# Anti-inflammatory effects of exendin-4, a glucagon-like peptide-1 analog, on human peripheral lymphocytes in patients with type 2 diabetes

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**ABSTRACT**

**Aims/Introduction:** Type 2 diabetes is characterized by dysregulation of immunity, oxidative stress and reduced incretin effects. Experimental studies suggest that glucagon-like peptide (GLP-1) might have immunomodulating effects. We hypothesize that GLP-1 receptor agonist, exendin-4, might reduce inflammatory response in type 2 diabetes.

**Materials and Methods:** Using peripheral blood mononuclear cells (PBMC) sampled from 10 type 2 diabetes and 10 sex- and age-matched control subjects and supernatants from PBMC culture, the expression of phospho-mitogen activated protein kinase (MAPK) signaling pathways in CD4+ T helper lymphocytes and monocytes was analyzed using flow cytometry. Cytokines/chemokines and superoxide anion before and after treatment with exendin-4 were measured by cytometric bead array and chemiluminesence assay, respectively.

**Results:** Compared with control subjects, PBMC from type 2 diabetes patients showed activated MAPK (P38, c-Jun NH2-terminal protein kinase and extracellular signal-regulated kinase) signaling pathway, elevated superoxide anion, increased pro-inflammatory cytokines (tumor necrosis factor-$\alpha$, interleukin-1$\beta$, interleukin-6) and chemokines (CCL5/regulated on activation normal T-cell expressed and secreted and CXCL10/interferon-$\gamma$-induced protein 10). These changes were attenuated by exendin-4, possibly through the suppression of p38 MAPK.

**Conclusions:** These results suggest that exendin-4 might downregulate pro-inflammatory responses and reduce oxidative stress by suppressing MAPK signaling pathways in type 2 diabetes. (J Diabetes Invest, doi: 10.1111/jdi.12063, 2013)

**KEY WORDS:** Diabetes, Exendin-4, Inflammation

## INTRODUCTION

Type 2 diabetes is characterized by decreased $\beta$-cell mass and impaired insulin secretion. Several mechanisms, such as endoplasmic reticulum (ER) stress, oxidative stress, glucotoxicity, lipotoxicity and amyloid deposition have been implicated[1–3]. Intriguingly, all these factors might induce an inflammatory response that contributes to $\beta$-cell dysfunction and death. In patients with type 2 diabetes, insulitis characterized by islet infiltration with immune cells and increased release of cytokines/chemokines has been reported[4,5]. In the same vein, antagonists of pro-inflammatory cytokines, such as interleukin (IL)-1$\beta$ antagonists, have been shown to improve glycemia, preserve $\beta$-cell function and prevent diabetes development[6–8].

Inflammation is associated with the cellular activation of mitogen-activated protein kinase (MAPK) signaling pathways through serine or threonine phosphorylation of transcription factors, which bind to promoter regions of a gene network to mediate immune responses, resulting in pathophysiological changes and clinical consequences[9]. In mammalian cells, p42/p44 extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK) and p38 MAPK are the three main kinases that can be activated by pro-inflammatory cytokines (such as tumor necrosis factor (TNF)-$\alpha$, IL-1$\beta$ and IL-6), mitogens and physical stress[9]. Previous studies showed abnormal ERK and p38 MAPK activities in monocytes of patients with type 2 diabetes[10]. In experimental models, specific inhibition of MAPK pathways can suppress pro-inflammatory responses with delayed onset of diabetes[11]. Importantly, cytokines, such as TNF-$\alpha$ and IL-1$\beta$, can activate membrane-bound nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase to

generate superoxide in endothelial cells (ECs). Inhibitor of NADPH oxidase has been shown to abrogate superoxide production in response to TNF-α in ECs. This might imply that inflammation can induce oxidative stress, which can contribute to and perpetuate the pathological process of type 2 diabetes[12].

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted by enteroendocrine L cells in the gut. Apart from promoting β-cell proliferation and inhibiting apoptosis, stimulating insulin secretion and reducing blood glucose[13], GLP-1 might possess extraglycemic activity including anti-inflammatory effects[14,15]. In *ex vivo* studies, exendin-4, a GLP-1 analog, has been shown to improve β-cell function by reducing pro-inflammatory cytokines (interferon-γ, IL-17, IL-1β and IL-2) and caspase-3 activation in human islets[16]. Inhibitors of dipeptidyl peptidase-IV, known to increase circulating levels of GLP-1, reversed new onset diabetes in non-obese diabetic (NOD) mice by reducing insulitis, modulating inflammation and improving islet function[14].

Despite these experimental findings, it remains uncertain whether exendin-4 shows any modulating effects in the human immune system. Herein, we investigated the effects of exendin-4 on inflammatory responses and oxidative stress in human lymphocytes and monocytes in peripheral blood mononuclear cells of type 2 diabetes patients.

## METHODS
### Blood Samples from Patients with Type 2 diabetes and Control Subjects
A total of 10 Chinese patients with type 2 diabetes were recruited from the Department of Pediatrics and the Diabetes and Endocrine Center of the Prince of Wales Hospital, Hong Kong. Type 2 diabetes was diagnosed according to the 1985 World Health Organization criteria using diagnostic values of fasting plasma glucose (PG) ≥ 7.0 mmol/L and/or 2-h (or random) PG ≥ 11.1 mmol/L with or without 75-g oral glucose tolerance test, depending on the presence or absence of symptoms. All patients were non-smokers and free from infection for 4 weeks preceding the study. Bodyweight and body height were measured for determination of body mass index (BMI). A total of 10 sex- and age-matched healthy Chinese volunteers were recruited as healthy controls (CTL). From each subject, 20 mL of venous peripheral ethylenediamine tetra-acetic acid (EDTA) blood was obtained, followed by immediate fractionation of peripheral blood mononuclear cells (PBMC) for *ex vivo* study. In brief, PBMCs were prepared by centrifuging 20 mL EDTA venous blood using a Ficoll–Paque plus density gradient (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). Plasma and serum were stored in 300-μL aliquots at −70°C until analysis. The aforementioned protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong-New Territories East Cluster Hospitals. Informed consent was obtained from all participants according to the Declaration of Helsinki.

### Flow Cytometric Analysis of Intracellular Activated (Phosphorylated) MAPK Signaling Molecules
Flow cytometric analysis was applied to determine the activation of MAPK signaling pathways indicated by phospho-ERK, phospho-p38 MAPK and phospho-JNK in CD4+ T helper lymphocytes and monocytes in PBMC from patients and controls. Briefly, PBMC (viability > 95%) were prepared by Ficoll–Paque plus density gradient centrifugation. The PBMC culture was incubated with or without exendin-4 (Sigma–Aldrich, St. Louis, MO, USA) at 50 nmol/L for 10 mins at 37°C in a 5% $CO_2$ atmosphere. PBMC was then fixed/permeabilized by Cytofix/Cytoperm™ buffer (BD Biosciences, Mississauga, ON, Canada) at room temperature for 15 min. Cells were then washed with Perm/Wash™ buffer twice and resuspended in BD Pharmingen™ stain buffer (BD Pharmingen Corp, San Diego, CA, USA) at $1 \times 10^7$ cells/mL. PE-conjugated anti-human phospho-ERK, phospho-p38 MAPK, phospho-JNK antibody or mouse immunoglobulin G (IgG) isotypic antibody (BD Pharmingen) was added to each tube and incubated at room temperature for 45 min in the dark. Cells were then washed and resuspended with stain buffer (BD Pharmingen) for flow cytometric analysis using a BD FACSCalibur flow cytometer (BD Biosciences Corp, San Diego, CA, USA). During the flow cytometric analysis of intracellular MAPK, PE-conjugated antibodies against CD4 and CD14 cell surface markers were used for gating of CD4+ T helper lymphocyte (CD4+) and monocytes (CD14+) in PBMC. Mouse IgG isotypic antibodies were used to normalize the background signal. Results were expressed as mean fluorescence intensity (MFI) for the expression of intracellular phospho-p38 MAPK, phospho-ERK and phospho-JNK in 10,000 cells using BD CellQuest™ software (BD FACSCalibur; BD Biosciences Corp).

### Measurement of Clinical Biomarkers
Blood samples were collected by venepuncture after an overnight fast. Serum was centrifuged (1500 *g* for 15 min at 4°C) and stored at 4°C until measurement. Biochemical markers, such as insulin, plasma glucose and glycated hemoglobin (HbA$_{1c}$), were measured at the Department of Chemical Pathology of the Prince of Wales Hospital, The Chinese University of Hong Kong, with an externally accredited quality assurance program.

### Measurement of Concentrations of Cytokines, Chemokines, Insulin, C-peptide and Autoantibodies (Autoantibodies to Glutamic Acid Decarboxylase and Protein Tyrosine Phosphatase)
The PBMC culture was incubated with or without exendin-4 (50 nmol/L) for 24 h at 37°C in a 5% $CO_2$ atmosphere. The cell-free supernatant was harvested and stored at −80°C for subsequent assays. The supernatant concentration of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-10 and IL-12p70, and chemokines CXCL8/IL-8, CCL5/regulated on activation normal T-cell expressed and secreted (RANTES), CCL2/

**Table 1** | Demographic and clinical characteristics of patients with type 2 diabetes and control subjects

| Variables | Type 2 diabetes | Controls |
|---|---|---|
| *n* | 10 | 10 |
| Sex (male/female) | 6/4 | 8/2 |
| Age (years) | 32 (27–33) | 26.5 (25–31.5) |
| Duration of disease (years) | 1 (1–2) | NA |
| Fasting plasma glucose (mmol/L) | 8.6 (5.9–12.1) | 4.9 (4.5–5.2) |
| Body mass index (kg/m$^2$) | 28 (26.7–33.2) | 21.1 (19.3–22.6)* |
| Fasting serum C-peptide (pmol/L) | 980.2 (470.5–1561.4) | 232 (170.9–275.7)* |
| Anti-GAD | Negative | Negative |
| Anti-IA2 | Negative | Negative |
| HOMA-IR | 29.2 (20.1–42.6) | 7.9 (5.8–13.5)* |
| HOMA-β% | 193.8 (123.7–505.8) | 416.4 (340.7–664.6) |
| Treatment with metformin | | |
|   Patients, *n* (%) | 8 (80%) | NA |
|   Daily dose (mg) | 2100 (1700–3000) | NA |
| Treatment with simvastatin | | |
|   Patients, *n* (%) | 3 (30%) | NA |
|   Daily dose (mg) | 26.7 (20–40) | NA |
| Treatment with gliclazide | | |
|   Patients, *n* (%) | 2 (20%) | NA |
|   Daily dose (mg) | 125 (90–160) | NA |
| Treatment with lisinopril | | |
|   Patients, *n* (%) | 2 (20%) | NA |
|   Daily dose (mg) | 12.5 (12.5–12.5) | NA |
| Cytokines/chemokines | | |
|   TNF-α (pg/mL) | 56 (7–236) | 22 (8–58)* |
|   IL-1β (pg/mlL) | 111 (6–184) | 35 (22–61)* |
|   IL-6 (pg/mL) | 114 (8–205) | 55 (20–105)* |
|   RANTES (pg/mL) | 330 (53–694) | 32 (15–98)* |
|   CXCL10 (pg/mL) | 76 (10–156) | 24 (16–38)* |

*$P < 0.05$. CXL10, interferon-γ-induced protein 10; GAD, glutamic acid decarboxylase; HOMA-β, homeostatic model assessment of β-cell finction; HOMA-IR, homeostatic model assessment of insulin resistance; IA2, protein tyrosine phosphatase; IL, interleukin; NA, not applicable, RANTES, regulated on activation normal T-cell expressed and secreted; TNF, tumor necrosis factor.

monocyte chemoattractant protein-1 (MCP-1), CXCL10/interferon-γ-induced protein 10 (IP-10) and CXCL9/monokine induced by interferon-γ were measured using the inflammatory cytokine and chemokine cytometric bead array (CBA) reagent kits (BD Pharmingen), respectively. Samples were analyzed using a multifluorescence BD flow cytometer (FACSCalibur™) with BD CellQuest™ software and BD™ CBA software. The coefficients of variation for all cytokine and chemokine assays were less than 10%. Serum insulin/C-peptide concentrations were measured by enzyme-linked immunosorbent assay (ELISA; Mercodia AB, Sylveniusgatan, Sweden). The status of diabetes was further confirmed by enzyme immunoassay for the determination of autoantibodies to glutamic acid decarboxylase (anti-GAD) and protein tyrosine phosphatase (anti-IA2) in serum (Medizym, Dahlewitz/Berlin, Germany).

### Quantification of Superoxide Anion by Chemiluminescent Assay

The superoxide anion oxidizes luminol in a reaction that produces photons of light that are readily measured with a standard luminometer. The superoxide anion detection kit (Calbiochem Corp., San Diego, CA, USA) utilizes an enhancer that increases the sensitivity of the assay by amplifying the chemiluminescence. Cells ($5 \times 10^5$) were suspended in 100 uL luminol-enhancer assay medium and incubated for 30 mins at room temperature. $O_2^-$ oxidized luminol in a reaction that produced photons of light that were measured with a luminometer (VICTOR$^3$ multi-label plate reader; Perkin Elmer, Waltham, MA, USA).

### Statistical Analysis

All analyses were carried out using SPSS for Windows, version 16.0, (SPSS Inc., Chicago, IL, USA). Between-group comparison (basal and *ex vivo* culture supernatant concentrations) was made by Mann–Whitney *U*-test, whereas within-group compar-
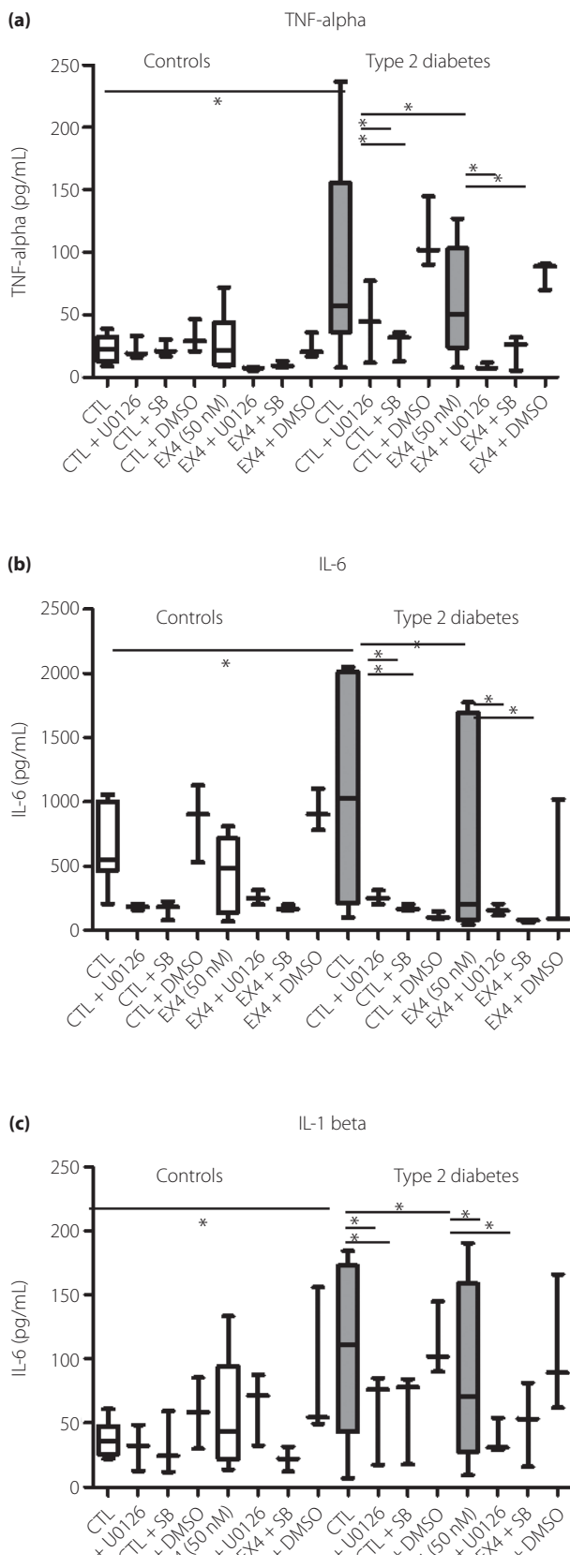
**(a)**

TNF-alpha

Controls          Type 2 diabetes

TNF-alpha (pg/mL)

*x*-axis labels: CTL, CTL + U0126, CTL + SB, CTL + DMSO, EX4 (50 nM), EX4 + U0126, EX4 + SB, EX4 + DMSO, CTL, CTL + U0126, CTL + SB, CTL + DMSO, EX4 (50 nM), EX4 + U0126, EX4 + SB, EX4 + DMSO*

**(b)**

IL-6

Controls          Type 2 diabetes

IL-6 (pg/mL)

**(c)**

IL-1 beta

Controls          Type 2 diabetes

IL-6 (pg/mL)

**Figure 1** | Effects of exendin-4 on the *ex vivo* production of pro-inflammatory cytokines of controls (CTL) and patients with type 2 diabetes. Peripheral blood mononuclear cells from controls and type 2 diabetes patients were pretreated with U0126 (U0126 10 μmol/L), SB203580 (SB 10 μmol/L) for 1 h followed by incubation with or without exendin-4 (Ex4; 50 nmol/L) for 24 h. Release of (a) tumor necrosis factor (TNF)-α, (b) interleukin (IL)-1β and (c) IL-6 were determined by cytometric bead array using flow cytometry. Dimethyl sulfoxide (DMSO) (0.1%) was used as the vehicle control. Results are presented with box-and-whisker plots. *$P < 0.05$.

ison (before and after exendin-4 treatment) was made by Wilcoxon signed-rank test. A *P*-value less than 0.05 (two-tailed) was considered significant.

## RESULTS

### Clinical Profile of Type 2 Diabetes Patients and Controls
None of the patients with type 2 diabetes were treated with insulin, and all had disease duration less than 5 years without clinical complications. Compared with age- and sex-matched controls, type 2 diabetes patients had significantly higher fasting plasma glucose, BMI, insulin and C peptide levels than control subjects (Table 1).

### Exendin-4 Suppressed TNF-α, IL-1β, IL-6 Expression from PBMC in Type 2 Diabetes Patients
Previous studies reported elevated serum levels of inflammatory cytokines including TNF-α, IL-6, IL-1β and IL-6 in newly diagnosed type 2 diabetes patients[17–19]. In this experiment, after 24 h of incubation with PBMC culture, we observed significantly higher levels of inflammatory cytokines (TNF-α, IL-6 and IL-1β) in PBMC supernatants from patients with type 2 diabetes than controls (Figure 1). Treatment with exendin-4 (50 nmol/L), U0126 (specific inhibitor of ERK signaling pathway) and SB 203580 (specific inhibitor of p38 MAPK signaling pathway) for 24 h significantly reduced cytokine levels in PBMC supernatants from patients with type 2 diabetes (all $P < 0.05$), but not in control subjects (all $P > 0.05$).

### Exendin-4 Suppressed CXCL10 and CXCL5 Production from PBMC in Type 2 Diabetes Patients
These findings together with the anti-inflammatory effects of GLP-1 prompted us to further investigate the immunoregulatory role of exendin-4 on production of chemokines from PBMC in type 2 diabetes patients. The normal physiological plasma concentration of GLP-1 is approximately 10 pg/mL, but the local concentration of GLP-1 can be 100–1000 fold higher than that of the circulating level[20]. In this *ex vivo* experiment, the exendin-4, used as the GLP-1 mimic, is expected to produce a concentration similar to that in the local tissues. Here in, chemokines recruit activated immune cells to sites of inflammation and are important mediators of insulitis. A suppressor of cytokine-induced chemokine and Fas expression has been shown to
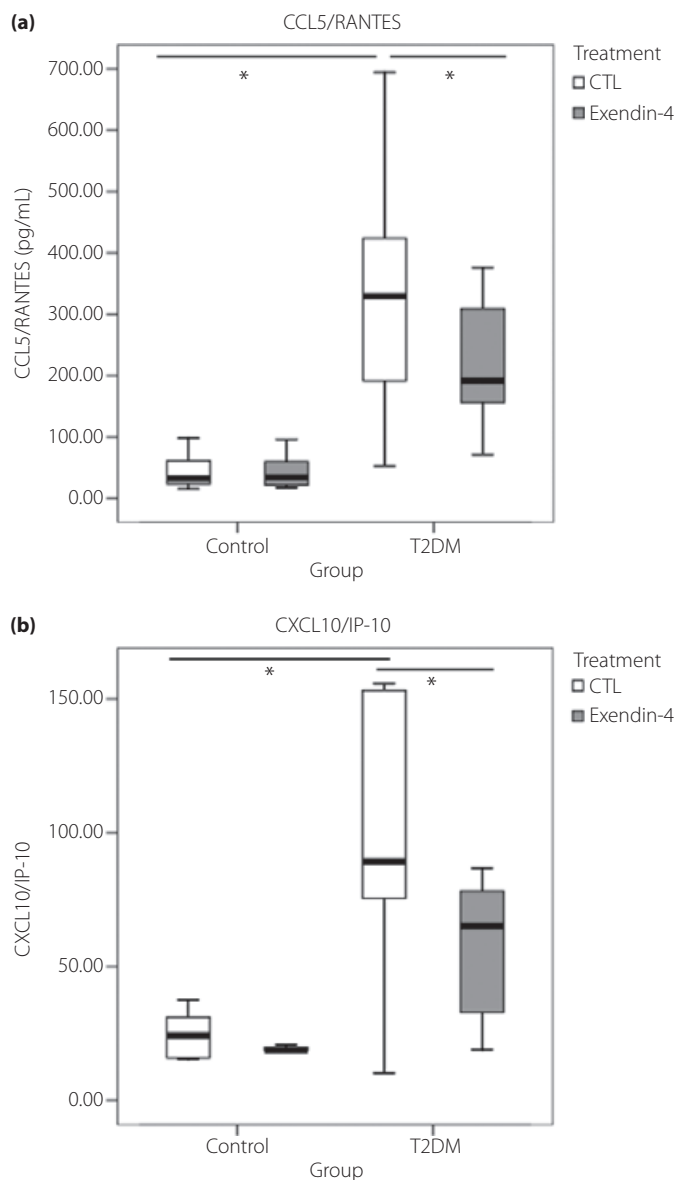
**(a)** CCL5/RANTES

**(b)** CXCL10/IP-10

**Figure 2** | (a) CCL5/regulated on activation normal T-cell expressed and secreted (RANTES) and (b) CXCL10/interferon-γ-induced protein 10 (IP-10) levels in culture supernatants of peripheral blood mononuclear cell from patients with type 2 diabetes (T2DM) and normal controls (CTL) with or without treatment of exendin-4 (50 nmol/L) for 24 h at 37°C. Results are presented with box-and-whisker plots. *$P < 0.05$.

inhibit apoptosis-mediated β-cell death[21]. As shown in Figure 2, exendin-4 significantly suppressed the release of CCL5/RANTES and CXCL10/IP-10 from PBMC in the type 2 diabetes group compared with the control group (all $P < 0.05$).

### Effects of Exendin-4 on the Activation of Intracellular ERK and p38 MAPK but not JNK in CD4+ T Helper Lymphocytes and Monocytes

To characterize the intracellular mechanism for the release of cytokines and chemokines from PBMC, we investigated the

phosphorylation of intracellular ERK and p38 MAPK in CD4+ T lymphocytes and monocytes. As shown in Figure 3, the representative histograms of flow cytometric analysis illustrated increased levels of phospho-ERK, phospho-p38, and phospho-JNK in CD4+ T lymphocytes and monocytes in type 2 diabetes patients compared with control subjects. Figure 4 showed higher phosphorylation of ERK and p38 at baseline in type 2 diabetes patients than control subjects (all $P < 0.05$). Exendin-4 suppressed phosphorylation of ERK and p38 MAPK (all $P < 0.05$) in CD4+ T lymphocytes and monocytes from type 2 diabetes patients ($P > 0.05$).

### Exendin-4 Suppressed Oxidative Stress by Decreasing Superoxide Anion Production

Using the chemiluminescence assay, we detected higher levels of superoxide ions in supernatant of PBMC cultures from type 2 diabetes patients than control subjects, suggestive of oxidative stress, which could be inhibited with 24-h exendin-4 treatment, and U0126 (specific inhibitor of ERK signaling pathway) and SB 203580 (specific inhibitor of p38 MAPK signaling pathway; Figure 5).

### DISCUSSION

In the present study, for the first time we have confirmed the activation of monocytes and CD4+ T cells with increased activation of MAPK signaling molecules including ERK, JNK and p38, as well as elevated production of pro-inflammatory cytokines, chemokines and superoxide anions in PBMC from patients with type 2 diabetes compared with healthy subjects. Interestingly, most of these immunological changes were reversed or attenuated by *ex vivo* treatment with exendin-4. These findings lend support to the dysregulation of immunity in type 2 diabetes, which might be ameliorated by direct immunomodulating effects of exendin-4 treatment.

There is now emerging evidence showing the importance of immune dysregulation in type 2 diabetes[17]. Chemokines belong to a family of more than 50 proteins that play key roles in recruitment of leukocytes at the site of inflammation[22]. In diabetes, CD4+ T lymphocytes, once recruited by chemokines such as CCL5/RANTES to the islets, will differentiate into T helper1 (Th1) to secret Th1 and pro-inflammatory cytokines that activate other immune cells to cause islet inflammation and β-cell disorder[22]. In mammalian cells, the MAPK molecules are serine and threonine protein kinases, which can be activated by pro-inflammatory cytokines to regulate a wide range of cellular responses[23,24]. Although ERK signaling is involved in cell proliferation, differentiation, transformation and cytokine production[25,26], activation of p38 MAPK in immune cells can lead to apoptosis, stress responses and inflammation[27]. Phosphorylation of p38 MAPK also participates in the production of inflammatory cytokines and chemokines, including CCL2, IL-1β, IL-6 and TNF-α, from human mesangial cells[23], endothelial cells[24], memory T cells[27] and eosinophils[28,29].
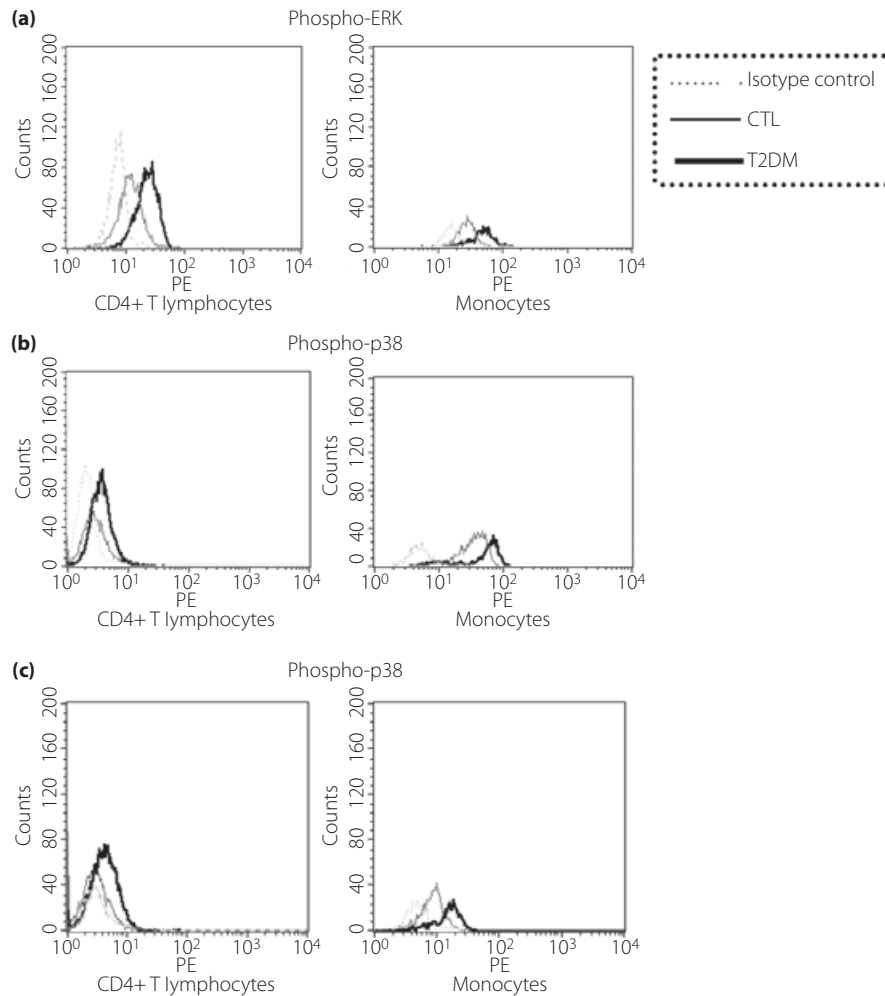
**Figure 3** | Representative histograms of flow cytometry analysis of phospho-mitogen-activated protein kinases including (a) phospho-extracellular signal-regulated kinase (ERK), (b) phospho-p38, and (c) phospho-c-Jun NH2-terminal protein kinase (JNK) in CD4+ T lymphocytes and monocytes from peripheral blood mononuclear cells in patients with type 2 diabetes (T2DM) and the control group (CTL). Triplicate experiments were carried out with essentially identical results and representative figures are shown. Immunoglobulin G1 isotypic control antibodies, which have no specificity for target cells within a particular experiment yet retain all the non-specific characteristics of the antibodies used in the experiment. Inclusion of this antibody is to confirm the specificity of primary antibody binding and exclude non-specific fragment crystallizable receptor binding to cells or other cellular protein interactions.

Hitherto, it remains uncertain whether MAPK pathways are implicated in the production of pro-inflammatory cytokines/chemokines from human PBMC in type 2 diabetes. In the present study, we were able to confirm the mechanisms of activation of MAPK signaling in both monocytes and CD4+ T helper lymphocytes in type 2 diabetes.

Type 2 diabetes is characterized by reduced incretin effect and GLP-1 hyposecretion[30]. However, it is now widely accepted that GLP-1 secretion does not differ between patients with type 2 diabetes and normal controls[31]. For the first time, using human PBMC, we have shown that exendin-4 treatment suppressed the secretion of IL-1β, TNF-α and IL-6, possibly through the suppression of phosphorylation of p38 MAPK and ERK. Apart from augmenting insulin release during meal time,

GLP-1/exendin-4 exerts extrapancreatic effects including suppressing inflammatory cytokines and chemokines release from human islets and β-cell lines[15,32]. In support of this notion, exendin-4 has been shown to prevent encephalomyocarditis virus-induced β-cell destruction by reducing inflammatory immune response of macrophages[15].

In type 2 diabetes, exendin-4 has been consistently shown to reduce serum inflammatory markers, such as high sensitivity C-reactive protein, and cytokines such as CCL2/MCP-1, compared with the placebo treatment[33]. In obese mice, treatment with dipeptidyl peptidase-4 inhibitor, sitagliptin, reduced local inflammation in adipose tissue and pancreatic islets[34]. In the same vein, in the colon of mice with T-cell-induced inflammatory bowel disease resembling Crohn's disease and ulcerative
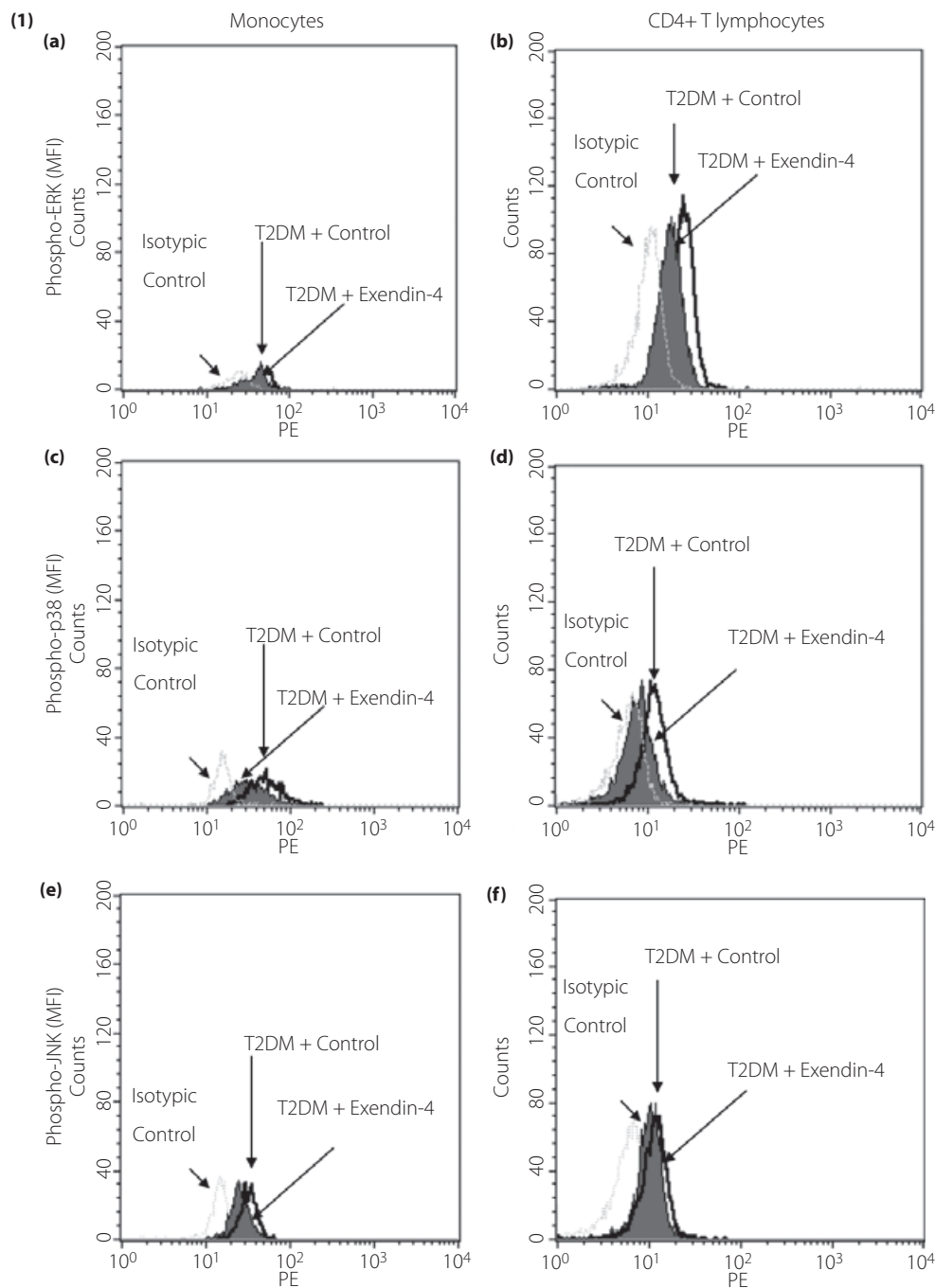
**Figure 4** | Basal and *ex vivo* expression of phospho-mitogen-activated protein kinases (MAPK) in exendin-4 treated CD4+ T lymphocytes and monocytes from peripheral blood mononuclear cells in patients with type 2 diabetes (T2DM) and healthy subjects. (4-1) Representative histograms show the intracellular expression of (a,b) phospho-extracellular signal-regulated kinase (ERK), (c,d) phosphor-p38 and (e,f) phosphor-c-Jun NH2-terminal protein kinase (JNK) in peripheral blood mononuclear cells incubated without or with exendin-4 (50 nmol/L) for 10 min. (4-2) Results are expressed in bar charts. (a) Phosphorylation of ERK, (b), phosphorylation of p38 MAPK and (c) phosphorylation of JNK in monocytes and CD4+ T lymphocytes from patients with type2 diabetes and control subjects were analyzed by flow cytometry. Results are presented with box-and-whisker plots.

colitis, there was marked reduction in proglucagon-derived intestinal peptides including GLP-1, GLP-2 and glicentin[35]. Apart from the detection of messenger ribonucleic acid transcripts of

GLP-1 receptor in ribonucleic acid isolated from murine spleen, thymus and lymph nodes[36], other researchers have reported the expression of GLP-1 receptor on natural killer T (NKT) cells
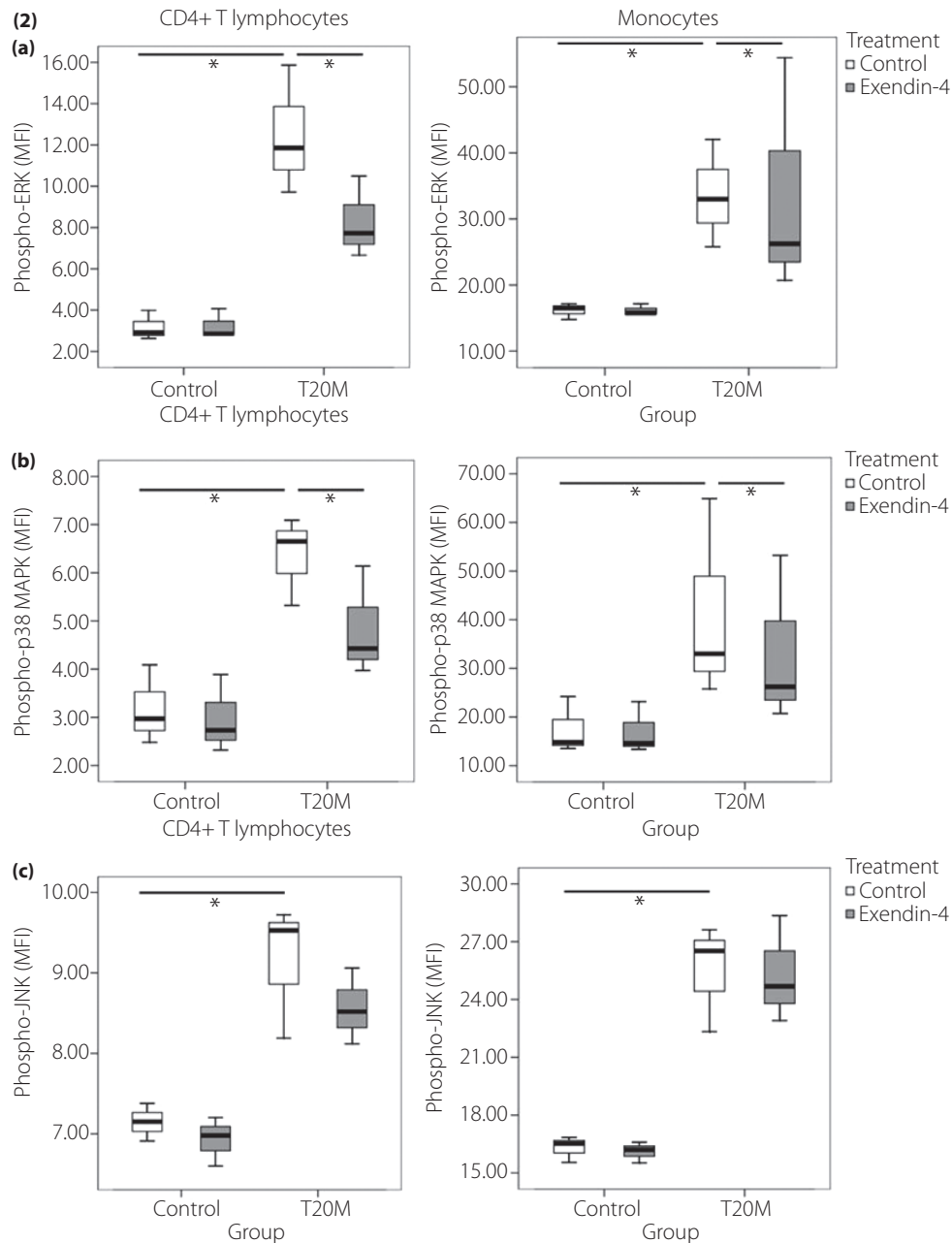
**Figure 4** | *(Continued)*

with GLP-1, inducing a dose-dependent inhibition of cytokine secretion from NKT cells in a case report of two patients with type 2 diabetes and psoriasis[37]. In streptozotocin-induced rats, GLP-1 receptor agonist protected against renal damage and polyneuropathy through its anti-inflammatory action[38].

Given the circulating and ubiquitous nature of monocytes, our results suggested that the anti-inflammatory effects of exendin-4 might be mediated partly through its direct effects on immune cells. In experimental studies, treatment with exendin-4 reduced monocyte/macrophage accumulation in the arterial wall by inhibiting the inflammatory response in macrophages[39]. In the present study, we confirmed the signaling pathways associated with immune activation of PBMC. In this regard, our group also showed that exendin-4 protected human islet amyloid polypeptide-induced apoptosis of rat β insulinoma cell line, INS1-E, through similar pathways although p-JNK was not affected[40]. Taken together, the immunomodulating effects of exendin-4 on multiple immune effectors were in part mediated by its inhibition on the activation of p38 MAPK, cytokines and chemokine release.
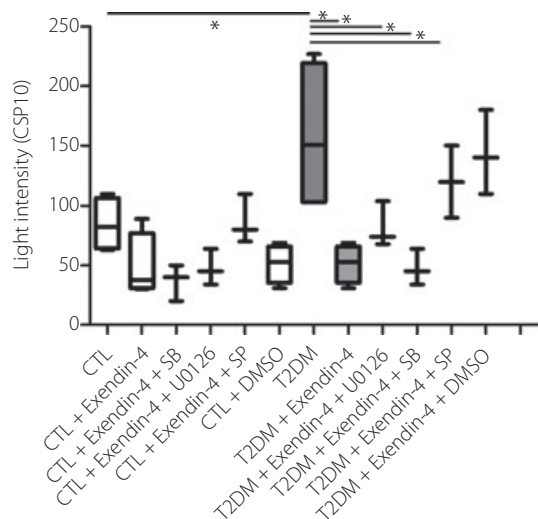
**Figure 5** | Effect of exendin-4 on the *ex vivo* production of superoxide anion in culture supernatant of peripheral blood mononuclear cells (PBMC) from type 2 diabetes patients and control subjects. PBMC culture was incubated with or without exendin-4 at 50 nmol/L for 24 h, with or without pretreatment with U0126 (U0126 10 μmol/L), SP600125 (SP 10 μmol/K) and SB203580 (SB 10 μmol/L) for 1 h. Culture supernatants were then harvested for the assay of superoxides. Results are presented with box-and-whisker plots. *$P < 0.05$. CTL, control; DMSO, dimethyl sulfoxide; T2DM, type 2 diabetes.

Activation of cytokine release can lead to increased oxidative stress as a result of membrane-bound NADPH oxidase activation[12]. In pancreatic islets, activation of immune cells and cytokine release increased oxidative stress with the loss of β-cells, which was prevented by anti-oxidant compounds[41]. With the onset of hyperglycemia, which promotes oxidative stress[42], a vicious cycle of inflammation, oxidative stress, β-cell loss and hyperglycemia can be set up. In support of this notion[43], in the present *ex vivo* study, we confirmed increased production of superoxide ions in PBMC from patients with type 2 diabetes, which could be attenuated by the treatment with exendin-4.

Several studies have reported the ameliorating effects of exendin-4 on diabetic vascular complications, such as nephropathy, by decreasing oxidative stress[44,45]. In primary neuronal cultures and human SH-SY5Y cells, exendin-4 reduced amyloid β toxicity and oxidative stress[46]. Interestingly, superoxide dismutase activity was enhanced in recombinant human GLP-1-pretreated streptozotocin-induced diabetic mice with improved hyperglycemia[47]. In the present experiment, exendin-4 treatment inhibited superoxide anion in PBMC in type 2 diabetes, which might add to the anti-inflammatory effects of exendin-4 and break the vicious cycle.

Using PBMC from patients with type 2 diabetes, we have shown, for the first time, the activation of monocytes and CD4+ T cells indicated by increased MAPK signaling pathways, cytokines/chemokines release and oxidative stress. Apart from adipokines and cytokines, metabolic triggers such as blood glucose and lipids, notably free fatty acids[17], might trigger an inflammatory cascade to induce cellular dysfunction. In this connection, our novel findings regarding the direct immuno-modulating effects of exendin-4 in PBMC though suppression of phosphorylation of p38 MAPK and ERK, and production of superoxide anion, suggest that the beneficial effects of exentin-4 might extend beyond diabetes to include other autoimmune or inflammatory diseases.

## ACKNOWLEDGEMENTS

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