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Interleukin-6 released from differentiating human beige adipocytes improves browning

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Abbreviations

analysis of variance; ANOVA, bone morphogenetic protein; BMP, brown adipose tissue; BAT, CCAAT/Enhancer binding protein beta; CEBPB, cell death-inducing DFFA-like effector A; CIDEA, 3',5'-cyclic adenosine monophosphate; cAMP, cytochrome c1; CYC1, deep neck; DN , Dulbecco's modified Eagle's medium; DMEM, ELOVL fatty acid elongase 3; ELOVL3, enzyme-linked immunosorbent assay; ELISA, extracellular acidification rate; ECAR, fetal bovine serum; FBS, fibroblast growth factor; FGF, human adipose-derived mesenchymal stem cell; hADMSC, interleukin-6 receptor; IL-6R, IL-6-receptor alpha; IL6RA, Janus kinase; Jak, leptin; LEP, low-density lipoprotein receptor relative 11; LR11, monocyte chemoattractant protein; MCP, oxygen consumption; OC, oxygen consumption rate; OCR, peroxisome proliferator-activated receptor gamma; PPARγ, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PGC1A, PR/SET Domain 16; PRDM16, signal transducer and activator of transcription 3; Stat3, subcutaneous neck; SCN, T-Box 1; TBX1, uncoupling protein 1; UCP1, white adipose tissue; WAT, Zic family member 1; ZIC1

Abstract

Brown and beige adipocytes contribute significantly to the regulation of whole body energy expenditure and systemic metabolic homeostasis not exclusively by thermogenesis through mitochondrial uncoupling. Several studies have provided evidence in rodents that brown and beige adipocytes produce a set of adipokines („batokines") which regulate local tissue homeostasis and have beneficial effects on physiological functions of the entire body. We

observed elevated secretion of Interleukin (IL) -6, IL-8 and monocyte chemoattractant protein (MCP) -1, but not tumor necrosis factor alpha (TNFα) or IL-1β pro-inflammatory cytokines, by *ex vivo* differentiating human beige adipocytes (induced by either PPARγ agonist or irisin) compared to white. Higher levels of IL-6, IL-8 and MCP-1 were released from human deep neck adipose tissue biopsies (enriched in browning cells) than from subcutaneous ones. IL-6 was produced in a sustained manner and mostly by the adipocytes and not by the undifferentiated progenitors. Continuous blocking of IL-6 receptor by specific antibody during beige differentiation resulted in downregulation of brown marker genes and increased morphological changes that are characteristic of white adipocytes. The data suggest that beige adipocytes adjust their production of IL-6 to reach an optimal level for differentiation in the medium enhancing browning in an autocrine manner.

Keywords: Interleukin-6, beige adipocyte, batokine, thermogenesis, obesity

1. INTRODUCTION

Studies in nuclear medicine detected high abundance of thermogenic adipose tissue, which can dissipate energy directly into heat as a result of a cold challenge, in healthy adult humans mostly in the supraclavicular, deep neck (DN) and paravertebral regions [1-4]. The activity of these depots was predicted to account for up to 5% of basal metabolic rate in adults, highlighting the possibility of therapeutic application of browning in the treatment of obesity and type 2 diabetes mellitus [5]. There are at least two types of thermogenic adipocytes, classical brown and beige, which have different origins and distribution as demonstrated in rodents [6]. In humans, however, the origin, development and distinct functions of the brown and beige types of adipocytes remain elusive [6]. It is becoming evident that brown and beige adipocytes are not only heat-generating cells. They secrete specific adipokines termed "batokines" which may have an autocrine, paracrine, or endocrine activity [6,7]. There is, however, only limited information about the secreted factors by thermogenic adipocytes and their effects in humans [7].

There is growing evidence supporting that Interleukin (IL)-6 not only acts as a central mediator of inflammatory processes but also serves as an endocrine modulator of metabolism for the entire body [8]. IL-6, released by myocytes, targets several tissues including liver, pancreas, brain, white and brown adipose tissue (WAT and BAT), and balances exercise-associated catabolic pathways in order to mediate glycemic control during recovery [9-12]. It was also shown that IL-6 secreted by brown adipocytes was required for the profound effects of BAT on the glucose homeostasis and insulin sensitivity in mice [13]. Therefore, IL-6 can mediate some of the long-term systemic beneficial effects of physical training and non-shivering thermogenesis. However, the effect of IL-6 on the differentiation of human thermogenic adipocytes remains elusive.

Previously, we demonstrated that human adipocyte progenitors from abdominal subcutaneous fat could be differentiated into beige adipocytes *ex vivo* in response to long-term rosiglitazone (Peroxisome proliferator-activated receptor gamma (PPARγ)-driven browning protocol) or irisin administration. Contrarily, Bone morphogenetic protein (BMP) -7 treatment resulted in a functional browning in parallel with a classical brown-like gene expression pattern [14]. In this study, we investigated the release of selected cytokines by primary human adipocytes and adipose tissue specimens from different anatomical origins. We found that more IL-6, IL-8

and monocyte chemoattractant protein (MCP)-1 were secreted to the differentiation media of beige adipocytes as compared to white adipocytes. Irisin treatment slightly enhanced IL-6, IL-8 and MCP-1 release during the differentiation program. BMP7 administration, however, prevented the secretion of these cytokines.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals were from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

2.2 Ethics statement and obtained samples

Human adipose-derived mesenchymal stem cells (hADMSCs) were isolated from subcutaneous abdominal, subcutaneous neck (SCN) and DN adipose tissue of volunteers (BMI<29.9) aged 20–65 years who underwent a planned surgical treatment. In case of abdominal surgeries only a subcutaneous fat biopsy was collected from one individual as described previously [14-17]. In case of thyroid surgeries a pair of DN and SCN adipose tissue samples was obtained to rule out inter-individual variations. Portions were snap-frozen in liquid nitrogen for RNA extraction. Patients with known diabetes, malignant tumor or with abnormal thyroid hormone levels at the time of surgery were excluded. Written informed consent was obtained from all participants before the surgical procedure. The study protocol was approved by the Medical Research Council of Hungary (20571-2/2017/EKU). All experiments were performed in accordance with the approved ethical guidelines and regulations.

2.3 Isolation, differentiation and treatments of hADMSCs

Mycoplasma-free [15] hADMSCs were isolated and cultivated; white and beige adipocytes were differentiated from hADMSCs according to described protocols [14,15]. Human recombinant Irisin (Cayman Chemicals, Ann Arbor, MI, USA) at 250 ng/ml or human recombinant BMP7 at 50 ng/ml (R&D systems, Minneapolis, MN, USA) was administered during the white differentiation procedure [14]. Where indicated, cells were treated with Human IL-6R alpha (R&D Systems, MAB227) or IgG1 Isotype Control (M5284) antibody at 5μg/ml [18] every day during the whole differentiation. To investigate the response of differentiated adipocytes to thermogenic induction, cells received a single bolus of dibutyril-cAMP at 500 μM concentration for 4h [15,19]. RNA, DNA preparation, TaqMan real-time RT-PCR and mitochondrial DNA quantification by Q-PCR were carried out as described previously [14,15].

2.4 Determination of cytokine release

10-20 mg of SCN and DN tissue samples from the same donors were floated for 24h in 10% FBS-containing DMEM-F12-HAM medium [20]. In cell culture experiments, supernatants were either harvested daily and stored for cytokine measurements or the media from the same donor's differentiating cells were pooled from day 1 to 7 and from 8-14. The concentration of IL-6, IL-1β, IL-8, tumor necrosis factor alpha (TNFα) and MCP-1 was measured from the collected culture media using ELISA DuoSet Kit (R&D Systems) [16,17].

2.5 Immunofluorescence staining, quantification of browning

hADMSCs were plated and differentiated on Ibidi eight-well μ-slides; vital and immunofluorescence stainings were carried out as described previously [14,17]. Sample scanning was done by iCys Research Imaging Cytometer (iCys, Thorlabs Imaging Systems, Sterling, VA, USA). The images were processed and analyzed by our high-throughput automatic cell-recognition protocol using the iCys companion software (iNovator Application Development Toolkit version 7.0, CompuCyte Corporation, Westwood, MA, USA), Cell Profiler and Cell Profiler Analyst (The Broad Institute of MIT, MA, USA). See reference ref. 14 and 21 for further details about the analysis.

2.6 Determination of cellular oxygen consumption (OC) and extracellular acidification rate (ECAR)

OC and ECAR were measured using an XF96 oxymeter (Seahorse Biosciences, North Billerica, MA, USA). Cells were seeded and differentiated in 96-well XF96 assay plates. On the day of measurement, after recording the baseline OC and ECAR for 30 min, adipocytes received a single bolus dose of dibutyryl-cAMP (at 500 μM final concentration) modelling adrenergic stimulation. Then, stimulated OC was measured every 30 minutes. The final reading took place at 5 h post-treatment. Next, proton leak respiration was recorded after adding oligomycin at 2 μM concentration to block ATP synthase activity. As a last step, cells received a single bolus dose of Antimycin A (10 μM final concentration) for baseline correction. The oxygen consumption rate (OCR) and ECAR was normalized to protein content and normalized readings were shown. For statistical analysis, the fold change of OC and ECAR levels were determined comparing basal, cAMP stimulated and oligomycin inhibited (both in unstimulated and stimulated cells) OCRs/ECARs of each sample to the basal OCR/ECAR of untreated white adipocytes [15].

2.7 Statistical analysis

Results are expressed as the mean ± s.d. for the number of assays indicated. The normality of distribution of the data was tested by Kolmogorov-Smirnov test. For multiple comparisons of groups statistical significance was calculated and evaluated by one-way ANOVA followed by Tukey post-hoc test. In comparison of two groups two-tailed Student's t-test was used.

3. RESULTS

3.1 Beige but not classical brown-like adipocytes secrete increased levels of IL-6, IL-8 and MCP-1

Primarily, hADMSCs were cultivated from abdominal subcutaneous fat and differentiated to white, beige or brown-like adipocytes according to previously described protocols [14]. Media were collected during the regular replacement of the adipogenic cocktails and secreted cytokines were measured after samples from the first and second week of differentiation were pooled. We found that IL-6 **(Fig. 1A)**, IL-8 **(Fig. 1B)** and monocyte chemoattractant protein (MCP)-1 **(Fig. 1C)** secretion was significantly higher in the differentiation media of beige cells induced by either rosiglitazone or irisin from abdominal hADMSCs compared to adipocytes which were differentiated to white. BMP7 treatment, however, was less or not effective in inducing secretion of these cytokines and was even inhibitory when added during

the beige protocol. TNFα or IL-1β pro-inflammatory cytokines were not released (data not shown). To our knowledge, the secretion of TNFα or IL-1β by brown and beige adipocytes was not detected by any other study so far. In the heterogeneous populations of progenitors and mature adipocytes derived from abdominal hADMSC cultures, mostly the differentiated adipocytes but not the preadipocytes were stained positive for IL-6 **(Fig. 2A** and **B**, **Supplementary Fig. 1A)**. In parallel with our previous findings [14], there was no significant difference in regard with the differentiation capacity between white and browning adipocytes **(Fig. 2C)**. Secondary antibody control images are displayed on **Supplementary Fig. 1B**.

To extend our observations further, we have obtained pairs of DN and SCN adipose tissue biopsies from the same donors; isolated and differentiated hADMSCs from these sites as described above. Total tissue lysates of DN fat expressed higher levels of the thermogenic markers **(Fig. 2D)** and were enriched in thermogenic adipocytes as compared to SCN biopsies [19,22-24]. Stromal-vascular fractions [25] or single-cell clones of progenitors that were isolated from DN adipose tissue were able to differentiate into functional brown or beige adipocytes at a higher extent than those which were cultivated from SCN depots [26,27]. In our experiments, the biopsies were floated in culture media for 24h [20]. We found that higher levels of IL-6 **(Fig. 2E)**, MCP-1 **(Supplementary Fig. 2A)** and IL-8 (**Supplementary Fig. 3A)** were released by DN biopsies than from SCN ones. When hADMSCs were cultivated and differentiated from these sites, we found that, except for the irisin-induced beige adipocytes, the anatomical origin did not affect IL-6 **(Fig. 2F)** and MCP-1 **(Supplementary Fig. 2C)** production of the adipocytes. More IL-8 was secreted by DN than SCN-derived adipocytes **(Supplementary Fig. 3C)**. TNFα or IL-1β cannot be detected from these samples as well (data not shown).

Next, we examined cytokine secretion of abdominal subcutaneous adipocytes in the time-course of differentiation, therefore collected and replaced media daily. We found that MCP-1 **(Supplementary Fig. 2B)** and IL-8 **(Supplementary Fig. 3B)** secretion was induced at the end of the first week of PPARγ-driven beige differentiation and then it declined. On the other hand, IL-6 release increased in time of differentiation and did not subside **(Fig. 2G)**. Comparing the daily measurements with those collected and pooled over longer periods of time revealed that the two did not differ substantially either in the first or second weeks of the differentiation **(Figures 1A** and **2G)**. This means that after daily replacement of the medium, browning cells quickly adjusted their production to meet a set level of IL-6, suggesting that this cytokine may have a role in the regulation of the differentiation process.

3.2 Continuous inhibition of the IL-6-Receptor resulted in a downregulation of the browning marker genes and extracellular acidification during beige differentiation

As a next step, we investigated the consequence of blocking of IL-6-receptor alpha (encoded by *IL6RA* gene) with an antibody [18]. In accordance with our former studies [14,15], the expression of several brown marker genes, including the beige-selective *TBX1* [22], was significantly elevated in response to the PPARγ-driven browning protocol as compared to white adipocytes **(Fig. 3)**. The inhibition of IL-6-receptor resulted in a significantly weaker induction of *UCP1*, *CIDEA* and *ELOVL3* **(Fig. 3A)** browning marker genes [6] compared to adipocytes treated with the isotype control antibody. Contrarily, the white adipogenic marker, *LEP* was upregulated **(Fig. 3B)**. We found downregulation of *TBX1*, while the expression of *ZIC1*, a classical brown marker [19] was not altered as a result of IL-6-receptor blocking **(Fig. 3C)**. While the inhibition of IL-6-receptor did not affect the overall differentiation rate **(Fig. 3D)**, mitochondrial DNA content **(Fig. 3E)** and expression of *CYC1*, a mitochondrial-

enrichment marker was significantly decreased. The expression of transcriptional co-factors which drive browning [6] (*PGC1A*, *PRDM16*), general adipogenic markers (*CEBPB*, *PPARG*) and *IL6RA* did not change in response to the antibody administration **(Supplementary Fig. 4)**.

To investigate how morphological characteristics of beige adipocytes change as a result of IL-6-receptor inhibition a slide-based image-cytometry approach was applied. This method combines texture analysis and detection of Ucp1 protein content in single adipocytes [14,21]. When the blocking antibody was applied on top of the PPARγ-driven beige protocol the texture sum variance increased **(Fig. 4A)** reflecting larger lipid droplets while the median Ucp1 intensity decreased **(Fig. 4B)**, that is, differentiation shifted toward producing more white adipocytes **(Fig. 4C)**.

When the functional properties of the adipocytes treated with anti-IL-6-receptor were measured, the responsiveness to cAMP **(Fig. 5A)** and basal, stimulated or proton-leak OC did not decrease significantly **(Fig. 5B)**. However, basal and cAMP-stimulated extracellular acidification were lowered in a significant degree in response to sustained IL-6-receptor inhibition **(Fig. 5C)**.

4. DISCUSSION AND CONCLUSIONS

The term "batokine" can be used for those bioactive molecules which are secreted to a larger extent by brown and beige adipocytes as compared to white or whose production is enhanced in response to thermogenic activation of brown and beige adipocytes [7]. Several "batokines" were described in rodents. Out of those, fibroblast growth factor (FGF) 21 [28], the soluble form of the low-density lipoprotein receptor relative 11 (sLR11) [29], adenosine [30] or prostaglandin E2 [31] were studied in human samples. Meteorin-like [32] and FGF21 [22] are produced preferentially by beige, while BMP8b is mainly secreted by mature classical brown adipocytes [33]. The secretome of human brown and beige adipocytes and the distinct "batokine" profile of classical brown and beige cells, however, remain elusive.

In the present study, we detected IL-6, IL-8 and MCP-1 release from human adipose tissue samples and differentiating primary adipocytes –more pronouncedly by beige cells– without the secretion of the general pro-inflammatory cytokines, TNFα and IL-1β **(Fig. 1)**. Out of the investigated cytokines, only the secretion of IL-6 was maintained till the end of the differentiation **(Fig. 2G)**.

IL-6 has multiple effects on the metabolic homeostasis of the entire body. Overproduction of IL-6 has a pivotal role in the recruitment of immune cells into WAT which can lead to chronic inflammation during weight gain [8,34]. This remodeling process, especially in visceral fat, is also marked by TNFα and IL-1β secretion by adipocytes and macrophages, respectively, and contributes to the development of insulin resistance, type 2 diabetes and inhibited browning [34-38]. On the other hand, IL-6 can upregulate IL-4 receptor α chain in macrophages [39]. This sensitizes them to IL-4 action and promotes M2 polarization that renders mice less susceptibe to develop insulin resistance [39,40]. Of note, it was first reported then contradicted that M2 macrophages in adipose tissues produce catecholamines during the thermogenic process, in response to IL-4 stimulation [41,42].

IL-6$^{-/-}$ mice develop obesity during aging mostly because of the missing effects of the cytokine on the central nervous system [43]. Recently, it was shown that these mice have reduced core body temperature and energy expenditure during long-term cold exposure [44]. Mice overexpressing IL-6 and the soluble IL-6R are significantly smaller and have a decreased body weight including markedly reduced body fat in all regions as compared to wild type mice [45]. Moreover, daily intraperitoneal injections of IL-6 upregulated Ucp1 expression in subcutaneous WAT [46]. In humans, IL-6-blockade therapy is used to treat rheumatoid arthritis or Crohn's disease [47]. Mild to severe weight gain was observed in patients during the pharmacological blockade of IL-6 by tocilizumab, a humanized monoclonal antibody which hardly penetrates the blood-brain barrier therefore has to have an effect outside of the central nervous system [47,48]. The weight gain might be explained because of the reduced browning enhanced by the auto/paracrine IL-6 signaling.

In line with the aforementioned *in vivo* studies, we found that inhibition of the auto/paracrine effect of IL-6 resulted in decreased beige phenotype of human subcutaneous adipocytes (**Figures 3** and **4**). The inhibition of IL-6-receptor did not result in significant changes when it was applied during the white adipocyte differentiation. Other studies showed that this pleiotropic cytokine was increasingly expressed and secreted by brown adipocytes in response to thermogenic stimuli or by skeletal myocytes as a result of exercise [7-9,49]. IL-6 serum levels are also elevated as a result of *in vivo* cold exposure [50] or transplantation of BAT into the visceral cavity of mice [13]. IL-6 produced as a "batokine" or myokine strongly contributes to reduced weight gain and insulin resistance in mice [7,8,13]. When the BAT biopsies were transplanted from IL-6$^{-/-}$ mice into wild type animals that were maintained on high-fat diet; the intervention failed to improve fat mass, glucose homeostasis and insulin sensitivity [13].

It was shown in a recent study that mouse adipocyte progenitor cells treated with IL-6 at 100 ng/ml concentration induced Stat3 phosphorylation by the Jak family of tyrosine kinases, Jak1 and Jak2 [51]. This signaling inhibits *TGFB3* expression and promotes the commitment of both murine and human adipocyte progenitors to thermogenic beige differentiation [51]. The auto/paracrine TGFβ signaling can prevent browning [52]. This can be resolved by two independent mechanisms: while β3-adrenergic agonists, IL-11, MCP-1 or IL-6 induce the aforementioned Jak/Stat3 pathway [51,53]; BMP7 inhibits ROCK and represses the G-actin-regulated transcriptional coactivator myocardin-related transcription factor A, MRTFA [54].

Based on the above observations, IL-6, IL-8 and MCP-1 release during the differentiation of human beige adipocytes induced by either rosiglitazone or irisin can be a positive auto/paracrine feed-back mechanism that facilitates the commitment of adipocyte precursors towards beigeing and enhances the thermogenesis capacity. These secreted cytokines do not seem to have a significant effect in the BMP7-induced browning. Therefore, it is likely that in addition to other currently unknown mechanisms, a reduced thermogenesis capacity might be one of the reasons why IL-6$^{-/-}$ mice or patients receiving IL-6-blockade therapy gain weight.

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Figure 1. Human beige adipocytes secrete high levels of IL-6, IL-8 and MCP-1. (A) IL-6, (B) IL-8 and (C) MCP-1 secretion by *ex vivo* differentiated abdominal subcutaneous adipocytes, n=7. Abdominal subcutaneous ADMSCs were differentiated for two weeks to white (W) or beige (B) adipocytes. 250 ng/ml irisin or 50 ng/ml BMP7 was administered during the whole differentiation process. Conditioned differentiation media were collected and pooled during the 1st and 2nd week of differentiation and secreted IL-6 was measured by sandwich ELISA. Results are expressed as the mean ± SD for the number of assays (adipocytes of different donors) indicated. For multiple comparisons of groups statistical significance was evaluated by one-way ANOVA followed by Tukey post-hoc test. *$p<0.05$.

Figure 2. IL-6 secretion by adipocytes and adipose tissues of different anatomical origin. (A) Pattern of IL-6 expression in human primary beige adipocytes and undifferentiated progenitors of abdominal subcutaneous origin. Cells were differentiated as in Figure 1, treated with 100 ng/ml brefeldin A to block the secretion of IL-6 for 12 h before immunostaining. Nuclei were labelled with 50 µg/ml Hoechst 33342 for 1 h. Gallery images (N=9 cells, recognized based on their nuclei) were captured by an iCys Research Imaging Cytometer. Left panel: IL-6 staining. Middle panel: bright field images of the same cells (lipid droplets of differentiated cells are visible, undifferentiated cells are invisible). Right panel: Hoechst 33342 staining of the same cells (brighter and shrunk nuclei belong to differentiated cells, while pail and larger nuclei are undifferentiated). In every experiment, 2000 cells per each sample was recorded and measured. The experiment was repeated four times with ADMSCs from independent healthy donors. (B) IL-6 protein content of adipocytes per cell. (C) Ratio of adipocytes matured over total number of cells. (D) Expression of browning marker genes in paired subcutaneous neck (SCN) and deep neck (DN) adipose tissue biopsies of 5 different donors. Total RNA was isolated from snap frozen tissue portions using TRIzol Reagent. Normalized gene expression was determined by RT-qPCR, target genes were normalized to *GAPDH*. (E) Amount of IL-6 in the conditioned media of paired SCN and DN biopsies of 8 different donors after 24 h incubation. (F) IL-6 release by SCN and DN adipocytes. ADMSCs isolated from paired SCN and DN biopsies of 6 different donors were differentiated as in Figure 1. Conditioned differentiation media were collected and pooled during the 1st and 2nd week of differentiation and secreted IL-6 was measured by sandwich ELISA. Results are expressed as the mean ± SD for the number of assays (adipocytes of different donors) indicated. (G) Daily IL-6 production of *ex vivo* differentiated abdominal subcutaneous adipocytes of one representative donor. Conditioned differentiation media were collected every day and secreted IL-6 was measured by sandwich ELISA (SD means the experimental error of the technical replicates). For multiple comparisons of groups statistical significance was evaluated by one-way ANOVA followed by Tukey post-hoc test; in comparison of two groups two-tailed Student's t-test was used. *$p<0.05$

Figure 3. Blocking of IL-6-Receptor results in inhibition of browning ex vivo. Abdominal subcutaneous hADMSCs were differentiated for two weeks to white or beige adipocytes. 5 µg/ml anti-IL-6-receptor α blocking (white bars) or isotype control (black bars) antibody was administered during the whole adipogenic differentiation process. (A) Expression of browning marker genes. (B) Expression of a white adipocyte marker. (C) Expression of a beige-

selective and a classical brown marker gene. The experiment was repeated five times with hADMSCs from independent healthy donors. Normalized gene expression was determined by RT-qPCR, target genes were normalized to *GAPDH*. (D) Ratio of differentiated adipocytes over total number of cells. Images were collected with an iCys Research Imaging Cytometer, the experiment was repeated five times with ADMSCs from independent healthy donors. (E) Relative mitochondrial DNA amount of human adipocytes determined by qPCR in 5 different donors. For comparison of two groups two-tailed Student's t-test was used. **p<0.01, *p<0.05.

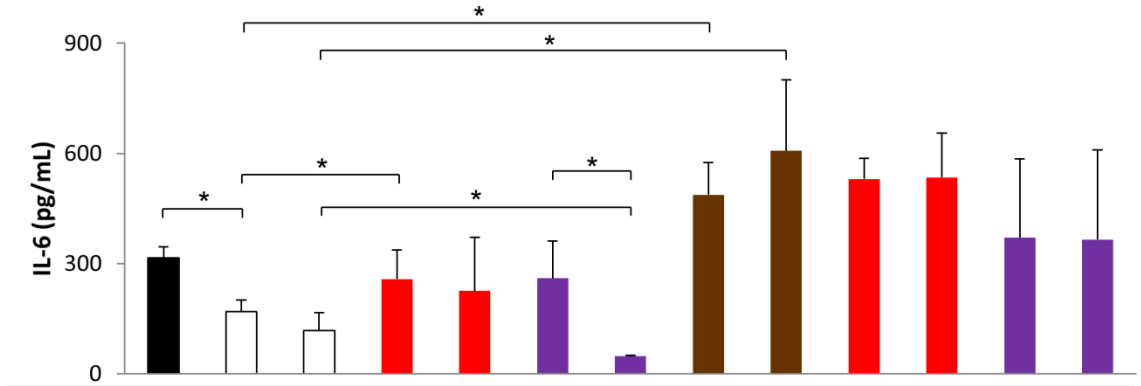
Figure 4. Morphological features of the primary adipocytes differentiated in the presence of anti-IL-6-receptor. Abdominal subcutaneous hADMSCs were differentiated and treated as in Figure 3. Cells were washed and fixed in 4% paraformaldehyde for 5 min followed by blocking in 5% skimmed milk for 2 h and staining with anti-Ucp1 primary antibody for 6 h at room temperature. Alexa 488 goat anti-rabbit IgG was applied as a secondary antibody. Antibodies were used and additional washing steps between and after Ab usage were carried out in the presence of 0.1% saponin in PBS for effective cell permeabilization. Images were collected with an iCys Research Imaging Cytometer. In every experiment, 2000 cells per each sample was recorded and measured. The experiment was repeated five times with ADMSCs from independent healthy donors. (A) Texture sum variance (as compared to untreated beige adipocytes). (B) Median Ucp1 protein content of adipocytes per cell. (C) Contour plot figures which show single cell data of texture sum variance and Ucp1 protein content of differentiated adipocytes of one representative donor based on browning adipocytes, which contain small lipid droplets and high levels of Ucp1, are identified. For multiple comparisons of groups statistical significance was evaluated by one-way ANOVA followed by Tukey post-hoc test; in comparison of two groups two-tailed Student's t-test was used. **p<0.01, *p<0.05.

Figure 5. Functional properties of the primary adipocytes differentiated in the presence of anti-IL-6-receptor. Abdominal subcutaneous hADMSCs were differentiated and treated as in Figure 3. (A) Effect of short-term cAMP treatment on the expression of *UCP1* gene in adipocytes. Cells received a single bolus of dibutyril-cAMP at 500 μM concentration for 4 hours. The experiment was repeated five times with ADMSCs from independent healthy donors. Normalized gene expression was determined by RT-qPCR, target gene was normalized to *GAPDH*. (B) Basal, cAMP stimulated and oligomycin inhibited oxygen consumption (OC) levels (as compared to basal OCR of white adipocytes) in 4 different hADMSC-derived adipocyte donors. OC of adipocytes was measured with an XF96 oxymeter. After recording the baseline OC, cells received a single bolus dose of dibutyril-cAMP. Proton leak respiration was determined after adding oligomycin to block ATP synthase activity. (C) Basal and cAMP-stimulated extracellular acidification levels (as compared to basal ECAR of isotype control (ITC)-treated white adipocytes) in 4 different adipocyte donors measured with an XF96 oxymeter. For multiple comparisons of groups statistical significance was evaluated by one-way ANOVA followed by Tukey post-hoc test; in comparison of two groups two-tailed Student's t-test was used. *p<0.05

Highlights

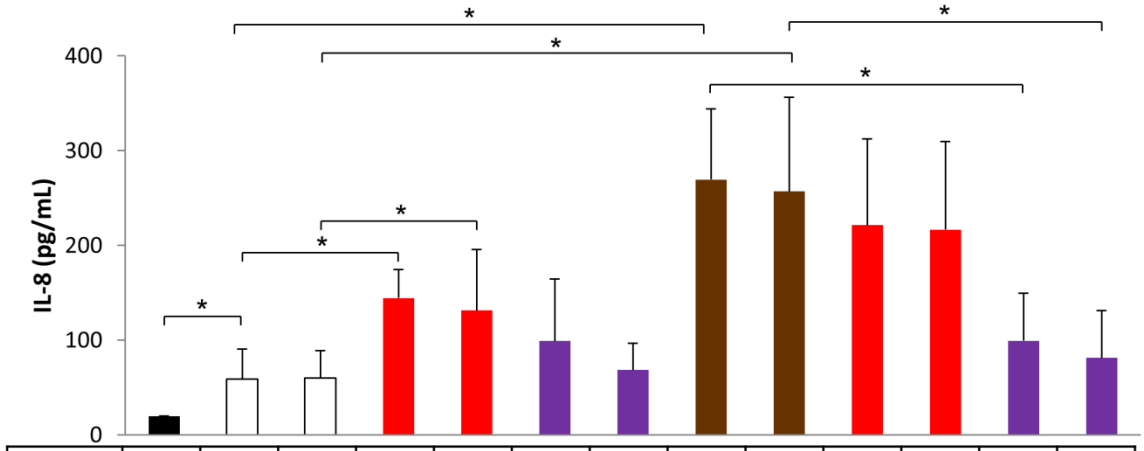
- IL-6, MCP-1 and IL-8 secretion is higher by beige compared to white adipocytes.
- Differentiating adipocytes do not secrete TNF-α and IL-1β, excluding inflammation.
- Blocking of IL-6 receptor results in changes that are characteristic of white adipocytes.
- IL-6 levels reach an optimum in the medium enhancing browning in an autocrine manner.

A



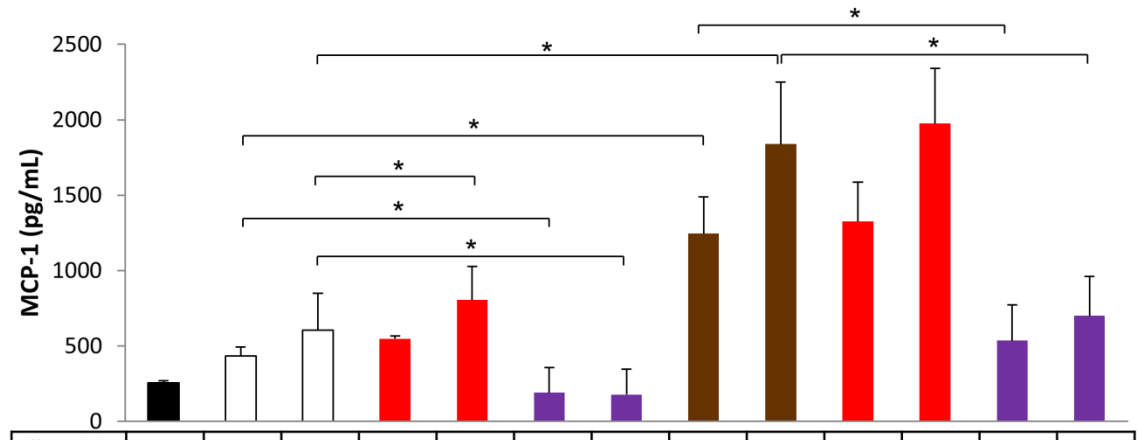
Differentiation	-	W	W	W	W	W	W	B	B	B	B	B	B
Week	-	1	2	1	2	1	2	1	2	1	2	1	2
Irisin	-	-	-	+	+	-	-	-	-	+	+	-	-
BMP7	-	-	-	-	-	+	+	-	-	-	-	+	+

B

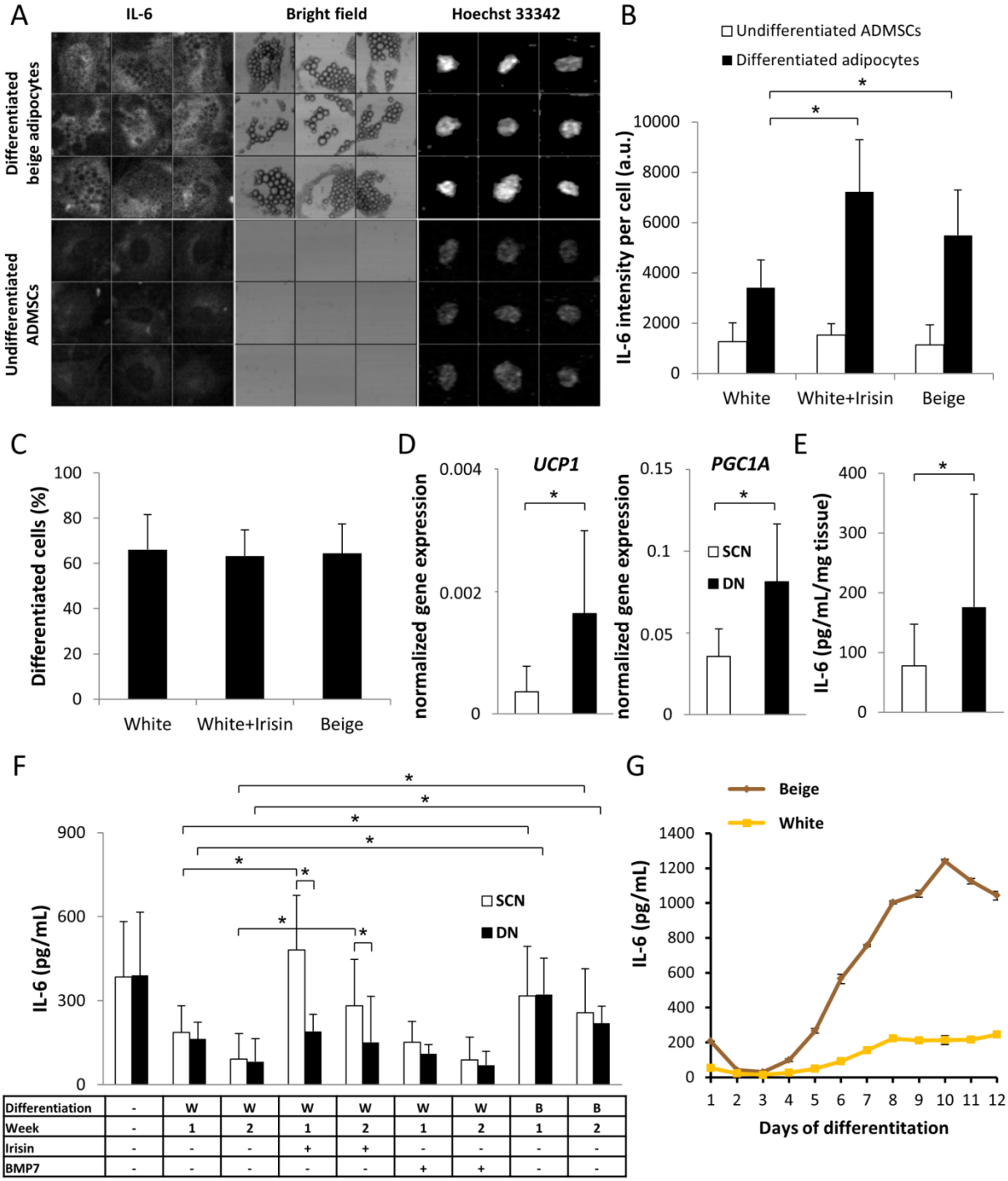


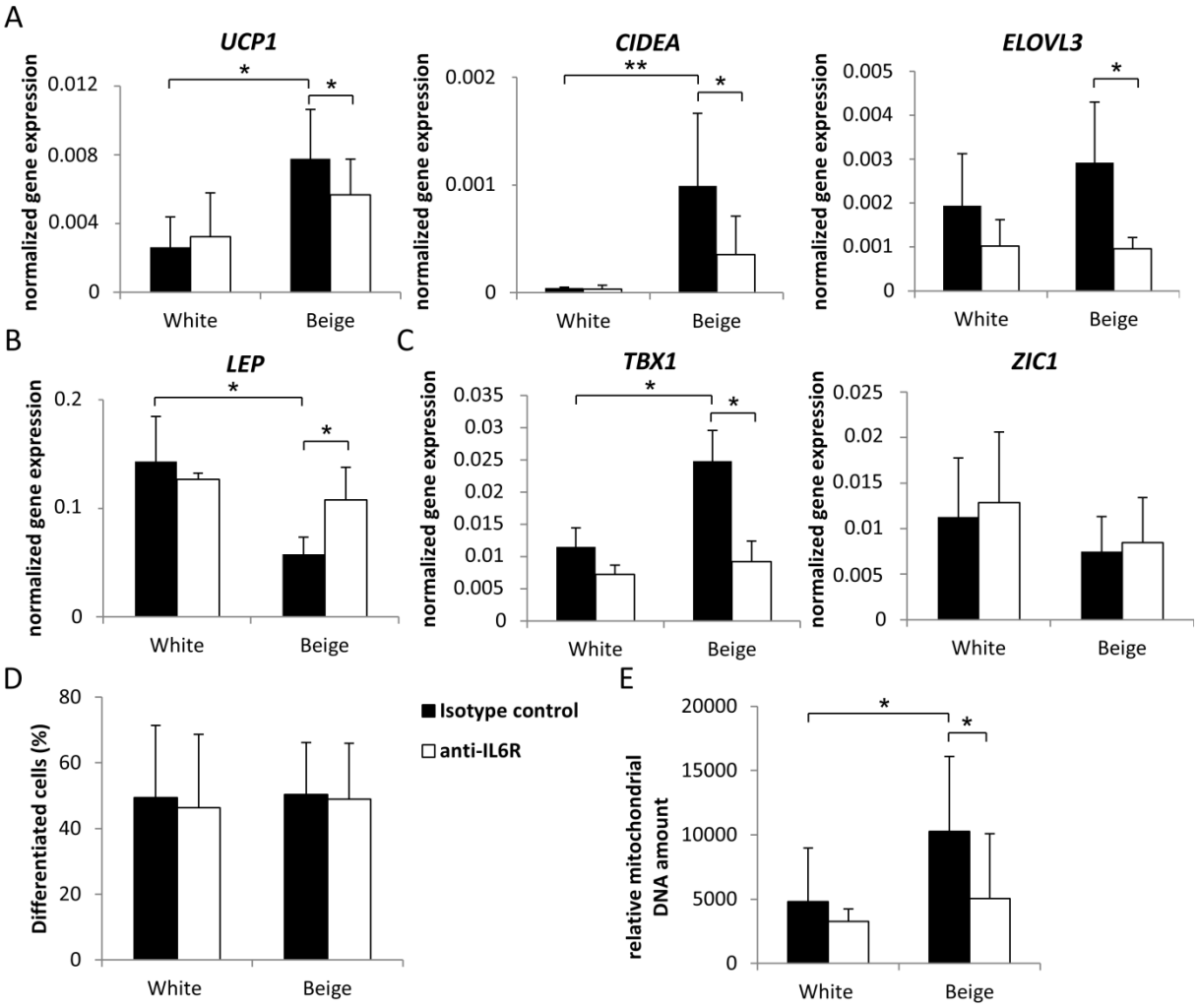
Differentiation	-	W	W	W	W	W	W	B	B	B	B	B	B
Week	-	1	2	1	2	1	2	1	2	1	2	1	2
Irisin	-	-	-	+	+	-	-	-	-	+	+	-	-
BMP7	-	-	-	-	-	+	+	-	-	-	-	+	+

C

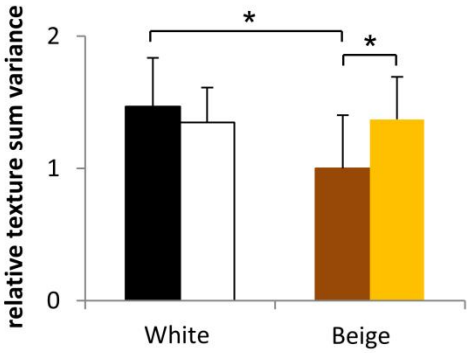


Differentiation	-	W	W	W	W	W	W	B	B	B	B	B	B
Week	-	1	2	1	2	1	2	1	2	1	2	1	2
Irisin	-	-	-	+	+	-	-	-	-	+	+	-	-
BMP7	-	-	-	-	-	+	+	-	-	-	-	+	+


A
IL-6
Bright field
Hoechst 33342
Differentiated beige adipocytes
Undifferentiated ADMSCs
B
Undifferentiated ADMSCs
Differentiated adipocytes
IL-6 intensity per cell (a.u.)
White
White+Irisin
Beige
C
Differentiated cells (%)
White
White+Irisin
Beige
D
UCP1
PGC1A
normalized gene expression
SCN
DN
E
IL-6 (pg/mL/mg tissue)
F
IL-6 (pg/mL)
SCN
DN
Differentiation - W W W W W W W B B
Week - 1 2 1 2 1 2 1 2
Irisin - - - + + - - - -
BMP7 - - - - - + + - -
G
Beige
White
IL-6 (pg/mL)
Days of differentitation


A
UCP1
CIDEA
ELOVL3
normalized gene expression
White
Beige
B
LEP
C
TBX1
ZIC1
D
Differentiated cells (%)
Isotype control
anti-IL6R
E
relative mitochondrial DNA amount

A



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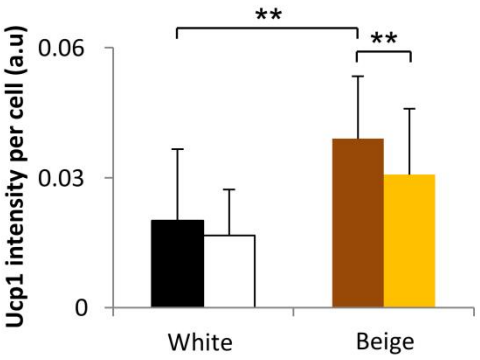

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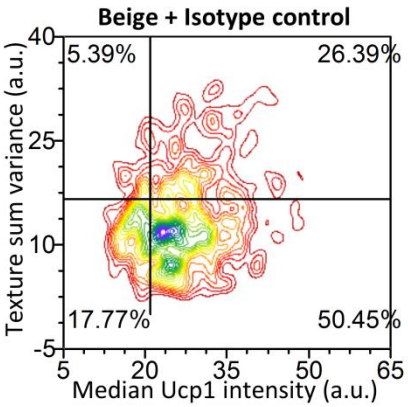
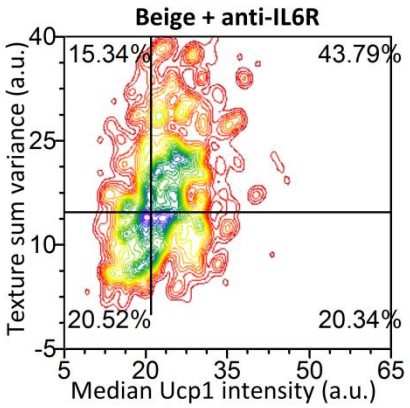
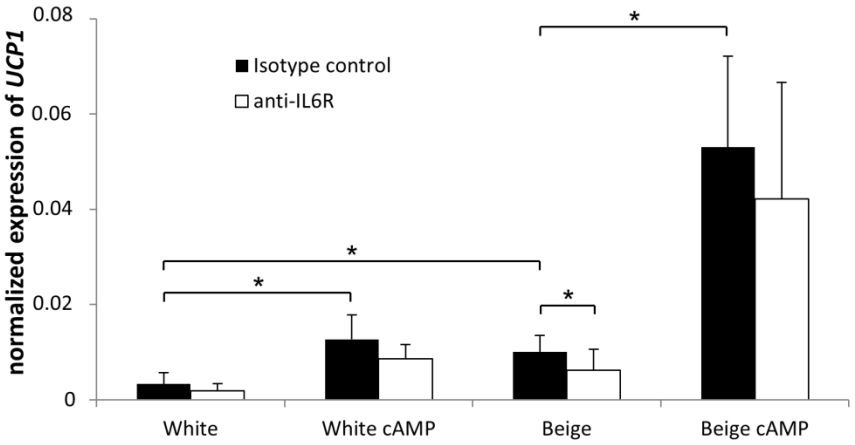
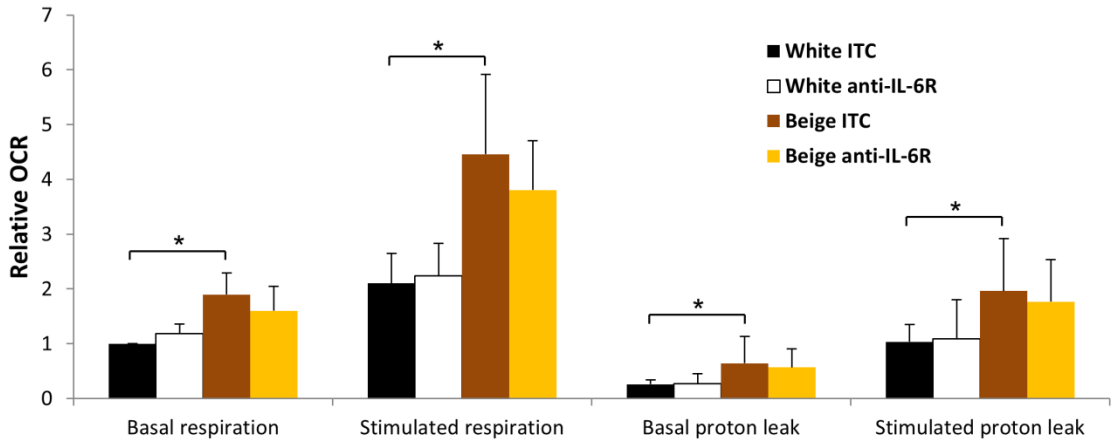




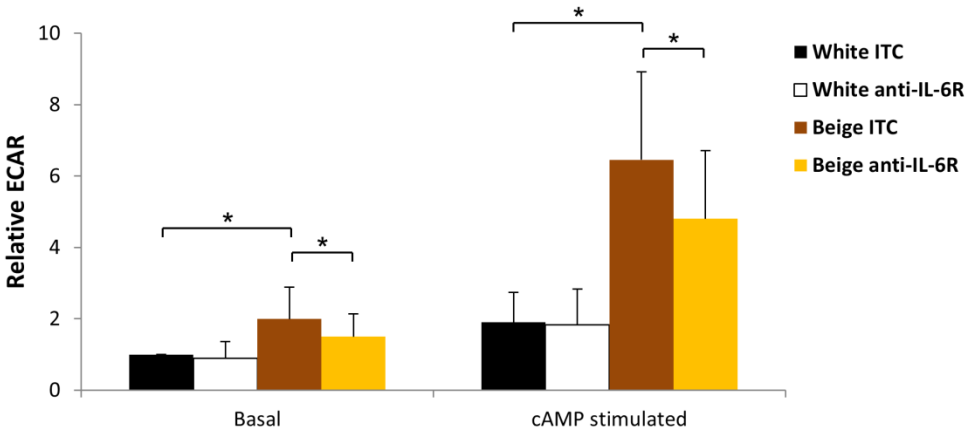
A


normalized expression of UCP1
0.08
0.06
0.04
0.02
0
Isotype control
anti-IL6R
*
*
*
*
White
White cAMP
Beige
Beige cAMP

B


Relative OCR
7
6
5
4
3
2
1
0
White ITC
White anti-IL-6R
Beige ITC
Beige anti-IL-6R
*
*
*
*
Basal respiration
Stimulated respiration
Basal proton leak
Stimulated proton leak

C


Relative ECAR
10
8
6
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2
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White ITC
White anti-IL-6R
Beige ITC
Beige anti-IL-6R
*
*
*
*
Basal
cAMP stimulated