16]. Significantly more PEC-C2D macrophage cells co-cultured *in vitro* with adipocytes  
308 expressed Mac-2 than PEC-C2D macrophage cells cultured alone or with preadipocytes (Figure  
309 3B,  $P<0.05$ ). We observed a significant decrease ( $P<0.05$ ) in the number of PEC-C2D  
310 macrophage cells expressing CD11b and Mac-2 when incubated with preadipocytes (Figure 3B).

311

### 312 *3.2 Assessment of C2D macrophage cells after trafficking into WAT and BAT*

313 We previously found that C2D macrophage cells could be isolated from gonadal WAT  
314 [15]. However, macrophage trafficking to BAT has not been well characterized. We used  
315 confocal microscopy to visualize and count C2D macrophages in WAT and BAT. We counted  
316 an average of 97 C2D macrophages/mm<sup>2</sup> in BAT compared to 146 C2D macrophages/mm<sup>2</sup> in  
317 WAT ( $P>0.05$ , T test,  $n\geq 9$  fields scored per tissue; Figure 2C). We also assessed if C2D  
318 macrophage immigration to WAT and BAT induced inflammation. We did differential staining  
319 of white blood cells isolated from the adipose tissue 36 hrs after *i.p.* injection of C2D  
320 macrophage cells. This survey revealed distinct cell distributions. The white cell distribution in  
321 WAT was 8±1% PMN, 60±2% macrophage/monocyte and 33±1% lymphocytes compared to

322 3±2% PMN, 79±2% macrophage/monocytes and 18±1% lymphocytes in BAT ( $P<0.01$ ;  $\chi^2$  test).  
323 We also assessed C2D macrophage cell localization within WAT and BAT. WAT-C2D  
324 appeared between adipocytes and some appeared to spread around the adipose cells (Figure 2C,  
325 panels a, c, e). In contrast, BAT-C2D appeared only between adipocytes and generally had a  
326 round appearance (Figure 2C, panels b, d, f).

327 To determine if the C2D macrophage cells isolated from BAT and WAT maintained the  
328 same phenotype they expressed in the peritoneum or if they responded to the different tissue  
329 environments, we compared BAT-C2D and WAT-C2D for the expression of Ly-6C, Mac-2,  
330 CD11b and F4/80. Cells were labeled with CFDA and the CFDA-SE positive cells were  
331 assessed (Figure 4A). We observed that 33% of the WAT-C2D expressed Ly-6C, while there  
332 were very few Ly6C-positive (<1%) BAT-C2D. A significantly higher number of WAT-C2D  
333 macrophage cells expressed ( $P<0.05$ ) Mac-2 compared to BAT-C2D macrophage cells. We  
334 observed over 30% of the WAT-C2D macrophage cells expressed F4/80, but almost no BAT-  
335 C2D expressed F4/80 (Figure 4A). Significantly more WAT-C2D cells expressed CD11b than  
336 BAT-C2D (Figure 4A). When we compared C2D macrophage phenotype to the phenotype of  
337 the recipients' macrophages, we observed that a higher percentage of WAT-C2D cells expressed  
338 F4/80 compared to the recipient's macrophages (CFDA negative cells; Figure 4B). We detected  
339 no significant differences in the % positive cells that expressed Ly-6C, Mac-2 or CD11b when  
340 we compared WAT-C2D and recipient macrophages (Figure 4B).

341 To further characterize the impact of the adipose microenvironment on recently migrated  
342 macrophages, we measured transcript levels in C2D macrophages that were isolated and sorted  
343 from the peritoneal cavity, WAT or BAT. We measured the expression of an array of  
344 inflammatory chemokines and cytokines and their receptor genes by quantitative PCR. As

345 shown in Figure 5, the overall expression of chemokine, cytokine and receptor genes was  
346 dramatically down-regulated in BAT-C2D cells relative to the gene expression of C2D cells  
347 maintained *in vitro*, compared to those of PEC-C2D and/or sorted WAT-C2D cells. When  
348 WAT-C2D cells were compared to PEC-C2D macrophage cells, transcript levels for several  
349 chemokines were lower (Figure 5A). These included MIP-3b/CCL19 (3 vs. 9 fold), NAP-  
350 3/CXCL1 (-0.5 vs. 5 fold), CCL11 (1 vs. 6 fold), CXCL5 (2 vs. 4 fold), CXCL9 (3 vs. 7) and  
351 CXCL12 (4 vs. 12 fold). MIP-1a/CCL3 and MCP-5/CCL12 were also down-regulated in PEC-  
352 C2D compared to C2D macrophages grown *in vitro*.

353 Interestingly we found that two cytokines receptor genes had higher transcript levels in  
354 PEC-C2D macrophage cells compared to WAT-C2D macrophage cells; CD121a/IL 1r1 (8 vs. 2  
355 fold) and CD130/IL6st (6 vs. 3 fold). Additionally, C3 (5 vs.1 fold) had higher expression in  
356 PEC-C2D cells compared to WAT C2D cells (Figure 5E).

### 357 *3.3 Macrophages gene expression in response to WAT and BAT in vitro*

358 C2D macrophage cells exhibited distinct phenotypes in response to WAT or BAT  
359 adipose environments. Therefore, to confirm that C2D macrophage behavior reflected that of  
360 primary macrophages, we indirectly co-cultured BM-Mo in transwell plates (top) with  
361 collagenase-digested WAT and BAT (bottom). RNA from the BM-Mo was isolated and the  
362 transcript levels of *TNF- $\alpha$* , *IL-6*, *IL-1 $\beta$* , *Arg-1*, *Ym-1* and *Fizz-1* were assessed using qRT-PCR  
363 (Table 1). WAT incubated BM-Mo macrophages had higher *TNF- $\alpha$*  and *IL-6* transcript levels  
364 than BAT-BM Mo (Table 1). This would be consistent with the observations seen with C2D  
365 macrophages. In contrast, there were no differences in *IL-1 $\beta$*  or the anti inflammatory genes  
366 *Arg-1*, *Ym-1* and *Fizz-1* between BM-Mo inoculated in WAT or BAT (Table 1).

367

368 **4. Discussion**

369 Adipose tissue contains a heterogeneous array of cells including preadipocytes and  
370 adipocytes along with resident and inflammatory macrophages constituting up to 40 percent of  
371 the cell population [34]. Additionally, the trafficking of C2D macrophage cells to both WAT  
372 and BAT provided a unique opportunity to determine the impact of these distinct adipose  
373 environments on recently immigrating macrophages and how different microenvironments affect  
374 macrophage plasticity. We used the C2D macrophage cell line to investigate this question. This  
375 is a powerful model because the cells are phenotypically defined both *in vitro* and *in vivo* and the  
376 phenotypic change of the C2D macrophages in response to WAT or BAT paralleled the *in vitro*  
377 response of primary macrophages {Table 1; also see reference [35]}. Therefore, by knowing the  
378 characteristics of the C2D macrophages before and after exposure to the different adipose tissues  
379 we know exactly what changes are due to their immediate exposure to different  
380 microenvironments. Indeed, the finding that preadipocytes allowed C2D macrophage cells to  
381 spread regardless of their differentiation state, while white adipocytes inhibited macrophage  
382 spreading supports the hypothesis that macrophage phenotype is dependent on the adipose tissue  
383 microenvironment.

384 WAT has been well characterized. There are differences in CD68<sup>+</sup> macrophages between  
385 visceral and subcutaneous WAT [36]. “Obese” WAT has increased proinflammatory cytokine  
386 transcripts [37,38] and secreted cytokines such as TNF [39], angiotensinogen, PAI-1,  
387 PGAR/FIAF, IL-6, leptin, and resistin [40-42]. In particular, isolated adipocytes secrete TNF-  
388 alpha, IL-6, IL-8, IL-1Ra, IL-10, leptin, adiponectin, resistin [41] and visfatin [40,43] and  
389 various populations of CD14<sup>+</sup> CD31<sup>+</sup> adipose tissue macrophages (ATMs) [40] or MGL1<sup>+</sup>  
390 ATMs have increased *IL10*, *Arg1*, and *Pgc1b* transcript levels [44] or secrete MCP-1, MIP-1 $\alpha$

391 and IL-8 [43]. *Nos2* and *IL1b* transcripts also go up in MGL1<sup>-</sup> CCR2<sup>+</sup> macrophage populations  
392 around necrotic adipocytes [44]. One explanation for the differences may be the origins of the  
393 tissues. Brown adipose cells may be more closely related to muscle cells than white adipose  
394 cells [45,46]. Macrophages in normal muscle are angiogenic or anti-inflammatory [47]. In  
395 addition, BAT and WAT express and secrete different autocrine, paracrine and endocrine  
396 signals. WAT has been recognized as an endocrine organ. It produces and secretes a plethoric  
397 collection of adipokines [48-50]. Among them for example, adipisin, leptin and adiponectin are  
398 highly expressed in WAT, whereas their production in BAT is associated only with  
399 thermogenically inactive BAT cells [51]. In contrast, BAT has been reported to express other  
400 cellular mediators, such as basic fibroblast growth factor [52] and prostaglandins E2 and F2 $\alpha$   
401 [53]. BAT cells also produce T4 thyroxine deiodinase type II [54] and nitric oxide synthase  
402 enzymes (eNOS and iNOS) [55] which enables it to produce T3 and NO, respectively.  
403 Uncoupling protein, unique to BAT, also exhibits chloride channel properties [56]. Chloride  
404 channels can regulate NADPH oxidase membrane depolarization [57] and can regulate  
405 phagocyte cell function [58]. Therefore, it is possible that BAT can regulate the C2D  
406 macrophage cell phenotype because of UCP's unique ability to regulate oxidative metabolism.  
407 While it is not clear at present what specific molecular mechanisms are responsible for the  
408 distinct phenotypic differences between WAT-C2D and BAT-C2D, we have shown that there are  
409 significant interactions between adipocytes and macrophages that is mediated by cytokines and  
410 cell-cell contact that affects the differentiation and function of both macrophages and adipocytes  
411 [35]. This current study extends the macrophage interaction to include brown adipocytes by  
412 showing that C2D macrophages traffic to the BAT and they acquire a phenotype unique to that



413 tissue. The data support the hypothesis that macrophage plasticity is dependent upon  
414 environmental signals and is not predetermined as some have suggested [10].

415  
416 It is possible that the adoptive transfer technique or procedures used in recovering  
417 macrophages for our study may have impacted the results. First, adoptive transfer could have  
418 induced a peritonitis or inflammation in the adipose tissue. We do not believe this to be the case.  
419 Although, we detected some neutrophils (28% PMNs in PEC, 3% in BAT and 10% in WAT), the  
420 lack of an acute inflammatory response where one would expect a large PMN inflammation  
421 (>80% PMN) [59] and extensive macrophage activation [60] suggests that we did not induce a  
422 peritonitis or abnormal inflammation in these tissues. We found that the recipient host  
423 macrophages that were isolated from the SVF had a similar cell surface phenotype to the WAT-  
424 C2D cells. These data suggest that the C2D macrophages were acquiring a “resident  
425 macrophage phenotype” as opposed to a proinflammatory phenotype which would be expected  
426 of recently immigrating macrophages in obese mice [18,43]. This hypothesis is supported by  
427 the fact that we saw inconsistent evidence of a proinflammatory phenotype in WAT-C2D  
428 because both M1 (e.g. TNF- $\alpha$ ) and M2 (IL-10) [61,62] markers were up regulated. The  
429 differential counts of the cells in WAT and BAT also did not reflect an inflammatory milieu.  
430 The CD11b expression on WAT-C2D macrophages would also be reflective of cells which are  
431 undergoing normal cell trafficking [63].

432 Ruan *et al.* found that the isolation of adipocytes with collagenase for 2 hours led to the  
433 activation of the adipocytes when they were assayed *in vitro* [64]. Our macrophages were  
434 isolated with a 40 minute collagenase treatment and that exposure could have affected them and  
435 we cannot rule out this possibility. However, the adipocytes were separated from the

436 macrophages quickly and the expression of some of the genes of interest (e.g. TNF and TNFR)  
437 take several hours to upregulate [64]. In addition, if collagenase had a general activating action  
438 on the C2D macrophage cells [65], we probably would not have seen the general down  
439 regulation of C2D macrophage cell gene transcripts in BAT unless BAT had a suppressive  
440 environment.

441         Lastly, we were concerned that the 16 h incubation that we included before we assessed  
442 surface marker expression could have affected the macrophage phenotype [66]. We see changes  
443 in C2D macrophages when they are reintroduced to *in vitro* culture. However, two observations  
444 suggest that the cell surface expressions we report are an accurate sampling of the C2D  
445 macrophage phenotype. 1) The changes induced *in vivo* were still evident after an additional 16  
446 hours of *in vitro* culture and 2) the differential expression of surface markers such as CD11b in  
447 BAT and WAT paralleled the general changes in transcript level in those same tissues. The  
448 RNA used for those analyses was not subject to the 16 h. incubations.

449         In summary, the WAT microenvironment altered C2D macrophage cells differently than  
450 BAT. The changes in WAT were dependent upon the differentiation of both the macrophages  
451 and the adipocytes. In addition, WAT caused C2D macrophage cells to upregulate many genes  
452 and molecules compared to when they were isolated from BAT. To our knowledge, this is the  
453 first study to directly compare the macrophages that have recently trafficked to different adipose  
454 tissues in the absence of complicating chronic diseases or altered genetic states. The evidence  
455 that infiltrating macrophages begin to display unique tissue-specific phenotypes in normal mice  
456 reaffirms the adaptive nature of macrophages to their environment. Determining the properties  
457 of adipose tissue that make BAT and WAT so different may give us clues on how to regulate  
458 macrophages to prevent disease.

459

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470

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649 **Figure legends**

650

651 **Fig. 1. Gating strategies for cell sorting of CFDA-SE positive macrophage cells and effects**

652 **of *in vitro* culture and collagenase treatment on C2D macrophage phenotype.** A) C2D

653 macrophage cells were sorted based on negative expression of CFDA-SE, B) C2D CFDA-SE

654 macrophage cells were sorted based on positive CFDA-SE expression; C) Example of C2D

655 CFDA-SE<sup>+</sup> cells that were sorted from a mixed cell sample such as C2D CFDA-SE<sup>+</sup>

656 macrophages (region 2) co-cultured with 3T3L1 adipocytes (region 1); D) PEC-C2D

657 macrophages were treated with isotype control antibody (top) or anti Mac-2 antibody (middle

658 and bottom) then assessed by flow cytometry. Cells were treated with PBS (middle) or

659 collagenase (bottom) for 40 minutes before antibody probing.

660

661 **Fig. 2. Change in C2D macrophage cell morphology during co-cultured with adipocytes or**

662 **pre-adipocytes *in vitro* and after infiltration into BAT or WAT *in vivo*.** A) C2D macrophage

663 cells were labeled with CFDA-SE or B) C2D macrophage cells labeled with CFDA-SE and

664 isolated from peritoneal cavity (PEC-C2D) were cultured a) alone or co-cultured with b) 3T3L1

665 pre-adipocytes or c) adipocytes as described in the Materials and Methods. Panels a, b and c;

666 Cells viewed on the fluorescent microscope (Magnification x 200). Panels d, e and f are phase

667 contrast images of cells in a, b and c. C) WAT-C2D and BAT-C2D were collected from mice

668 two days after adoptive transfer. C2 D macrophages, WAT and BAT were processed as

669 described in Materials and Methods. Panels a and c images from the confocal microscope (x

670 100). Panels b and d are phase contrast images of the same fields.

671



672 **Fig. 3. Phenotype changes of C2D macrophage cells co-cultured with adipocytes or pre-**  
673 **adipocytes *in vitro*.** C2D or PEC-C2D cells labeled with CFDA-SE were cultured alone or co-  
674 cultured with 3T3L1 adipocytes or pre-adipocytes and the cell mixtures were immunostained for  
675 flow cytometry as described in Materials and Methods. C2D macrophage cell phenotypes were  
676 analyzed within CFDA-SE<sup>+</sup> population. A) C2D macrophage cells grown *in vitro* were cultured  
677 alone, co-cultured with 3T3L1 adipocytes or with pre-adipocytes. B) PEC-C2D macrophage  
678 cells were cultured alone, co-cultured with adipocytes or with pre-adipocytes. The data is  
679 presented as the mean  $\pm$  SEM (n= 3 independently collected samples per treatment group).  
680 Different letters indicate a significant difference between control, preadipocytes or adipocytes for  
681 CD11b (lower case) or Mac-2 (upper case) cell surface proteins. A *P* value of < 0.05 was  
682 considered significant.

683

684 **Figure 4. Phenotype changes in C2D macrophage cells isolated from WAT or BAT *in vivo***  
685 **after *i.p.* adoptive transfer.** C2D macrophage cells were isolated from WAT and BAT and  
686 immunostained to detect Ly-6C, Mac-2, CD11b and F4/80 by flow cytometry. A) Surface  
687 marker expression was assessed on CFDA-SE-positive cells isolated from WAT or BAT. B)  
688 Surface marker expression was assessed on CFDA-SE-positive cells (left) or CFDA-SE-negative  
689 cells (right) in the stromal vascular fraction isolated from WAT. The data is represented as mean  
690  $\pm$  SEM (n= 3-6 independently collected samples per adipose tissue type). Comparisons were  
691 done between samples stained for the same surface markers in panels A or B. \* indicates a  
692 significant difference with a *P* value of < 0.05.

693

694 **Fig. 5. Expression analysis of C2D macrophage cells isolated from WAT or BAT.** C2D  
695 macrophage cells were isolated from BAT and WAT by collagenase treatment as described in  
696 Materials and Methods. PEC-C2D (black bars), WAT-C2D (white bars) or BAT-C2D (grey bars)  
697 were purified by FACS and gene transcripts were quantified by qRT-PCR as described in the  
698 Materials and Methods. The data is presented as the mean  $\pm$  SEM (n=2 independent RNA  
699 samples; fat pads from 4 mice per pooled sample). Significant differences found between: \*  
700 PEC-C2D vs. WAT-C2D, PEC-C2D vs. BAT-C2D and WAT-C2D vs. BAT-C2D; † PEC-C2D  
701 vs. BAT-C2D and WAT-C2D vs. BAT-C2D; § PEC-C2D vs. WAT-C2D and PEC-C2D vs.  
702 BAT-C2D; ■ WAT-C2D vs. BAT-C2D; ○ PEC-C2D vs. WAT-C2D.

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## \*Highlights

- > C2D macrophage phenotype change rapidly in response to different microenvironments.
- > Macrophage phenotype responses are dependent on the differentiated stages of the macrophages and adipocytes.
- > Brown adipose tissue has distinct impact on macrophages compared to white adipose tissue.

Figure

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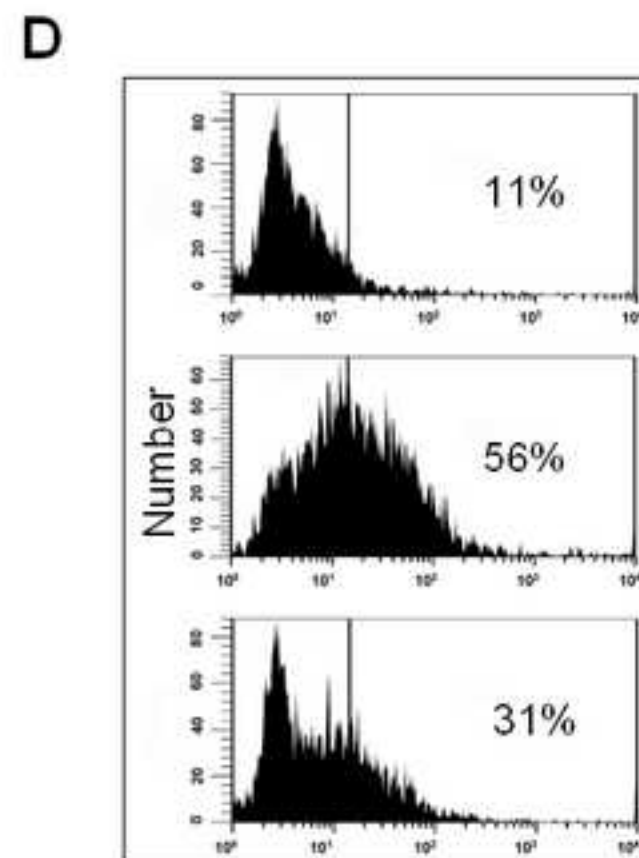
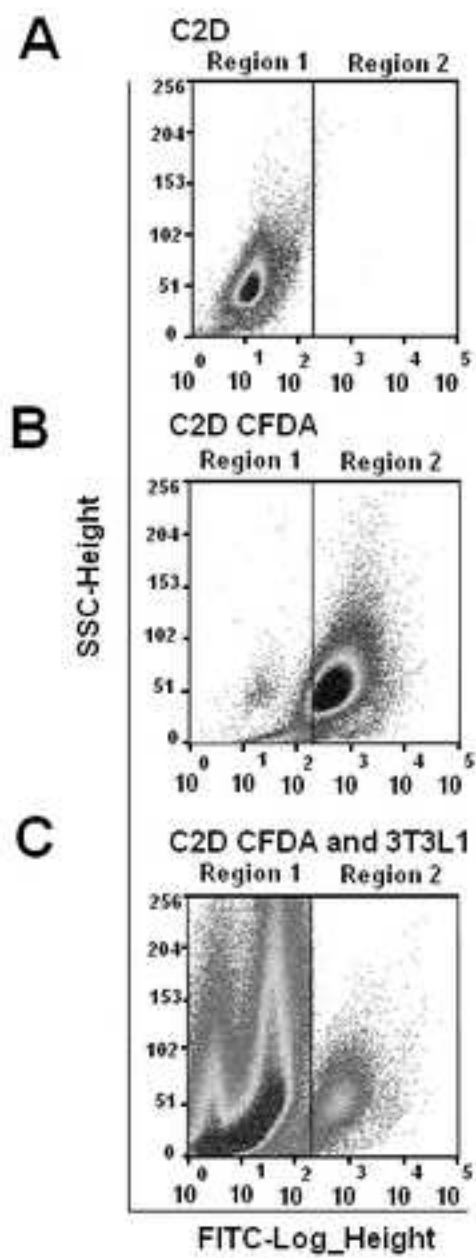
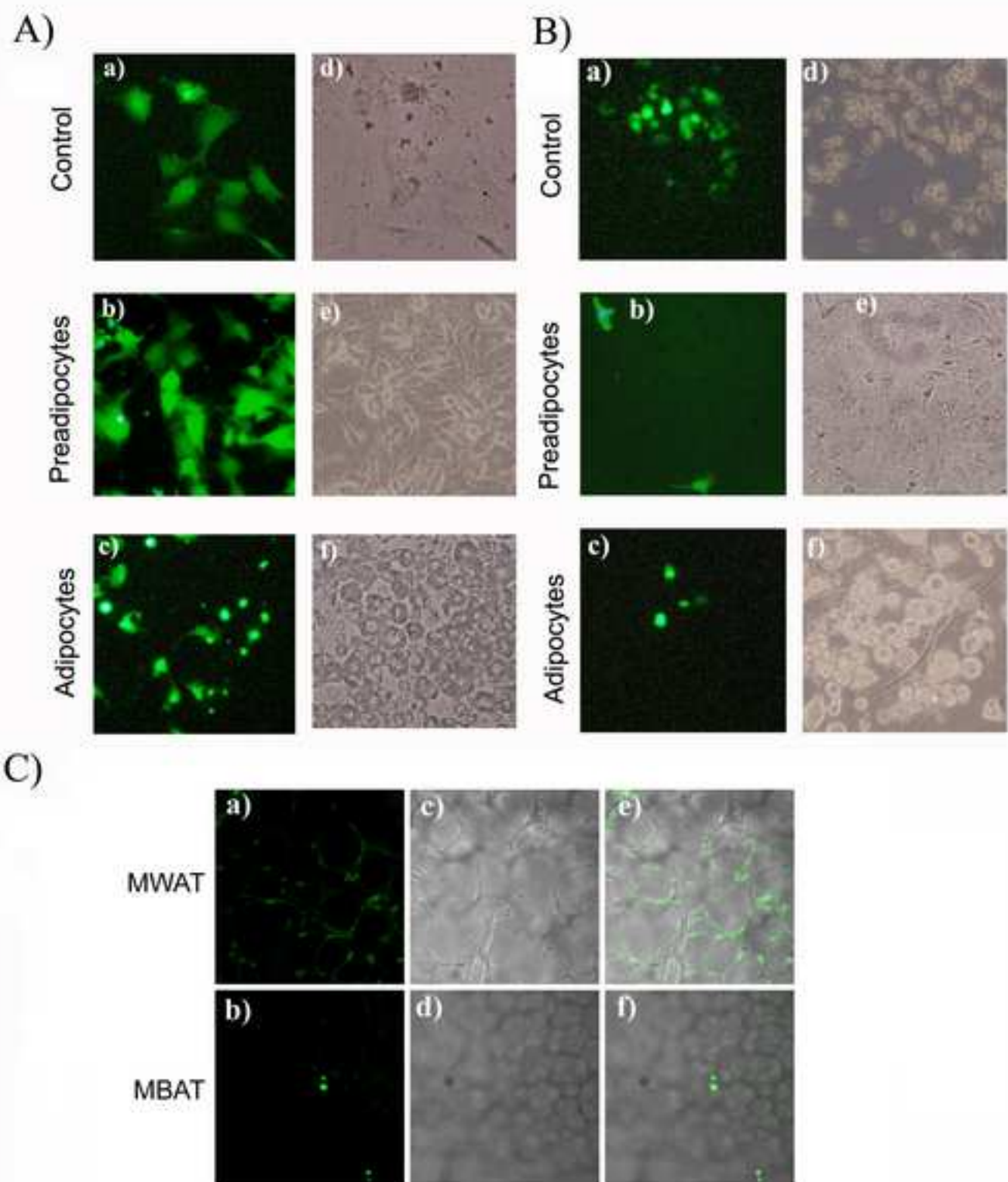
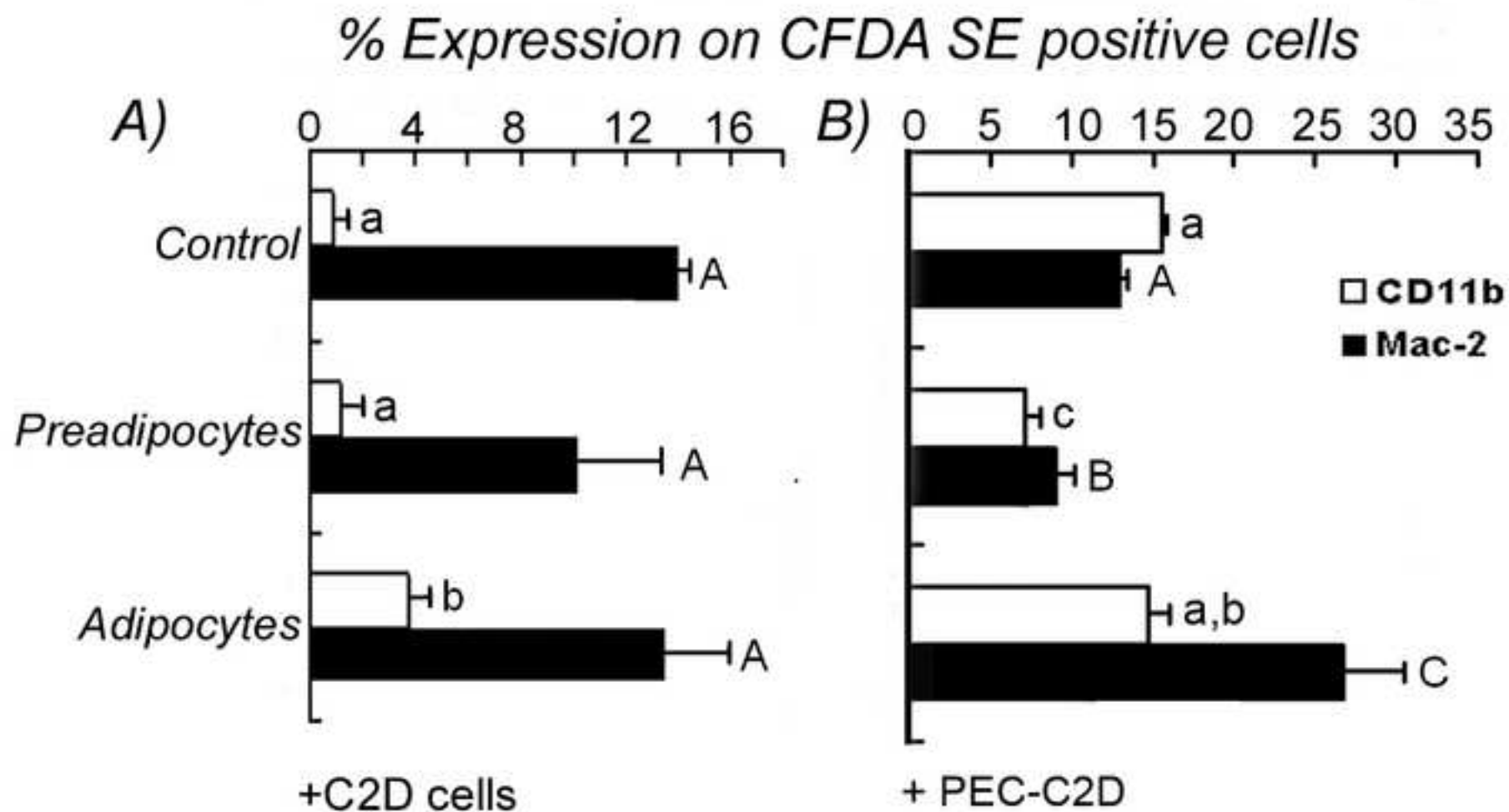


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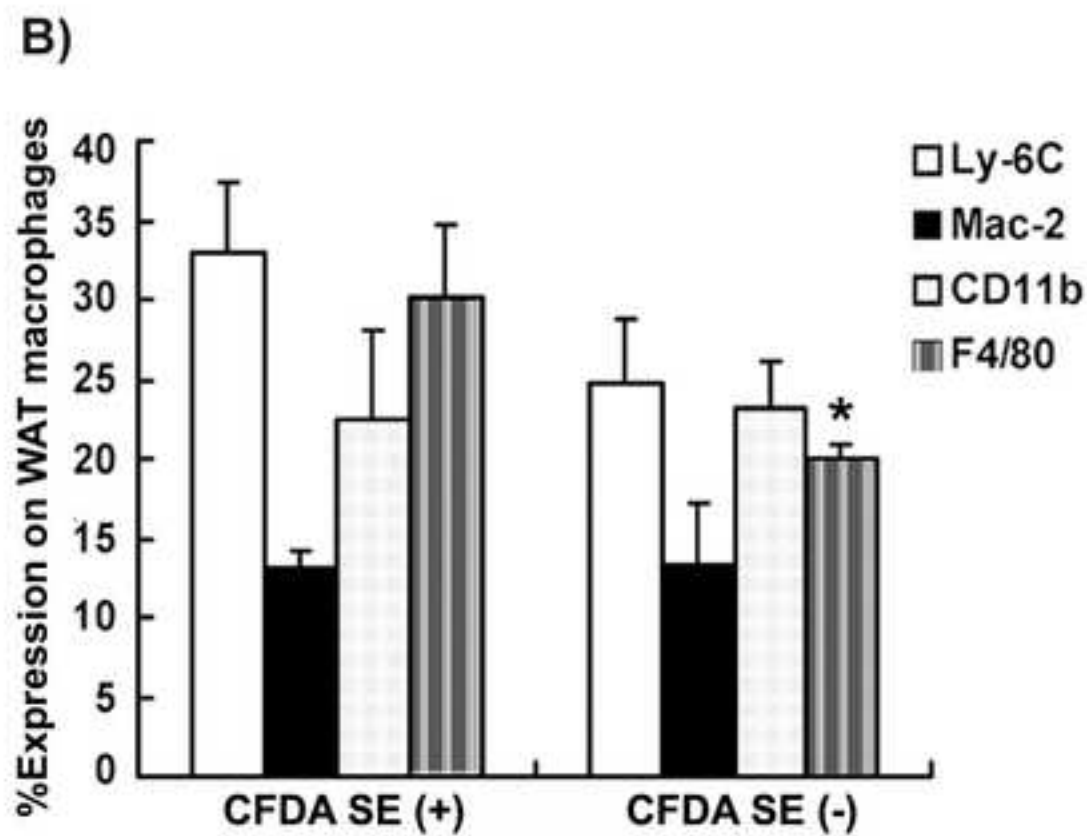
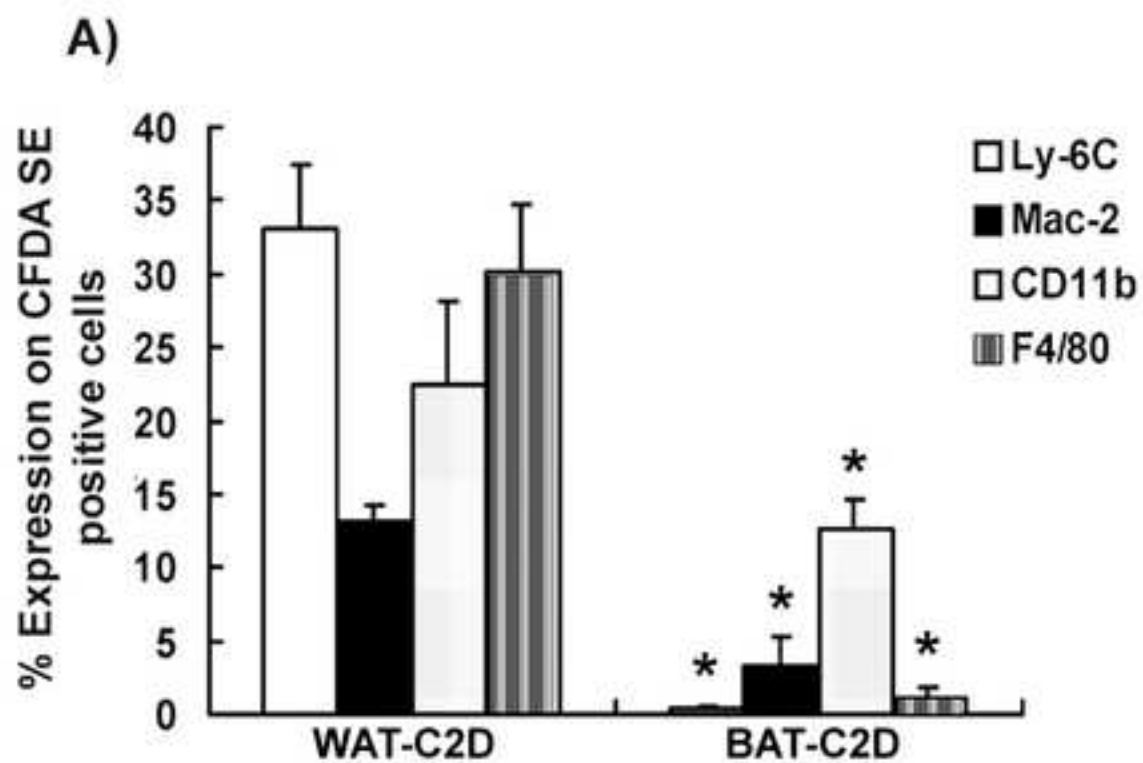
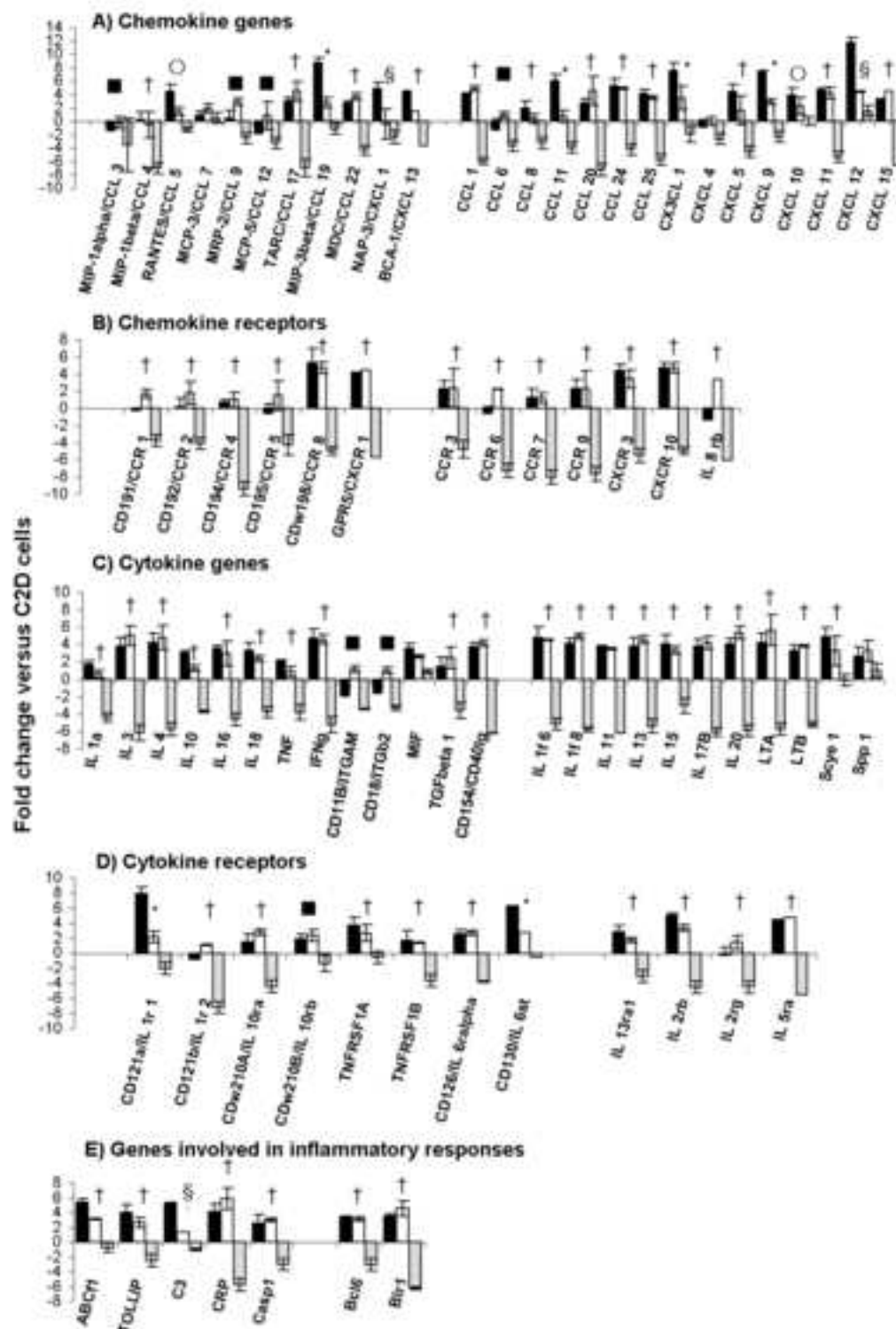


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**Table 1. Comparison of M1 and M2 gene transcripts in bone marrow derived macrophage cells after 24 h indirect co-culture with digested adipose tissues.**

Transcript	% gene transcripts compared to bone marrow derived macrophage cells	
	BAT-BM Mo <sup>1</sup>	WAT-BM Mo <sup>1</sup>
<i>TNF-<math>\alpha</math></i>	177 $\pm$ 39	370 $\pm$ 46†
<i>IL-6</i>	285 $\pm$ 47	564 $\pm$ 53†
<i>IL-1<math>\beta</math></i>	243,871 $\pm$ 154,323	170,380 $\pm$ 29,734
<i>Arg-1</i>	158 $\pm$ 105	168 $\pm$ 45
<i>Ym-1</i>	1 $\pm$ 1	5 $\pm$ 3
<i>Fizz-1</i>	163 $\pm$ 125	212 $\pm$ 35

1. Bone marrow derived macrophage cells were indirectly co-cultured (plate bottom) with digested fat pads (in transwell insert); WAT-BM Mo bone marrow derived macrophage cells co-cultured with paired gonadal fat pads; BAT-BM Mo bone marrow derived macrophage cells co-cultured with digested perispleen fat.
2. % gene transcript levels were calculated relative to bone marrow derived macrophages differentiated *in vitro* as described in the materials and methods.
3. Number represents average  $\pm$  standard error of the mean of 3 or 4 independent mouse samples. † indicates statistical difference compared to BAT-BM Mo,  $P < 0.05$ .