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SHORT COMMUNICATION

IL-33 stimulates expression of the GPR84 (*EX33*) fatty acid receptor gene and of cytokine and chemokine genes in human adipocytes

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RUNNING TITLE: IL-33 stimulates gene expression in human adipocytes

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ABSTRACT

Expression of GPCR fatty acid sensor/receptor genes in adipocytes is modulated by inflammatory mediators, particularly IL-1 β . In this study we examined whether the IL-1 gene superfamily member, IL-33, also regulates expression of the fatty acid receptor genes in adipocytes. Human fat cells, differentiated from preadipocytes, were incubated with IL-33 at three different dose levels for 3 or 24 h and mRNA measured by qPCR. Treatment with IL-33 induced a dose-dependent increase in GPR84 mRNA at 3 h, the level with the highest dose being 13.7-fold greater than in controls. Stimulation of *GPR84* expression was transitory; the mRNA level was not elevated at 24 h. In contrast to *GPR84*, IL-33 had no effect on *GPR120* expression. IL-33 markedly stimulated expression of the *IL1B*, *CCL2*, *IL6*, *CXCL2* and *CSF3* genes, but there was no effect on *ADIPOQ* expression. The largest effect was on *CSF3*, the mRNA level of which increased 183-fold over controls at 3 h with the highest dose of IL-33; there was a parallel increase in the secretion of G-CSF protein into the medium. It is concluded that in human adipocytes IL-33, which is synthesised in adipose tissue, has a strong stimulatory effect on the expression of cytokine and chemokine genes, particularly *CSF3*, and on the expression of GPR84, a pro-inflammatory fatty acid receptor.

Keywords:

Adipocyte

Adipose tissue

GPR84

Inflammation

Interleukin-33

1. Introduction

Several G-protein coupled receptors act as sensors/receptors for fatty acids [1-2]. GPR120 (or FFAR4), is a receptor for n-3 polyunsaturated fatty acids, and has been linked to the anti-inflammatory action of these lipids [2-3]. The primary ligands for GPR41 (FFAR3) and GPR43 (FFAR2) are short chain fatty acids, while GPR40 (FFAR1) is activated by medium and long chain saturated and mono-unsaturated fatty acids [1-2]. GPR84 (EX33), a pro-inflammatory receptor, is also activated by medium chain fatty acids [4-5]. Each of these GPCRs has a distinct tissue distribution, with GPR120 being strongly expressed in both adipocytes and macrophages (M1 and M2) in white adipose tissue, where it promotes adipogenesis [6] in addition to insulin sensitising and anti-inflammatory actions [2-3]. Adipocytes also express GPR84, and at least in the case of human fat cells GPR40, GPR41 and GPR43, albeit at low levels [7].

Recent studies have shown that the expression of *GPR84*, *GPR41* and *GPR120* is powerfully and differentially modulated by inflammatory mediators. Macrophage-conditioned medium, lipopolysaccharide, $\text{TNF}\alpha$ and $\text{IL-1}\beta$ have each been shown to strongly stimulate *GPR84* expression [4, 7-9]. In contrast, GPR120 expression is markedly reduced by these agents [7-8]. $\text{IL-1}\beta$ induces a particularly strong stimulation of GPR84 expression in human adipocytes and the expression of GPR41 is also increased [7]. The IL-1 superfamily of cytokines comprises other pro-inflammatory agonists in addition to $\text{IL-1}\beta$, including IL-18 and IL-33 [10], both of which are produced by human white adipocytes [10-11]. However, the main source of IL-33 in human adipose tissue appears to be endothelial cells [12]. IL-33 has multiple effects in inflammation and immunity, involving the stimulation of regulatory T (T_{reg}) cells and the production of Th2 cytokines and chemokines such as IL-5, IL-6 and MCP-1, including in adipose tissue [13].

In view of the link between inflammation and the production of GPCR fatty acid receptors in adipocytes, we have examined whether IL-33 modulates the expression of key fatty acid receptor genes in human adipocytes, and of cytokine and chemokine genes.

2. Materials and methods

2.1. Adipocyte cell culture

Human preadipocytes, isolated from subcutaneous adipose tissue, and proprietary cell culture media were purchased from PromoCell (Germany; Catalogue #C-12730, Lots #419Z023). The cells were plated into 12-well plates (5,000 cells/cm²), cultured to confluence, differentiated into adipocytes and then further cultured in nutrition medium (changed every 2-3 days) for up to 14 days, as previously [14]. The nutrition medium contained 3% foetal calf serum, dexamethasone

(400 ng/ml) and insulin (0.5 µg/ml). Differentiated adipocytes, characterised by fat droplets, were used at 12-14 days after differentiation was induced.

The adipocytes were divided into four groups which were incubated with either 0 (controls), 5, 25 or 100 ng/ml of recombinant IL-33 (Sigma, UK) for 3 or 24 h. The dose levels were based on previous studies [13]. Following the incubation period, the medium was aspirated and stored at -20°C, and the cells washed and frozen in TRI Reagent (Sigma) prior to storage at -80°C. Six individual sets of adipocytes were taken for each group.

2.2. RNA extraction and real-time PCR

The adipocytes were thawed and homogenised in the TRI Reagent in which they were stored, and RNA extracted using an RNeasy Micro Kit (Qiagen, UK). The purity of the extracted RNA was close to 2.0 (260/280 and 260/230 ratios by NanoDrop 1000, Wilmington, USA). The RNA was DNase-treated with a TURBO-DNA-free™ kit (Ambion, Life Technologies, USA) and 2 µg reverse transcribed using *Taqman*® reverse transcription reagents (Applied Biosystems, UK). cDNA (40-80 ng) was taken for real-time PCR which was performed in triplicates using a Gene Expression Master Mix and *TaqMan*® Gene Assays consisting of specific *Taqman*® probes (Applied Biosystems). The following human gene probes were employed: *GPR41* (Hs02519193_g1), *GPR84* (Hs01874713_s1), *GPR120* (Hs00699184_m1), *CCL2* (Hs00234140_m1), *IL1B* (Hs01555410_m1), *IL6* (Hs00985639_m1), *ADIPOQ* (Hs00605917_m1), *CSF3* (Hs00738432_g1), *CXCL2* (Hs00601975). *ACTB* (Hs99999903_m1) was the reference gene. PCR reactions were arranged in duplex format with the FAM-labelled *Taqman*® probe for each gene mixed with the VIC-labelled *Taqman*® probe for *ACTB*.

PCR amplification was performed with an ABI real-time PCR detection system (ABI StepOneplus, Applied Biosystems) with two-step thermal cycling: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analysed by the comparative $2^{-\Delta\Delta Ct}$ method, as previously [7, 14] and expressed as fold-changes in the target gene (normalized to *ACTB*) in adipocytes treated with IL-33 in relation to expression in the control cells (control cells =1).

2.3. Protein immunoassays

The concentration of IL-6 and G-CSF in the culture medium, which was centrifuged to remove any cell debris, was measured using sensitive MSD immunoassays (Meso Scale Discovery, USA). Plates were pre-coated with antibodies on discrete spots and the assay performed according to the manufacturer's instructions, as previously [7, 14]; data were analysed using Meso Scale software. The minimum level of detection was 0.06 pg/ml for IL-6 (interlot CV <20 %) and 1.5 pg/ml for CSF3 (interlot CV <18%).

2.4. Statistical analysis

The statistical significance of differences between groups was assessed by one-way ANOVA with a Bonferroni post-test (for selected groups) or with Student's unpaired *t* test. A value of $P < 0.05$ was taken as statistically significant.

3. Results

Human adipocytes, differentiated from fibroblastic preadipocytes in culture, were incubated with IL-33 for either 3 or 24 h to examine acute and prolonged responses to the cytokine. Low, medium and high concentrations of IL-33 were employed. IL-33 had no significant effect on GPR120 mRNA level at either 3 or 24 h, except for a small, but statistically significant, reduction for the middle concentration of the cytokine at the shorter time point (Fig 1b). There was also no effect of IL-33 on GPR41 mRNA level at 24 h; a small, dose-dependent, increase was evident at 3 h, the increase being statistically significant with the highest dose (Fig 1c). In contrast, IL-33 induced a marked, dose-dependent increase in GPR84 mRNA at 3 h, the level with the high dose being 13.7-fold greater than in controls (Fig 1a). This effect was, however, transitory; there was no significant change in GPR84 mRNA at 24 h (Fig 1a).

To assess the effects of IL-33 on the expression of classical inflammation-related genes, the mRNA level of a group of cytokine and chemokines was examined. IL-33 induced a marked, dose-dependent increase in mRNA of the *ILB*, *IL6*, *CCL2*, *CXCL2* and *CSF3* genes at 3 h (Fig 2a-e). With the highest dose of IL-33, there were 3.0-, 11.7-, 15.2- and 15.6-fold increases in mRNA level of the *CCL2*, *IL6*, *CXCL2* and *ILB* genes, respectively. *CSF3* exhibited a very substantial response to IL-33, the mRNA level with the highest dose of the cytokine being 183-fold higher than in the controls (Fig 2e).

As with *GPR84*, these responses to IL-33 were transitory; there was no effect of IL-33 on IL-1 β mRNA at 24 h (Fig 2a), and there was actually a small reduction in *CCL2* mRNA with the low and medium doses of the cytokine (Fig 2c). *CSF3*, IL-6 and *CXCL2* mRNA levels were each elevated at 24 h with the highest dose of IL-33 (3.9-, 2.5- and 3.3-fold, respectively); however, for *CSF3*, the increase was substantially lower than the 183-fold increase at 3 h.

In contrast to the other adipokine genes, expression of *ADIPOQ*, which encodes the key adipocyte hormone adiponectin, was not modified by IL-33, the mRNA level being unchanged at both 3 and 24 h with each dose of the cytokine (Fig 2f). *IL18* expression was also unaltered (results not shown).

To determine whether the changes in cytokine and chemokine gene expression were reflected by similar changes in secretion of the encoded protein, the effect of IL-33 on the

release of IL-6 and G-CSF was examined. Treatment with IL-33 resulted in a dose-dependent increase in the amount of the two adipokines in the culture medium at both 3 and 24 h (Fig 2g and 2h). The amount of both proteins was greater at 24 h than at 3 h, reflecting the delay between increases in mRNA level and release of the protein product. At 24 h, with the highest dose of IL-33, the amount of IL-6 was 7.9-fold greater than in the controls (4.7-fold at 3 h) and 23.9-fold higher (4.6-fold at 3 h) in the case of G-CSF.

4. Discussion

This study demonstrates that expression of the *GPR84* fatty acid receptor/sensor gene in human adipocytes is strongly and acutely stimulated by IL-33. This parallels the stimulatory effect of IL-1 β and TNF α , as well as other mediators, on the expression of this gene in fat cells [4, 7-9]. GPR84 is considered a pro-inflammatory receptor, its activation amplifying the production of IL-8 and IL-12 p40 in polymorphonuclear leukocytes and of TNF α by macrophages [5]. Thus IL-33 may contribute to the inflammatory response in adipose tissue in part through increased production of GPR84.

IL-33 did not have a suppressive effect on *GPR120* expression, in contrast to TNF α and IL-1 β [7-8]. The insulin sensitising and anti-inflammatory actions of GPR120, which is a receptor for n-3 polyunsaturated fatty acids [2-3], is unlikely to be modified by IL-33 contrary to what has been projected with IL-1 β and TNF α [7]. The intracellular signalling pathways linked to the IL-33 receptor, ST2 (and its co-receptor IL-1 receptor accessory protein) [15], which are expressed in human adipocytes [11], would appear to be implicated in the modulation of the transcription of the *GPR84* gene, but not *GPR120*. It is noted that while 3 and 24 h time-points were employed, reflecting potential acute and sustained effects of IL-33, the possibility cannot be excluded that some changes might occur at intermediate times, but if so these are likely to be limited.

IL-33 had marked effects on the inflammatory response in human adipocytes beyond GPR84, with a substantial stimulation of the expression of a selected group of cytokine and chemokine genes. Expression of *IL1B*, a co-member of the IL-1 superfamily, was strongly up-regulated by IL-33, as was *IL6* and *CXCL2* (encoding MCP-1) and *CSF3*. Stimulation of MCP-1, and IL-6 from the stromovascular fraction of murine adipose tissue by IL-33 has been noted previously [13]. Stimulation of *CSF3* expression was particularly marked in the present study, and this was accompanied by a major increase in the secretion of G-CSF into the medium. *CSF3* expression is also strongly up-regulated in human adipocytes by IL-1 β [14]. The substantial

increase in G-CSF production may be linked to neutrophil recruitment and function in adipose tissue, as well as a potential neutrophilic/neuroprotective role in inflammation in the tissue.

In contrast to the other adipokine genes examined, IL-33 had no effect on the expression of *ADIPOQ*. Since adiponectin, the adipocyte hormone encoded by *ADIPOQ*, has anti-inflammatory and insulin sensitising functions [see 7], IL-33 does not compromise the production of a factor with these actions, unlike IL-1 β and TNF α which inhibit *ADIPOQ* expression [7, 14]. As with GPR120, the possibility cannot be excluded of some small response to IL-33 at time-points intermediate to 3 and 24 h.

There are multiple potential sources of IL-33 within adipose tissue, since the *IL33* gene is expressed in adipocytes, macrophages and endothelial cells [12]. As such, the cytokine may have both autocrine and paracrine actions in the tissue. *IL33* expression is elevated in adipose tissue of obese humans where it is suggested that it may contribute to inflammation in obesity [12]. However, a protective effect of IL-3 has also been proposed [13, 16]; IL-33 reduces the inflammatory response in adipose tissue in obese mice through polarising macrophages to the M2 phenotype as well as reversing the overall recruitment of macrophages and the accumulation of pro-inflammatory cytokines [13, 16]. These changes may counter the direct pro-inflammatory effects of IL-33 on adipocytes.

4.1 Conclusions

IL-33 has selective actions on GPR fatty acid receptor gene expression in human adipocytes, stimulating expression of the pro-inflammatory receptor GPR84. IL-33 also has extensive effects on the production of key cytokines and chemokines in human adipocytes, with a powerful stimulatory action on the expression of *CSF3* and the secretion of its protein product G-CSF. IL-33 may be a significant modulator of the inflammatory response in adipose tissue.

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Conflicts of interest.

The authors declare that they have no competing interests.

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Legends to Figures

Fig. 1. Effect of IL-33 on (a) *GPR84*, (b) *GPR120*, and (c) *GPR41* gene expression in human adipocytes. The adipocytes were incubated with IL-33 (5, 20, or 100 ng/ml) for either 3 or 24 h. The results, which are expressed as fold-changes in mRNA level in cells treated with IL-33 relative to the respective control cells (no IL-33), are means $\pm$ SE (bars) for 4-6 sets of adipocytes. * $P<0.05$, *** $P<0.001$ compared with the control cells at the same time point.

Fig. 2. Effect of IL-33 on cytokine and chemokine gene expression (a-f) and protein secretion (g, h) in human adipocytes. The adipocytes were incubated with IL-33 (5, 20, or 100 ng/ml) for either 3 or 24 h. (a) *IL1B*, (b) *CXCL2*, (c) *CCL2*, (d) *IL6*, (e) *CSF3*, and (f) *ADIPOQ* gene expression. The gene expression results are fold-changes in mRNA level in cells treated with IL-33 relative to the respective control cells (no IL-33). The amount of protein was measured in the medium for IL-6 (g) and G-CSF (h). All results are means $\pm$ SE (bars) for 4-6 sets of adipocytes. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with the control cells at the same time point.

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Figure 1
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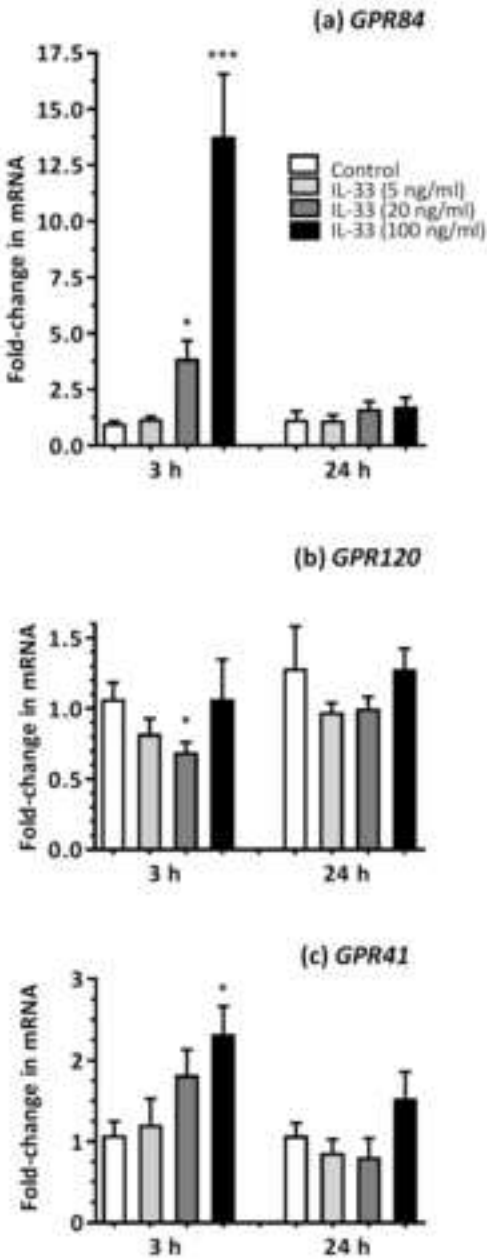
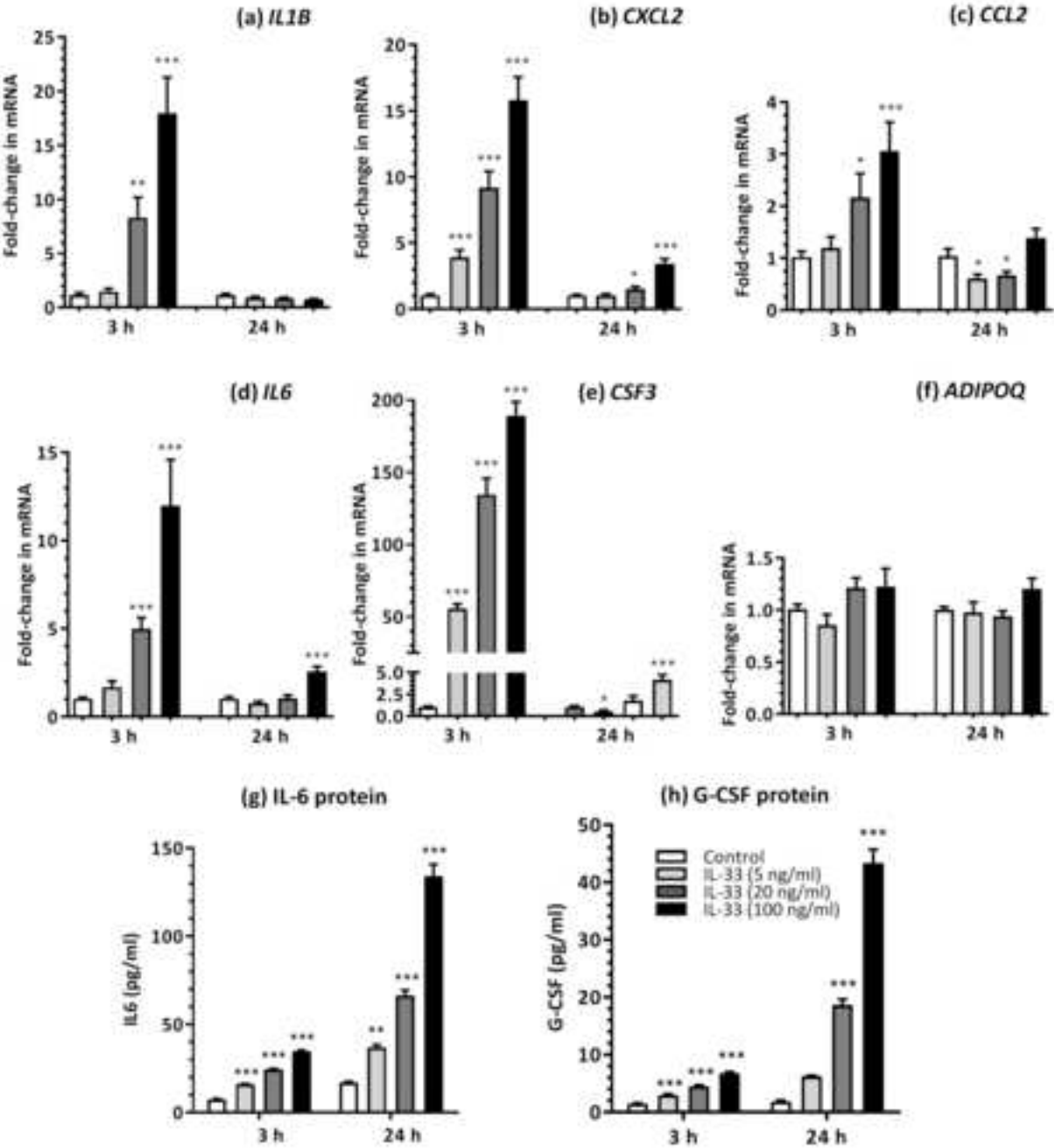


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