Diabetologia (2012) 55:2456–2468
DOI 10.1007/s00125-012-2592-3

ARTICLE

Glucagon-like peptide-1 inhibits adipose tissue macrophage infiltration and inflammation in an obese mouse model of diabetes

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Received: 15 February 2012 / Accepted: 24 April 2012 / Published online: 22 June 2012

Abstract
Aims/hypothesis Obesity and insulin resistance are associated with low-grade chronic inflammation. Glucagon-like peptide-1 (GLP-1) is known to reduce insulin resistance. We investigated whether GLP-1 has anti-inflammatory effects on adipose tissue, including adipocytes and adipose tissue macrophages (ATM).
Methods We administered a recombinant adenovirus (rAd) producing GLP-1 (rAd-GLP-1) to an *ob/ob* mouse model of diabetes. We examined insulin sensitivity, body fat mass, the infiltration of ATM and metabolic profiles. We analysed the mRNA expression of inflammatory cytokines, lipogenic genes, and M1 and M2 macrophage-specific genes in adipose tissue by real-time quantitative PCR. We also examined the activation of nuclear factor κB (NF-κB), extracellular signal-regulated kinase 1/2 and Jun N-terminal kinase (JNK) in vivo and in vitro.
Results Fat mass, adipocyte size and mRNA expression of lipogenic genes were significantly reduced in adipose tissue of rAd-GLP-1-treated *ob/ob* mice. Macrophage populations ($F4/80^+$ and $F4/80^+CD11b^+CD11c^+$ cells), as well as the expression and production of IL-6, TNF-α and monocyte chemoattractant protein-1, were significantly reduced in adipose tissue of rAd-GLP-1-treated *ob/ob* mice. Expression of M1-specific mRNAs was significantly reduced, but that of M2-specific mRNAs was unchanged in rAd-GLP-1-treated *ob/ob* mice. NF-κB and JNK activation was significantly reduced in adipose tissue of rAd-GLP-1-treated *ob/ob* mice. Lipopolysaccharide-induced inflammation was reduced by the GLP-1 receptor agonist, exendin-4, in 3T3-L1 adipocytes and ATM.
Conclusions/interpretation We suggest that GLP-1 reduces macrophage infiltration and directly inhibits inflammatory pathways in adipocytes and ATM, possibly contributing to the improvement of insulin sensitivity.

Keywords Adipose tissue · Adipose tissue macrophage · Anti-inflammatory · Cytokine · Glucagon-like peptide-1 · Inflammatory signalling · Insulin resistance · Lipogenic genes · M1 · M2 macrophage

Abbreviations

ATM	Adipose tissue macrophages
EMSA	Electrophoretic mobility shift assay

Electronic supplementary material The online version of this article (doi:10.1007/s00125-012-2592-3) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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ERK 1/2	Extracellular signal-regulated kinase ½
GLP-1	Glucagon-like peptide-1
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
NF-κB	Nuclear factor κB
rAd	Recombinant adenovirus
rAd-βgal	rAd producing β-galactosidase
rAd-GLP-1	rAd producing GLP-1
$\dot{V}O_2$	Oxygen consumption

Introduction

Obesity is associated with chronic, low-grade inflammation in adipose tissue, which contributes to the development of insulin resistance, a major cause of the metabolic syndrome, including type 2 diabetes [1]. The abundance of inflammatory cytokines, e.g. IL-6 and TNF-α, and chemokines is increased in fat cells of obese patients [2] and is correlated with insulin resistance [1]. Increased macrophage and T cell infiltration is observed in adipose tissue in obesity, and macrophages alter the levels of insulin signalling molecules and GLUT4, as well as inhibiting insulin action in adipocytes [3]. There is considerable evidence in support of the hypothesis that inflammation and immune cells play an important role in metabolic dysregulation. The inhibition of inflammatory signalling pathways or blocking of immune cell recruitment is correlated with improved insulin sensitivity. Myeloid-specific *Ikbkb* knockout mice (in which the gene encoding inhibitor of κB kinase β has been knocked out) are spared from obesity-induced insulin resistance [4]. *Mcp1* knockout or *Ccr2* knockout mice (in which the genes encoding monocyte chemoattractant protein [MCP]-1 and chemokine [C-C motif] receptor 2, respectively, have been knocked out) show reduced levels of adipose tissue macrophages (ATM) and are protected from obesity-induced insulin resistance [5, 6]. Treatment with anti-CD3 antibody to deplete T cells reduces Th1 cells and reverses insulin resistance [7].

Glucagon-like peptide-1 (GLP-1), which is secreted from intestinal L-cells in response to nutrient ingestion, is known to have many glucose-lowering actions, potentiating glucose-dependent insulin secretion, enhancing beta cell growth, and reducing food intake and body weight [8, 9]. In addition, GLP-1 improves insulin sensitivity in type 2 diabetic patients and animal models of the disease [10–12]. Further studies on mechanisms by which GLP-1 improves insulin sensitivity have shown that treatment of 3T3-L1 adipocytes with exendin-4, a GLP-1 receptor agonist, increases insulin-stimulated glucose uptake [13] and amplifies insulin signalling by upregulating basal insulin receptor, IRS-1 and GLUT4 abundance [14]. It has also been reported that exendin-4 increases adiponectin levels and prevents inflammatory adipokine production in 3T3-L1 adipocytes [15]. However, it is not known whether GLP-1 plays any role in adipose tissue inflammation, including ATM.

We have previously found that GLP-1 therapy improved insulin sensitivity, increased the activation of insulin signalling molecules in peripheral tissue and remitted diabetes in an obese mouse model of diabetes [10]. As obesity is associated with macrophage infiltration and inflammatory responses in adipose tissue that affect the development of insulin resistance, we investigated the role of GLP-1 in adipose tissue inflammation and macrophage infiltration. We show that GLP-1 reduces infiltration of ATM and has direct anti-inflammatory effects on adipocytes and ATM.

Methods

Animals Mice obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) were maintained at a facility at Gachon University. All animal experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Lee Gil Ya Cancer and Diabetes Institute, Gachon University.

Production of recombinant adenovirus producing GLP-1 (7-37) and treatment of ob/ob mice Recombinant adenovirus (rAd) producing GLP-1 (rAd-GLP-1) or rAd producing β-galactosidase (rAd-βgal) were produced as previously described [10]. Diabetic male *ob/ob* mice (blood glucose levels >13.9 mmol/l [250 mg/dl] for three consecutive days) were injected at 6 to 8 weeks of age with rAd-GLP-1 or rAd-βgal (4×10^9 plaque-forming units, i.v.). Intravenous injection of rAd-GLP-1 produces GLP-1 mainly in liver. Pair-fed and rAd-βgal-treated pair-fed control mice were given the same daily amount of food as that eaten by the corresponding rAd-GLP-1-treated group during the previous day. All animal groups were matched for body weight with the rAd-GLP-1-treated group at the beginning of each experiment.

Histological staining of adipose tissue and measurement of adipocyte size and number Quantitative evaluation of adipocyte size and number was performed on haematoxylin and eosin-stained sections using the UTHSCSA Image Tool program (http://compdent.uthscsa.edu).

Isolation of stromal vascular cells and flow cytometric analysis Stromal vascular cells were isolated from epididymal adipose tissue and stained with fluorescein isothiocyanate-conjugated anti-mouse F4/80 antibody, allophycocyanin-conjugated anti-mouse CD11c antibody and/or peridinin chlorophyll protein-conjugated anti-mouse CD11b antibody

(eBioscience, San Diego, CA, USA). Cells were analysed on a FACSCalibur (BD Bioscience, San Jose, CA, USA) with CellQuest software (BD Bioscience).

3T3-L1 cell differentiation and treatment 3T3-L1 preadipocytes were cultured and induced to differentiate as described previously [16, 17].

Real-time quantitative PCR PCR was carried out in a PCR system (7900HT fast real-time PCR; Applied Biosystems, Carlsbad, CA, USA). The PCR primers used are shown in Electronic Supplementary Material (ESM) Table 1. The relative copy number was calculated using the threshold crossing point (C_t) as calculated by the system's software, combined with the $2^{-\Delta\Delta C_t}$ calculations.

Western blot Western blots were performed with anti-AKT, anti-phospho-AKT (Ser473), anti-extracellular signal-regulated kinase (ERK)1/2, anti-phospho-ERK1/2 and anti-phospho-Jun N-terminal kinase (JNK) (Cell Signaling Technology, Beverly, MA, USA), and with anti-NF-κB p65 or anti-lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Electrophoretic mobility shift assay We performed electrophoretic mobility shift assay (EMSA) as described previously [18] using oligonucleotides for a specific NF-κB binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′).

Isolation of CD11b$^+$ macrophages Epididymal fat pads were excised from male C57BL/6 mice fed a high-fat diet (60% fat; Research Diets, New Brunswick, NJ, USA) for 8 to 10 weeks. Stromal vascular cells were collected and CD11b$^+$ cells isolated by immunoaffinity isolation using anti-CD11b antibodies conjugated to magnetic beads (MACS; Milenyi Biotec, Auburn, CA, USA). Isolated CD11b$^+$ cells were allowed to attach to a plate for 2 h at 37°C.

Measurement of NF-κB translocation ATMs were incubated with anti-NF-κB and DyLight 549-labelled goat anti-rabbit antibody (Thermo Scientific, Loughborough, UK). Fluorescent cell images were obtained on a confocal laser scanning microscope (LSM 700; Carl Zeiss MicroImaging, Jena, Germany) and an HCS reader (ArrayScan), and effective image analysis was done using Cellomics Technologies software (Thermo Scientific).

Energy balance A comprehensive animal metabolic monitoring system (CLAMS; Columbus Instruments, Columbus, OH, USA) was used for 72 h at 3 days after rAd-GLP-1 or rAd-βgal treatment. Energy expenditure and respiratory exchange ratios were calculated from gas exchange. The respiratory exchange ratio was computed as carbon dioxide output ($\dot{V}CO_2$) divided by oxygen consumption ($\dot{V}O_2$). Activity was measured on the *x* and *z* axes by counting the breaks in an infrared beam during a measurement period.

Statistical analysis Data are presented as means ± SD or means ± SEM. The statistical significance of the difference between two groups was analysed by unpaired Student's *t* test or ANOVA, followed by Tukey's honestly significant difference (HSD) test for multiple comparisons. A value of $p<0.05$ was accepted as significant.

Results

Improvement of insulin sensitivity and reduction of fat mass by rAd-GLP-1 treatment in diabetic ob/ob mice We had previously found that rAd-GLP-1 injection into diabetic *ob/ob* mice normalised blood glucose levels and improved insulin sensitivity [10]. To confirm these previous findings of a glucose-lowering effect of rAd-GLP-1, we injected rAd-GLP-1 (4×10^9 plaque-forming units) into the tail vein of diabetic *ob/ob* mice. We found that glucose levels decreased, GLP-1 was produced, insulin levels decreased, and glucose and insulin tolerance improved at 2 weeks after the rAd-GLP-1 injection (ESM Fig. 1), a finding consistent with our previous results [10].

Food intake in rAd-GLP-1-treated mice rapidly decreased and remained significantly lower than in the rAd-βgal-treated group over the 10 days of the experiment (ESM Fig. 2a). The rAd-GLP-1-treated, pair-fed and rAd-βgal-treated+pair-fed groups had reduced body weights compared with the group treated with rAd-βgal only (ESM Fig. 2b). We looked for differences in changes of fat mass between the rAd-GLP-1-treated and the pair-fed group. Analysis of fat mass by the ^{1}H minispec system showed that fat mass as a proportion of body weight was significantly reduced in rAd-GLP-1-treated *ob/ob* mice compared with untreated and rAd-βgal-treated *ob/ob* mice, but was not reduced in the pair-fed group (ESM Fig. 2c). To determine whether rAd-GLP-1 treatment reduces fat at specific sites, we measured the weight of epididymal, mesenteric, perirenal and subcutaneous fat of mice at 2 weeks after rAd-GLP-1 treatment. The weights of the first three were significantly reduced in rAd-GLP-1-treated *ob/ob* mice compared with rAd-βgal-treated mice, whereas subcutaneous fat in rAd-GLP-1-treated and pair-fed *ob/ob* mice did not differ in weight from that in rAd-βgal-treated mice. Mesenteric fat had a significantly reduced weight in the pair-fed group compared with the rAd-βgal-treated group (ESM Table 2).

Reduction of adipocyte size and expression of lipogenic genes in rAd-GLP-1-treated ob/ob mice To determine whether rAd-GLP-1 treatment affects the size of adipocytes, we prepared sections of epididymal adipose tissue at 2 weeks

after the rAd-GLP-1 injection, stained them with haematoxylin and eosin (Fig. 1a), and measured the adipocyte diameter and number. The size of adipocytes was significantly reduced (Fig. 1b) and the number of adipocytes in the same area was significantly increased (Fig. 1c) in rAd-GLP-1-treated *ob/ob* mice compared with untreated and rAd-βgal-treated mice. The size and number of adipocytes in the pair-fed mice were similar to untreated and rAd-βgal-treated mice. To investigate de novo lipogenesis in rAd-GLP-1-treated mice, we measured the mRNA expression of *Srebp1c* (also known as *Srebf1*), *Acc1* (also known as *Acaca*) and *Fas* (also known as *Fasn*), all genes that are involved in lipogenesis. We found that mRNA expression for these genes was significantly decreased in epididymal adipose tissue at 2 weeks after rAd-GLP-1 treatment (Fig. 1d–f). To investigate whether GLP-1 receptor stimulation directly affects lipogenesis and lipogenic gene expression, we treated 3T3-L1 pre-adipocytes with exendin-4 during differentiation and maturation, and examined triacylglycerol accumulation and mRNA levels of lipogenic genes. In contrast to the in vivo results, we found that triacylglycerol accumulation and the expression of *Srebp1c* and *Fas* mRNA were significantly increased in exendin-4-treated 3T3-L1 adipocytes (ESM Fig. 3).

Decreased macrophage infiltration and reduced M1 macrophage-specific mRNA expression in adipose tissue of rAd-GLP-1-treated mice To determine whether macrophage infiltration in white adipose tissue was changed by rAd-GLP-1 treatment, we analysed the F4/80$^+$ macrophage population in the stromal vascular fraction from epididymal fat by flow cytometric analysis at 2 weeks after treatment. The number of infiltrated cells was significantly decreased in epididymal fat of rAd-GLP-1-treated mice (Fig. 2a). The F4/80$^+$ macrophage population was also significantly decreased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 2b, c). As F4/80$^+$CD11b$^+$CD11c$^+$ cells are known to be specific targets for NEFA and high-fat feeding [19], we analysed the F4/80$^+$CD11b$^+$CD11c$^+$ cell population and found that it was significantly decreased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 2d, e). The F4/80$^+$CD11b$^+$CD11c$^-$ cell population was also significantly reduced in rAd-GLP-1-treated mice (Fig. 2f). Based on these results, we examined the

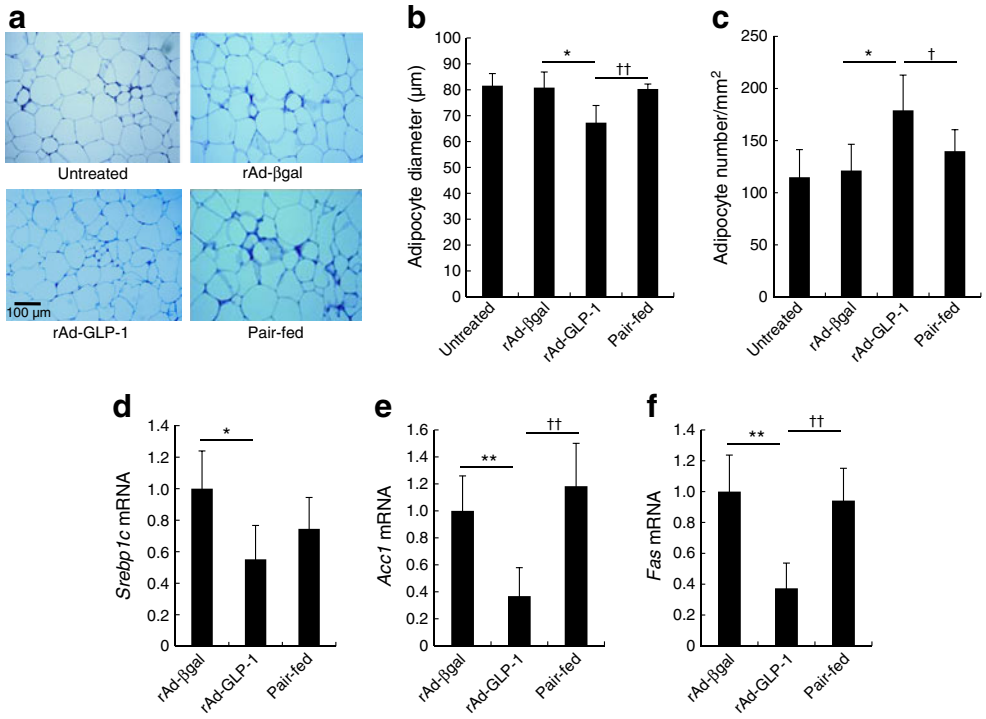

Fig. 1 Changes in adipocyte size and number, and expression of lipogenic genes in epididymal fat of rAd-GLP-1-treated *ob/ob* mice. Diabetic *ob/ob* mice were treated with rAd-GLP-1 or rAd-βgal and sections of epididymal adipose tissues prepared and stained as described in Methods. (**a**) Representative photomicrograph (magnification ×200). (**b**) The adipocyte diameter and (**c**) numbers of cells in the same area were measured with an image tool program (UTHSCSA); n=3–4 per group. Untreated and pair-fed diabetic *ob/ob* mice served as controls. (**d**) The expression of *Srebp1c*, (**e**) *Acc1* and (**f**) *FAS* mRNA in epididymal fat was analysed by real-time quantitative PCR and normalised to cyclophilin mRNA expression. The fold change was calculated as ratio of the expression level in rAd-βgal-treated mice; n=3–9 per group. Pair-fed diabetic *ob/ob* mice served as controls. Data (**b–f**) are means ± SD; *p<0.05 and **p<0.01 compared with rAd-βgal-treated mice; $^{\dagger}p$<0.05 and $^{\dagger\dagger}p$<0.01 compared with the pair-fed group

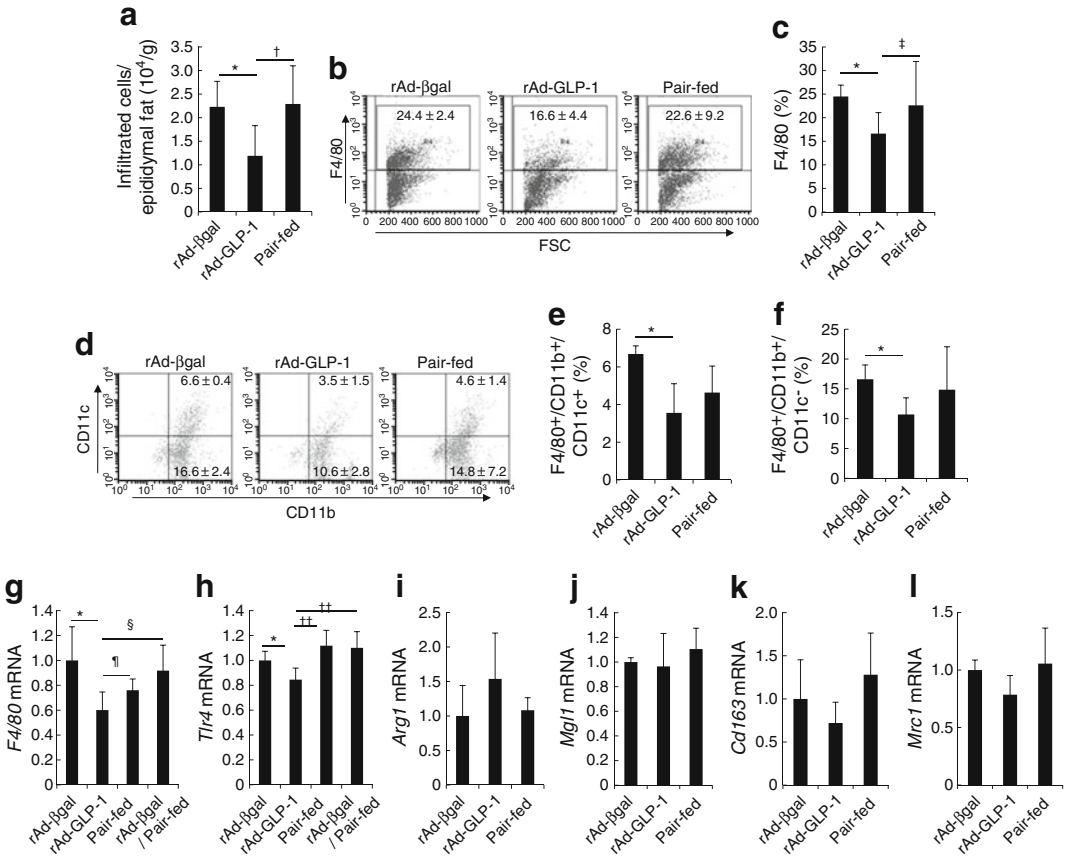

Fig. 2 Decrease of ATM infiltration in rAd-GLP-1-treated *ob/ob* mice. Diabetic *ob/ob* mice were treated with rAd-GLP-1 or rAd-βgal, and stromal vascular cells isolated from epididymal fat and analysed as indicated in Methods. (**a**) The number of infiltrated cells per g epididymal fat tissue; n=5–6 per group. (**b**) Dot plot representation of F4/80$^+$ cells. (**c**) Percentage of infiltrated F4/80$^+$ cells; ‡p=0.12. (**d**) Dot plot of relative expression of CD11b versus CD11c for FACS data obtained from F4/80$^+$ gated macrophages. (**e**) Percentage of F4/80$^+$/CD11b$^+$/CD11c$^+$ cells and (**f**) F4/80$^+$/CD11b$^+$/CD11c$^-$ cells; n=3–4 group. (**g**) At 2 weeks after treatment, epididymal fat tissue was removed and the expression of *F4/80*, (**h**) *Tlr4*, (**i**) *Arg1*, (**j**) *Mgl1*, (**k**) *Cd163* and (**l**) *Mrc1* mRNA was analysed by real-time quantitative PCR, with values normalised to cyclophilin expression. The fold change was calculated as ratio of the expression level in rAd-βgal-treated mice; n=3–6 per group. Pair-fed diabetic *ob/ob* mice served as controls. Data are means ± SD; *p<0.05 compared with rAd-βgal-treated mice; †p<0.05 and ††p<0.01 compared with the pair-fed group; §p=0.06; ¶p=0.09

expression of *F4/80* (also known as *Emr1*) and *Tlr4* mRNA, which are expressed in classically activated M1 macrophages. We also examined the mRNA expression of *Arg1*, *Mgl1* (also known as *Clec10a*), *Cd163* and *Mrc1*, which are expressed in alternatively activated M2 macrophages. The expression of *F4/80* and *Tlr4* mRNA was significantly decreased in rAd-GLP-1-treated *ob/ob* mice compared with rAd-βgal-treated mice, whereas that of *Arg1*, *Mgl1*, *Cd163* and *Mrc1* mRNA was not different among the groups (Fig. 2g–l).

Decrease of inflammatory cytokine mRNA expression and production in adipose tissue and peritoneal macrophages of rAd-GLP-1-treated mice As the M1 macrophage population was reduced in rAd-GLP-1-treated *ob/ob* mice, we examined whether the expression of pro-inflammatory adipokines or chemokines was inhibited in the adipose tissue of rAd-GLP-1-treated mice. Real-time quantitative PCR analysis of the expression of *Il6*, *Tnfa* (also known as *Tnf*) and *Mcp1* (also known as *Ccl2*) mRNA in adipose tissue showed, as expected, that the transcripts of these molecules were significantly decreased in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice (Fig. 3a–c). The production of IL-6, TNF-α and MCP-1 protein in adipose tissue was also significantly decreased in rAd-GLP-1-treated mice (Fig. 3d–f). There were no significant differences in inflammatory cytokine mRNA or protein abundance (except IL-6) between the rAd-βgal-treated, pair-fed, and rAd-βgal-treated+pair-fed groups (Fig. 3a–f). Examination of the expression of *Tnfa*, *Tlr4* and *Mcp1* mRNA in peritoneal macrophages at 2 weeks after rAd-GLP-1 treatment showed that the corresponding molecules were significantly decreased in rAd-GLP-1-treated *ob/ob* mice compared with rAd-βgal-treated mice (Fig. 3g–j). *Il6* mRNA expression was also decreased, but this was not

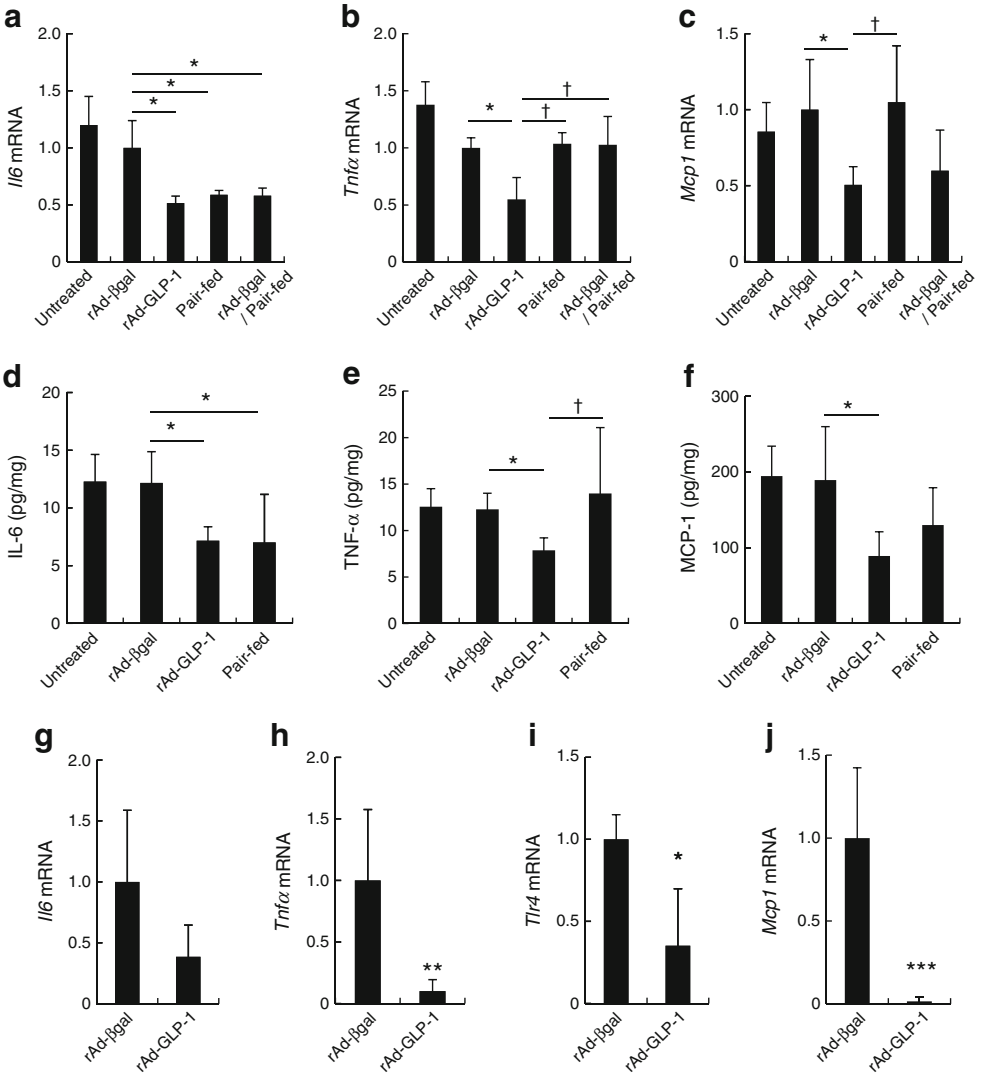

Fig. 3 Decrease of inflammatory cytokine mRNA expression and production in adipose tissue and peritoneal macrophages of rAd-GLP-1-treated *ob/ob* mice. Diabetic *ob/ob* mice were treated with rAd-GLP-1 or rAd-βgal and epididymal fat was removed as indicated in the Methods. (**a**) The expression of *Il6*, (**b**) *Tnfa* and (**c**) *Mcp1* mRNA, and protein levels of (**d**) IL-6, (**e**) TNF-α and (**f**) MCP-1 were measured (n=5–13 per group). Untreated and pair-fed diabetic *ob/ob* mice served as controls. For mRNA measurement, the fold change was calculated as the ratio of the expression in rAd-βgal-treated mice. (**g**) Peritoneal macrophages were isolated from diabetic *ob/ob* mice at 2 weeks after rAd-GLP-1 treatment, and *Il6*, (**h**) *Tnfa*, (**i**) *Tlr4* and (**j**) *Mcp1* mRNA was analysed as described above. Untreated mice served as controls. Data are means ± SD; *p<0.05, **p<0.005 and ***p<0.001 compared with rAd-βgal-treated *ob/ob* mice; $^{\dagger}p$<0.05 compared with pair-fed group

statistically significant (Fig. 3g). The serum concentrations of TNF-α and MCP-1 were not statistically different between treatments (ESM Fig. 4).

Reduction of NF-κB, ERK1/2 and JNK activation in adipose tissue of rAd-GLP-1-treated mice Activation of NF-κB is involved mainly in the expression of inflammatory responsive genes such as *Il6* and *Tnfa* [20, 21]. As pro-inflammatory cytokine production is reduced by rAd-GLP-1 treatment, we used EMSA to examine the activation of NF-κB in epididymal fat at 2 weeks after rAd-GLP-1 treatment. We found that NF-κB binding activity was reduced in adipose tissue of rAd-GLP-1-treated *ob/ob* mice compared with untreated, rAd-βgal-treated and pair-fed *ob/ob* mice (Fig. 4a). Because ERK1/2 and JNK are involved in NF-κB activation [21–23], we examined the activation of ERK1/2 and JNK in epididymal fat by western blot. We found that phosphorylation of ERK1/2 and JNK was decreased in rAd-GLP-1-treated *ob/ob* mice compared with untreated, rAd-βgal-treated and pair-fed *ob/ob* mice (Fig. 4b).

In vitro and in vivo inhibition of lipopolysaccharide-induced IL-6 and TNF-α production by exendin-4 To investigate whether GLP-1 receptor signalling directly inhibits pro-inflammatory cytokine mRNA expression in adipocytes, we pretreated differentiated 3T3-L1 adipocytes with exendin-4, stimulated them with lipopolysaccharide (LPS) and examined the expression of *Il6* and *Tnfa* mRNA. When 3T3-L1 adipocytes were stimulated with LPS alone, the expression of *Il6* mRNA was elevated at 2, 4 and 6 h after stimulation; that of *Tnfa* mRNA was elevated at 2 h after stimulation. However, when adipocytes were pretreated with exendin-4 before LPS stimulation, the expression of the genes encoding these cytokines was significantly reduced (Fig. 5a–b). Examination of IL-6 and TNF-α secretion in the culture medium at 12 h or 24 h after LPS stimulation revealed

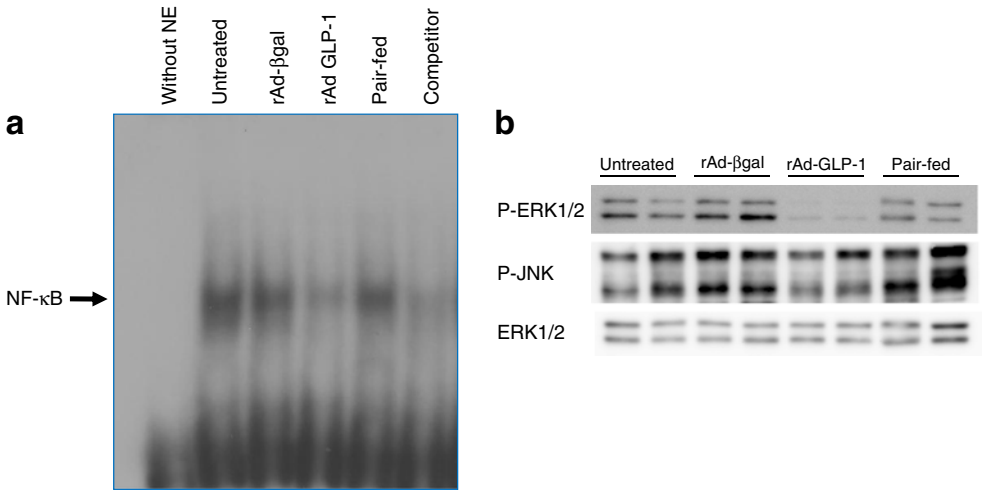

Fig. 4 rAd-GLP-1 treatment reduced NF-κB activation and phosphorylation of ERK1/2 and JNK in epididymal fat tissue. Diabetic *ob/ob* mice were treated with rAd-GLP-1 or rAd-βgal and epididymal fat was removed 2 weeks after treatment. (**a**) Autoradiograph of EMSA performed with ^{32}P-labelled NF-κB binding nucleotides and nuclear protein extracts (NE) of epididymal fat tissues from the indicated groups. Nuclear extracts from untreated mice incubated with an excess of the unlabelled probe (Competitor) served as a control. (**b**) Phospho(P)-ERK1/2 and phospho-JNK, as well as ERK1/2 were measured by immunoblotting of total protein extracts with respective antibodies. Untreated and pair-fed diabetic *ob/ob* mice served as controls. Results are representative of three independent experiments

that exendin-4 treatment inhibited LPS-induced IL-6 (12 h) and TNF-α (24 h) secretion compared with 3T3-L1 adipocyte cells treated with LPS alone (Fig. 5c–d). To confirm the in vitro effect of exendin-4 on the inhibition of LPS-induced inflammatory cytokine expression in vivo, we intraperitoneally injected C57BL/6 male mice with exendin-4 twice per day for 7 days, and then on day 7 injected mice with LPS. At 6 h after the LPS injection, the expression of *Il6* and *Tnfa* mRNA in adipose tissue was significantly suppressed by exendin-4 treatment compared with mice injected with LPS alone (Fig. 5e–f).

Inhibition of LPS-induced NF-κB activation by exendin-4 in 3T3-L1 adipocytes To determine whether the inhibition of LPS-induced *Il6* and *Tnfa* mRNA expression by exendin-4 was due to the inhibition of NF-κB activation, we used western blot to examine the LPS-induced translocation of NF-κB to the nucleus in exendin-4-treated 3T3-L1 adipocytes. We found that LPS-induced NF-κB/p65 nuclear translocation was suppressed by exendin-4 treatment (Fig. 6a). In addition, NF-κB DNA-binding activity was analysed by EMSA. The addition of excess unlabelled NF-κB oligonucleotide abolished NF-κB binding activity. Moreover, the addition of an antibody against the p65 subunit of NF-κB resulted in a supershift, indicating the specificity of the binding activity (Fig. 6b). We found that NF-κB binding activity was inhibited by exendin-4 treatment in 3T3-L1 adipocytes (Fig. 6c). ERK1/2 and phosphatidylinositol 3-kinase (PI3-K)/AKT are involved in LPS-induced NF-κB activation [22–24]. We therefore examined the effect of exendin-4 on LPS-induced activation of ERK1/2 and AKT in adipocytes. We found that LPS induced a notable phosphorylation of ERK1/2 and AKT at 10 min after treatment, and that exendin-4 treatment inhibited the LPS-induced phosphorylation of ERK1/2 and AKT in 3T3-L1 adipocytes (Fig. 6d).

Inhibition of inflammatory cytokine gene expression and NF-κB activation in exendin-4-treated ATM To investigate whether GLP-1 receptor signalling directly inhibits pro-inflammatory cytokine mRNA expression in ATM, we intraperitoneally injected exendin-4 into high-fat diet-induced obese mice, isolated CD11b$^+$ ATM from the epididymal fat 24 h later and examined the expression of *Il6*, *Tnfa* and *Mcp1* mRNA. We found that the mRNA expression of these cytokines was significantly reduced in exendin-4-treated mice (Fig. 7a–c). In addition, we isolated CD11b$^+$ ATM from the epididymal fat of obese mice, treated them with exendin-4 with or without LPS stimulation, and examined the expression of *Il6*, *Tnfa* and *Mcp1* mRNA. The expression of these mRNAs was significantly suppressed by exendin-4 treatment (Fig. 7d–i). Consistent with this result, LPS-induced NF-κB/p65 nuclear translocation was significantly inhibited by exendin-4 treatment (Fig. 7j, k).

Maintenance of a high level of energy expenditure in rAd-GLP-1-treated mice To investigate whether the reduction of fat mass is due to metabolic change induced by the rAd-GLP-1 treatment, we measured food intake, energy expenditure, locomotor activity, oxygen consumption and the respiratory exchange ratio. This was done with a metabolic monitoring system over 72 h, beginning 3 days after rAd-

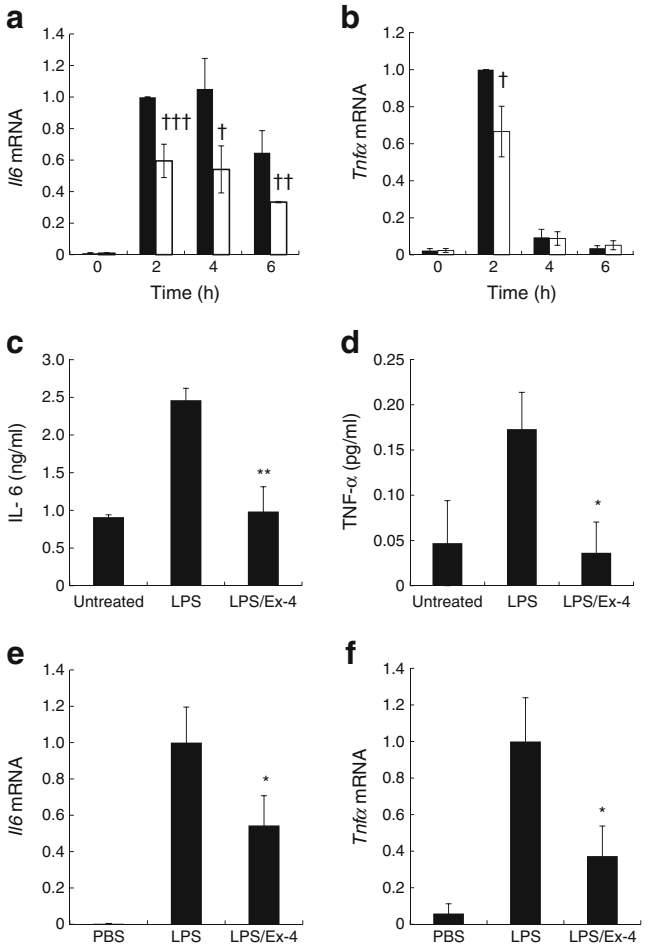

Fig. 5 Exendin-4 suppressed LPS-induced *Il6* and *Tnfa* mRNA expression and protein production in 3T3-L1 adipocytes and C57BL/6 mice. (**a**) 3T3-L1 adipocytes were incubated with (white bars) or without (black bars) 20 nmol/l exendin-4 (Ex-4) for 24 h, followed by treatment with LPS (100 ng/ml) for 0, 2, 4 or 6 h, and the expression of *Il6* and (**b**) *Tnfa* mRNA was analysed by real-time quantitative PCR, with values normalised to cyclophilin expression. The fold change was calculated as ratio of the expression in cells treated with LPS only for 2 h. $^{\dagger}p<0.005$, $^{\dagger\dagger}p<0.0005$ and $^{\dagger\dagger\dagger}p<0.0001$ compared with cells treated with LPS only. (**c**) Cell culture medium was collected at 12 or 24 h after LPS stimulation and the concentration of IL-6 and (**d**) TNF-α was determined by ELISA. (**e**) Male C57BL/6 mice were injected intraperitoneally twice a day for 7 days with exendin-4 (100 ng/mouse). As a control, the same volume of PBS was injected. At day 7, mice were injected with LPS (100 μg/mouse) and epididymal adipose tissue removed after 6 h. The expression of (**e**) *Il6* and (**f**) *Tnfa* mRNA was analysed by real-time quantitative PCR and normalised to cyclophilin expression. The fold change was calculated as ratio of the expression in mice injected with LPS only. (**a–f**) Results are representative of three independent experiments. Data are means ± SD; $n=5$ per group; (**c–f**) $^{*}p<0.05$ and $^{**}p<0.01$ compared with cells treated with LPS only

GLP-1 treatment. As expected, food intake was significantly reduced in rAd-GLP-1-treated mice compared with the rAd-βgal-treated group (Fig. 8a). Energy expenditure (Fig. 8b) and oxygen consumption (Fig. 8c) were not different in rAd-GLP-1-treated mice compared with the rAd-βgal-treated group, but showed a tendency to be higher than in the pair-fed group, in spite of the same energy intake. In addition, locomotor activity was significantly decreased in the rAd-GLP-1-treated group compared with the pair-fed group (Fig. 8d). The respiratory exchange ratio was significantly decreased in rAd-GLP-1-treated *ob/ob* mice and the pair-fed group compared with rAd-βgal-treated *ob/ob* mice (Fig. 8e).

Discussion

Obesity is linked to insulin resistance. One possible mechanism is that adipose tissue is infiltrated with immune cells that promote inflammatory responses, contributing to the pathogenesis of insulin resistance [1]. Blocking inflammatory pathways improves insulin sensitivity in high-fat diet-induced obesity or genetically obese diabetic mice [4, 25–27]. GLP-1 is known to improve insulin sensitivity [10–12], therefore this study investigated whether the suppression of adipose tissue inflammation might be a mechanism by which GLP-1 reduces insulin resistance. As GLP-1 has a short half-life, we used an rAd to constitutively produce GLP-1 in vivo, a strategy that successfully improved glucose levels and insulin sensitivity in a mouse model of type 2 diabetes [10].

Insulin resistance was significantly reduced in rAd-GLP-1-treated *ob/ob* mice compared with rAd-βgal-treated or pair-fed *ob/ob* mice, confirming findings of our earlier study [10]. Although the rAd-GLP-1-treated and the pair-fed group (fed the same amount as that eaten by the rAd-GLP-1 group) had reduced body weight gain, only the rAd-GLP-1 group had a significant reduction in the relative amount of fat, suggesting that GLP-1 specifically reduces fat accumulation. Furthermore, rAd-GLP-1 treatment significantly reduced abdominal fat, but not subcutaneous fat, consistent with previous results showing that abdominal fat deposition is associated with insulin resistance [28, 29].

The size of adipocytes in epididymal fat of rAd-GLP-1-treated *ob/ob* mice was smaller than that in rAd-βgal-treated and pair-fed *ob/ob* mice, suggesting that lipogenesis might be reduced by rAd-GLP-1 treatment. As expected, the expression of lipogenic gene transcripts such as *Srebp1c*, *Acc1* and *Fas* mRNA was significantly decreased in adipose tissue of rAd-GLP-1-treated *ob/ob* mice. However, exendin-4 treatment of 3T3-L1 adipocytes in vitro increased the expression of *Srebp1c* and *Fas* mRNA, and increased triacylglycerol accumulation, similar to previous results where GLP-1 stimulated fatty acid synthesis in explants of rat adipose tissue [30] and lipogenesis in isolated rat adipocytes [31]. Therefore, the decrease of lipogenic gene expression in adipose tissue of rAd-GLP-1-treated mice might be due to indirect effects resulting from other factors, including reduced food intake. As the expression of these genes in the

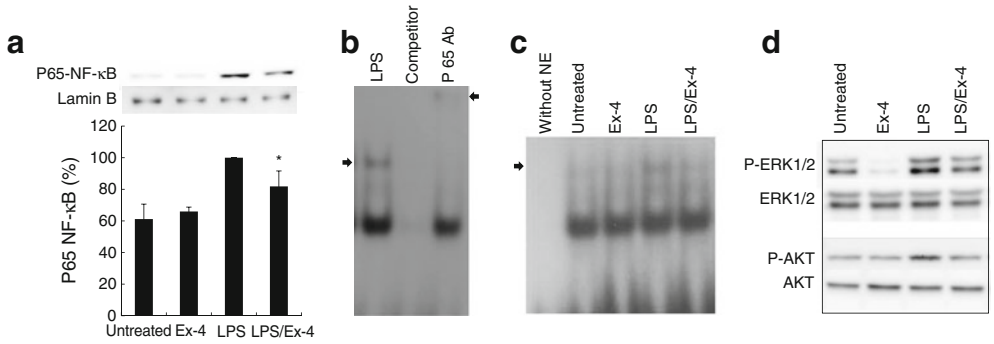

Fig. 6 Exendin-4 reduced LPS-induced NF-κB activation and phosphorylation of ERK1/2 and AKT in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured for 24 h without (untreated) or with 20 nmol/l exendin-4 (Ex-4), followed by treatment with LPS (100 ng/ml) for 1 h and preparation of nuclear extracts. (**a**) Western blot was performed with anti-NF-κB p65 antibody or anti-lamin B protein. The p65 NF-κB band intensity was normalised to lamin B. Data are percentages of p65 NF-κB levels in 3T3-L1 adipocytes treated with LPS only and are presented as mean ± SD; *$p<0.05$ compared with LPS-only treated cells. (**b**) Nuclear extracts from LPS-stimulated 3T3-L1 adipocytes were prepared and probed with ^{32}P-labelled NF-κB after competition with a molar excess of an unlabelled probe (Competitor) or by incubation with an antibody against p65 NF-κB (P65 Ab). Arrow left, p65 NF-κB band; arrow right, supershifted band. (**c**) Autoradiogram of EMSA performed with nuclear extract (NE) of untreated, exendin-4-treated (20 nmol/l), LPS-treated (100 ng/ml, 1 h), and exendin-4- (20 nmol/l) and LPS-treated (100 ng/ml, 1 h) 3T3-L1 adipocytes. Arrow, p65 NF-κB band. (**d**) 3T3-L1 adipocytes were cultured without (untreated) or with exendin-4 (20 nmol/l) for 24 h, followed by treatment with LPS (100 ng/ml) for 10 min. Cell extracts were prepared and analysed by western blot using anti-ERK1/2 and anti-phospho (P)-ERK1/2 antibodies, or anti-AKT and anti-phospho-AKT antibodies. Results are representative of three independent experiments

pair-fed group, which showed reduced body weight gain similar to that of rAd-GLP-1-treated mice, was not significantly different from that in the rAd-βgal-treated group, the reduction of lipogenic gene expression may not be simply due to the reduction of food intake. A recent report demonstrated that the inflammatory cytokine, TNF-α, induced the expression of genes involved in lipid metabolism in HepG2 cells [32]. Thus, the inhibition of inflammatory cytokines may decrease the expression of lipogenic genes and reduce adiposity in rAd-GLP-1-treated mice. In addition, the normalisation of glucose and insulin levels by rAd-GLP-1 treatment [10] might also result in decreased expression of lipogenic genes and reduced adiposity, as glucose and insulin coordinately regulate de novo lipogenesis [33, 34].

Obesity is associated with low-grade systemic inflammation and increased levels of pro-inflammatory cytokines such as IL-6 and TNF-α [2]. These cytokines are produced from adipocytes and ATM, contributing to insulin resistance [35, 36]. Thus, we examined whether rAd-GLP-1-treatment reduces macrophage infiltration and pro-inflammatory cytokine production in adipose tissue. We found that the proportion of F4/80$^+$ macrophages, as well as the mRNA expression and protein levels of IL-6, TNF-α and MCP-1, were significantly decreased in the adipose tissue of rAd-GLP-1-treated mice.

Recently, it has been shown that F4/80$^+$CD11b$^+$CD11c$^+$ triply positive cells, which have macrophage and dendritic cell-like features, are increased in adipose tissue during obesity and produce increased levels of inflammatory cytokines compared with F4/80$^+$CD11b$^+$CD11c$^-$ cells [19]. It has also been reported that the ablation of CD11c$^+$ cells decreases the inflammatory condition and protects against high-fat diet-induced insulin resistance [37]. We found that rAd-GLP-1 treatment of diabetic *ob/ob* mice significantly reduced this triply positive cell population, indicating that the reduced infiltration of triply positive cells contributes to the decrease of inflammatory cytokines by GLP-1.

Classically activated M1 macrophages produce inflammatory mediators, whereas alternatively activated M2 macrophages are generated by exposure to IL-4 and IL-13, and produce anti-inflammatory cytokines [38]. We found that the mRNA expression of M1 marker genes (*F4/80* and *Tlr4*) was significantly decreased by rAd-GLP-1 treatment, whereas the expression of M2 marker genes (*Arg1*, *Mgl1*, *Cd163* and *Mrc1*) was not changed. These results suggest that GLP-1 may affect the production of inflammatory, rather than that of anti-inflammatory, molecules. We also found that exendin-4 inhibited the LPS-induced expression of inflammatory cytokine genes in 3T3-L1 adipocytes and ATM in vitro, and that the expression of *Tnfa*, *Tlr4* and *Mcp1* mRNA was significantly reduced in peritoneal macrophages from rAd-GLP-1-treated *ob/ob* mice compared with rAd-βgal-treated mice. All of these results strongly support the hypothesis that GLP-1 inhibits the inflammatory pathways, possibly contributing to the improvement of insulin sensitivity.

The activation of NF-κB and JNK signalling pathways induces inflammatory cytokine and chemokine gene expression. Indeed, there is evidence that these pathways are linked to insulin resistance in type 2 diabetes. Hyperactivation of the NF-κB pathway has been reported in white adipose tissue from the Zucker diabetic fatty rat [22], an

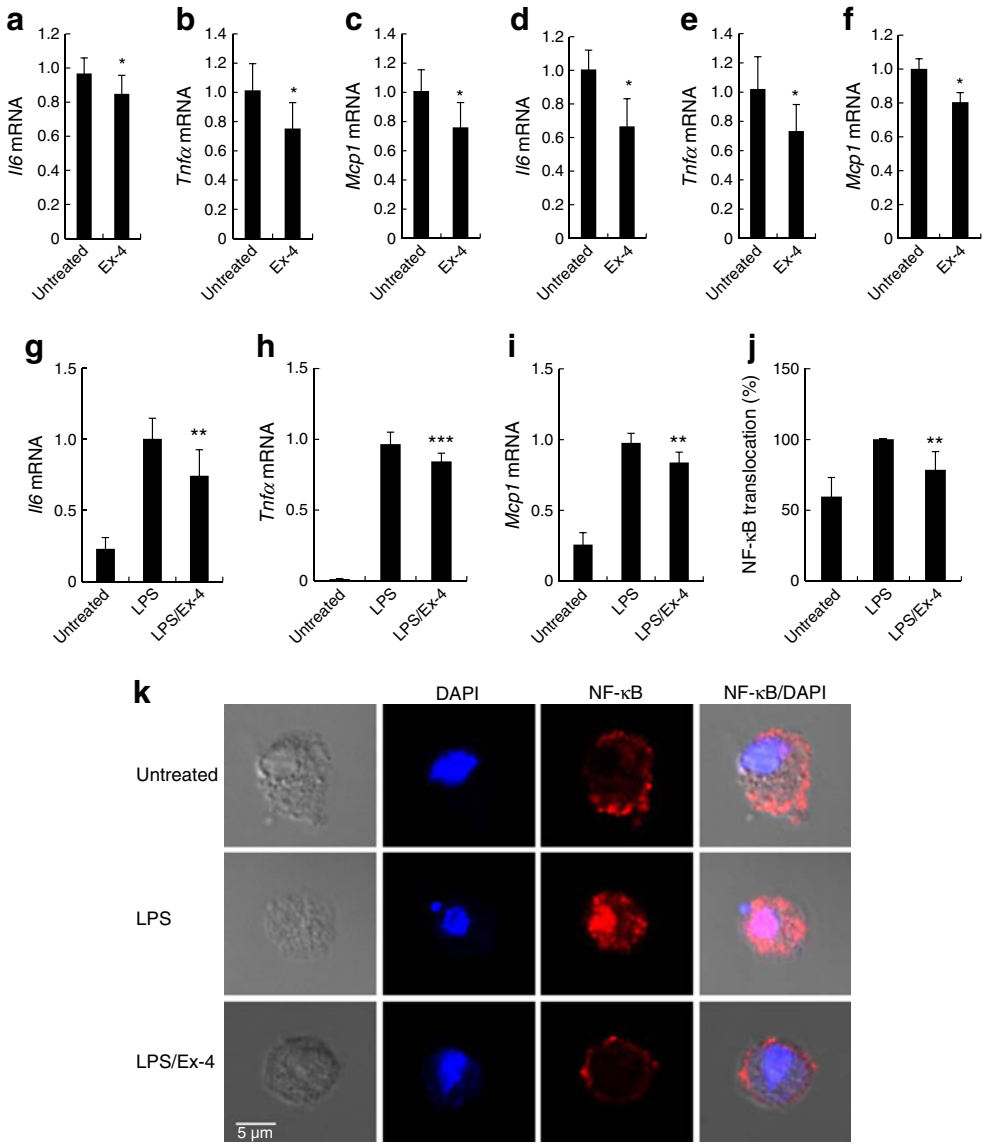

Fig. 7 Decrease of inflammatory cytokine gene expression and NF-κB activation in exendin-4-treated ATM. (**a**) C57BL/6 mice were fed a high-fat diet for 8 weeks and then injected with exendin-4 (Ex-4) (100 ng/mouse, twice at 12 h intervals). After 24 h, ATM were isolated from epididymal fat pads and the expression of *Il6*, (**b**) *Tnfa* and (**c**) *Mcp1* mRNA was measured. (**d**) ATM isolated from epididymal fat pads of male C57BL/6 mice fed a high-fat diet for 8 weeks were cultured for 24 h with or without 0.3 nmol/l exendin-4 and the expression of the pro-inflammatory cytokines (**d**) *Il6*, (**e**) *Tnfa* and (**f**) *Mcp1* was measured. (**g**) ATM were cultured for 24 h and then treated with 0.3 nmol/l exendin-4 for 1 h, followed by treatment with LPS (100 ng/ml) for 1 h, after which the expression of (**g**) *Il6*, (**h**) *Tnfa* and (**i**) *Mcp1* mRNA was analysed by real-time quantitative PCR. (**j**) NF-κB translocation was measured by ArrayScan HCS reader and (**k**) immunohistochemistry. Nuclei were stained with 4′-6-diamidino-2-phenylindole. The fold change (**g–j**) was calculated as ratio of the expression in LPS-only-treated groups. All results are representative of three independent experiments. Data (**a–j**) are means ± SD; *$p<0.05$, **$p<0.005$ and ***$p<0.001$ compared with LPS only-treated groups

animal model of type 2 diabetes. JNK1-deficient and NF-κB-deficient mice are protected from high-fat diet-induced insulin resistance [39, 40], due to the inhibition of inflammatory cytokine production [41, 42]. We found that the activity of NF-κB was markedly reduced in adipose tissue of rAd-GLP-1-treated mice and that the LPS-induced activation of NF-κB was inhibited in 3T3-L1 adipocytes and ATM treated with exendin-4 in vitro. This suggests that the normalisation of abnormally activated inflammatory signalling by GLP-1 contributes to the decreased production of inflammatory cytokines and chemokines. Consistent with our results, it was recently reported that exendin-4 treatment showed anti-inflammatory effects on glomerular endothelial cells and macrophages [43], and that liraglutide, a long-acting GLP-1 analogue, also inhibited NF-κB activation in human umbilical vein endothelial cells [44].

Energy intake in excess of energy expenditure may cause obesity and insulin resistance, while dysregulation of energy

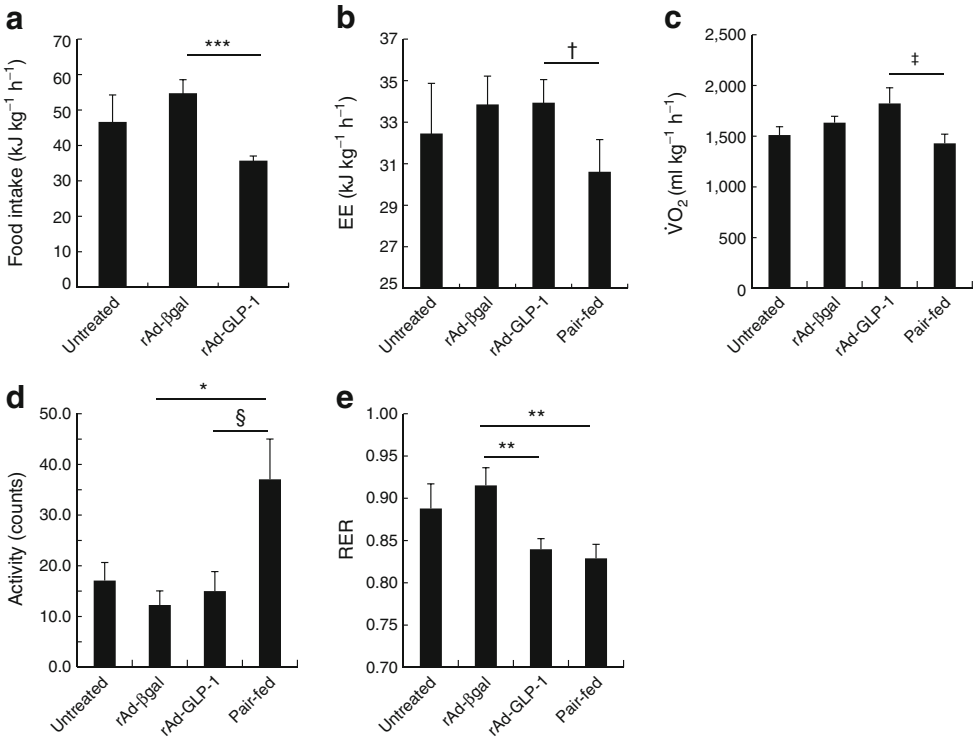

Fig. 8 Maintenance of a high level of energy expenditure in rAd-GLP-1-treated *ob/ob* mice. Diabetic *ob/ob* mice were treated with rAd-GLP-1 or rAd-βgal. (**a**) Food intake, (**b**) energy expenditure (EE), (**c**) $\dot{V}O_2$, (**d**) activity and (**e**) respiratory exchange ratio (RER) were measured by indirect calorimetry analysis for 72 h, beginning at 3 days after rAd-GLP-1 or rAd-βgal treatment. Untreated mice served as controls. Data are means ± SEM; n=7–8 per group; *p<0.05, **p<0.01 and ***p<0.0005 compared with rAd-βgal-treated mice; $^{\S}p$<0.005 compared with the pair-fed group; $^{\dagger}p$=0.103; $^{\ddagger}p$=0.055

balance could affect fat mass and adipocyte size [45]. A reduction of food intake in animals is usually accompanied by a reduction of energy use [46]. However, we did not observe any differences in energy expenditure in rAd-GLP-1-treated mice compared with rAd-βgal-treated mice, despite a significant decrease in food intake in the former. In addition, we observed a tendency for rAd-GLP-1-treated *ob/ob* mice to exhibit higher energy expenditure than the pair-fed group, which had the same amount of food intake as the rAd-GLP-1-treated group. Locomotor activity was significantly increased in the pair-fed group, perhaps as a result of food searching activity. This increased activity and possibly energy expenditure may have affected body weight, adipose tissue mass or IL-6 levels. The respiratory exchange ratio was significantly decreased in the rAd-GLP-1-treated and pair-fed groups compared with the rAd-βgal-treated group, suggesting that these groups used more fat than carbohydrate. We also found that fat oxidation was increased in exendin-4-treated C2C12 cells compared with untreated cells (unpublished data; Y.-S. Lee, J.-S. Choung, H.-S. Jun). These results suggest that increases in fat oxidation and energy expenditure may partly contribute to the reduction of fat mass in rAd-GLP-1-treated mice.

In conclusion, our studies show that GLP-1 attenuates macrophage infiltration and production of inflammatory cytokines in the adipose tissue of *ob/ob* mice. GLP-1 directly inhibits inflammatory signalling pathways, such as NF-κB, in adipocytes and ATM, resulting in a decrease of inflammatory cytokine levels in adipose tissue. In addition, GLP-1 might increase fat oxidation and energy expenditure, which would contribute to a reduction of fat mass with subsequent improvement of insulin sensitivity. All these actions of GLP-1 may therefore have a beneficial effect on insulin sensitivity in rAd-GLP-1-treated *ob/ob* mice.

Acknowledgements We thank A. Kyle (University of Calgary) for editorial assistance, M. Mamura and Y.-J. Kang (Lee Gil Ya Cancer and Diabetes Institute) for FACS analysis, and the Center of Animal Care and Use (Lee Gil Ya Cancer and Diabetes Institute) for provision of animal care.

Funding This work was supported by a grant from the Ministry of Korea Science and Engineering Foundation (20090071938), a grant from National Research Foundation of Korea (NRF: no. 2009-0079342) and a grant from the Innovative Research Institute for Cell Therapy (A062260).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement YSL contributed to the conception and design of the study, researched data and wrote the manuscript. MSP, JSC, SSK, HHO and SYH researched data and revised the manuscript. CSC, Y Kang and Y Kim participated in the analysis and interpretation of data, and in the revision of the manuscript. HSJ contributed to conception and design of the study, and wrote, reviewed and edited the manuscript. All authors approved the version to be published.

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