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Interleukin-17A inhibits adipocyte differentiation in human mesenchymal stem cells and regulates pro-inflammatory responses in adipocytes

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ABSTRACT

The immune system is closely linked to human metabolic diseases. Serum levels of IL-6 increase with obesity and insulin resistance. Not only does IL-6 decrease the insulin sensitivity of human cells such as adipocytes, but it also regulates the lineage commitment of naïve T cells into interleukin (IL)-17A-producing CD4(+) T (Th17) cells. Although IL-17A exerts a variety of effects on somatic tissues, its functional role in human adipocytes has not been identified. In this work, we show that IL-17A inhibits adipocyte differentiation in human bone marrow mesenchymal stem cells (hBM-MSCs), while promoting lipolysis of differentiated adipocytes. We find that IL-17A increases both mRNA and protein secretion of IL-6 and IL-8 during adipocyte differentiation in hBM-MSCs. IL-17A up-regulates cyclooxygenase (COX)-2 gene expression and thereby increases the level of prostaglandin (PG) E_2 in differentiated adipocyes. The suppression of anti-adipogenic PGE_2 by COX inhibitors such as aspirin and NS-398 partially blocked the effect of IL-17A on adipocyte differentiation in hBM-MSCs. Therefore, IL-17A exhibits its inhibitory effect in part via the COX-2 induction in differentiated adipocytes. In addition, treatment with anti-IL-17A antibody neutralizes IL-17A-mediated effects on adipocyte differentiation and function. These results suggest that IL-17A plays a regulatory role in both the metabolic and inflammatory processes of human adipocytes, similar to other pro-inflammatory cytokines such as IL-1, IFNγ, and TNFα.

1. Introduction

Studies on inflammation in obesity and other metabolic diseases have indicated a close relationship between adipose tissue and the immune system [1–7]. Adipocytes can produce various pro-inflammatory cytokines such as IL-6, IL-8, and tumor necrosis factor-α (TNFα), which modulate immune responses [8–12]. In a reciprocal manner, the increase of pro-inflammatory cytokines such as IL-1, interferon (IFN) γ, and TNFα, in both innate and adaptive immune responses can regulate the differentiation of pre-adipocytes to adipocytes, the metabolic states of adipocytes, as well as chemokine and adipokine production in adipocytes [8–12]. Moreover, low levels of chronic inflammation are known to be associated with obesity and insulin resistance. Macrophage infiltration into adipose tissue represents a well-defined inflammatory feature in obese conditions [1,2] and also in a mouse model of obesity-induced insulin resistance. Recent reports indicated that pro-inflammatory CD4(+) and CD8(+) T cells were found prior to macrophage infiltration [13–15]. These results suggest that T cells may play an important role at the onset of insulin resistance. Although both immune cell infiltration and inflammatory cytokine production increase in adipose tissue during the progression of obesity and insulin resistance, the mechanisms by which these tissues trigger inflammatory responses remain unclear [1–7].

In obese and insulin resistant conditions, adipose tissue contributes a significant proportion of circulating serum IL-6 [16]. It has recently been elucidated that IL-6 regulates the lineage commitment of naïve T cells to Th17 cells [16,17]. Th17 cells represent a distinctive lineage of helper T cells and differ from Th1 and Th2 cells [18–20]. IL-17A, IL-17F, and IL-22 are the major cytokines released from human Th17 cells. Since receptors for IL-17 and IL-22 are expressed across diverse somatic cell types, Th17 cells have been implicated in interactions between the immune system and somatic tissues [21–24]. IL-17A is known to regulate the physiological or pathological states of endothelial cells,

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Abbreviations: ADIPOQ, adiponectin; FABP4, fatty acid binding protein 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBM-MSCs, human bone marrow mesenchymal stem cells; PPARγ, peroxisome proliferator-activated receptor gamma; Q-RT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

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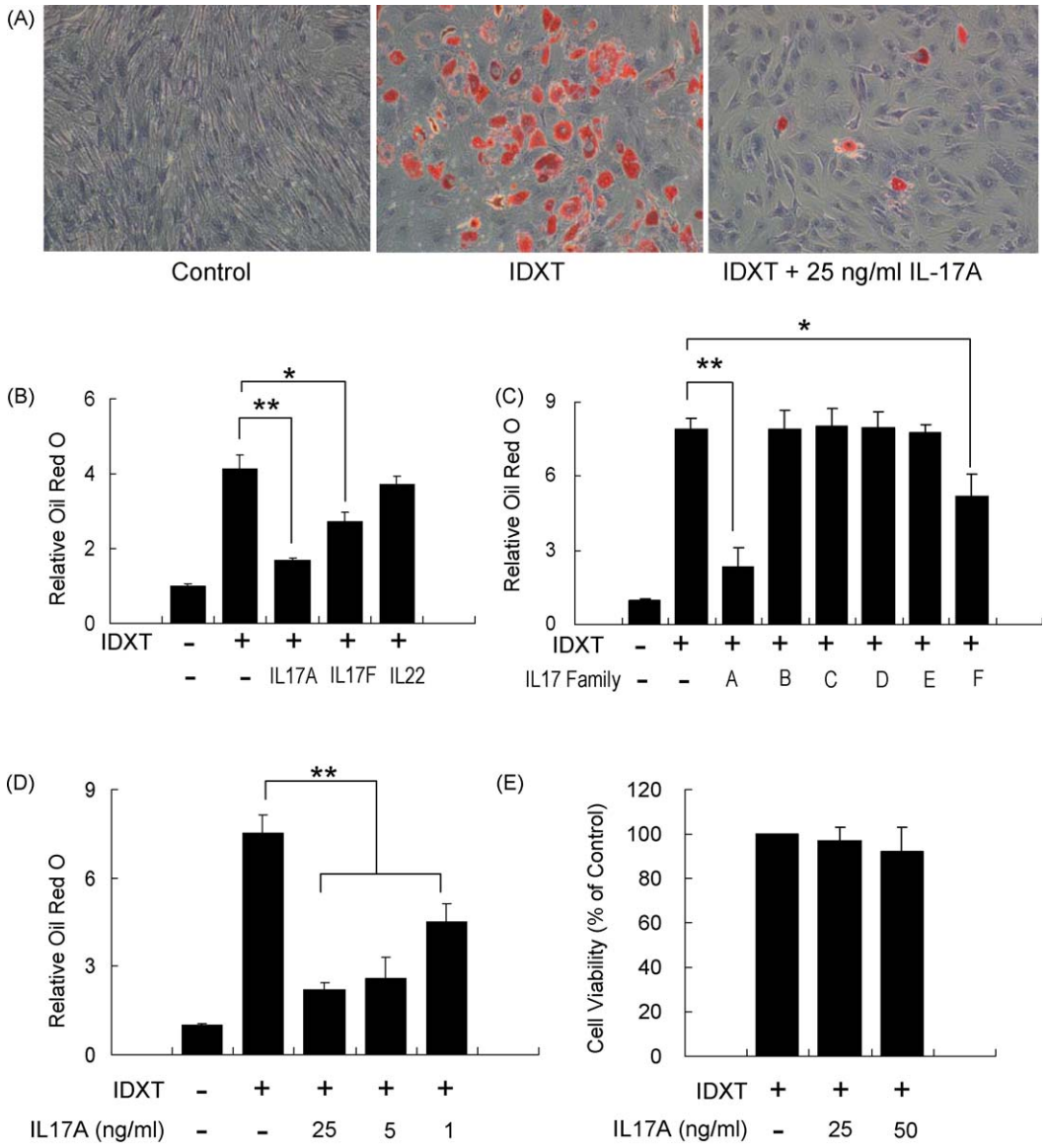

Fig. 1. IL-17A inhibits adipocyte differentiation of hBM-MSCs. hBM-MSCs were grown under IDXT conditions and co-treated with each cytokine, IL-17A (A and B), IL-17F (B), or IL-22 (B) (25 ng/mL each). Under the same condition as above, hBM-MSCs were co-treated with other IL-17 family members; IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F (25 ng/mL each) (C), respectively, or treated with different concentration of IL-17A (1, 5, and 25 ng/mL) (D). Fifteen days after adipogenic stimulation with IDXT, lipid droplets in adipocytes were stained with Oil Red O (ORO) and then after dissolving the ORO in isopropyl alcohol, the level of staining was quantified at 500 nm using a spectrometer. Data were normalized by setting the IDXT (−) control as 1. Results are the mean ± standard deviation (S.D.) of three measurements using independent hBM-MSCs from three different donors ($n = 3$). Cell viability was evaluated using the WST-1 assay after incubation of IL-17A for 17 days in hBM-MSCs (E). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with IDXT (+) condition.

epithelial cells, fibroblasts, and macrophages [21–24]. For instance, IL-17A increases production of IL-6 and various chemokines in vascular endothelial cells, pancreatic myoblasts, synoviocytes, osteoblasts, bronchial epithelial cells, and keratinocytes [25–28]. Although IL-17A induces IL-6 expression in many somatic tissue cells, it has been poorly addressed in human adipose tissues. It remains unclear whether IL-17A can modulate obesity and metabolic states, or whether the increase in IL-6 levels in the adipose tissues under conditions of obesity and insulin resistance could affect differentiation of naïve T lymphocytes.

Human bone marrow mesenchymal stem cells (hBM-MSCs) are proven to be useful for studying adipocyte differentiation and related metabolic diseases [29–31]. In obesity, the molecular mechanisms underlying adipocyte expansion in adipose tissue have not been clearly identified. It has been suggested that metabolic changes promote either pre-adipocytes or mesenchymal precursor cells to participate in a new adipocyte pool in the expanding adipose tissue [30]. During the expansion of adipocytes in obesity, immune cells are also recruited to the adipose tissue, a process that may affect terminal differentiation from mesenchymal precursors to adipocytes [3,4]. Consequently, the adipocyte differentiation of hBM-MSCs may represent a useful cell culture model for studying human metabolic diseases [30,31].

In the present study, we have evaluated the effects of Th17 cytokines, namely IL-17A, IL-17F, and IL-22, on adipocyte differentiation in hBM-MSCs. We observed mRNA expression of IL-17 receptors A and C in both hBM-MSCs and their differentiated adipocytes. Interestingly, we found that IL-17A significantly inhibited adipocyte differentiation in hBM-MSCs, as well as stimulating production of IL-6, IL-8, and PGE_2 in differentiated adipocytes. These findings suggest that IL-17A may play a role in

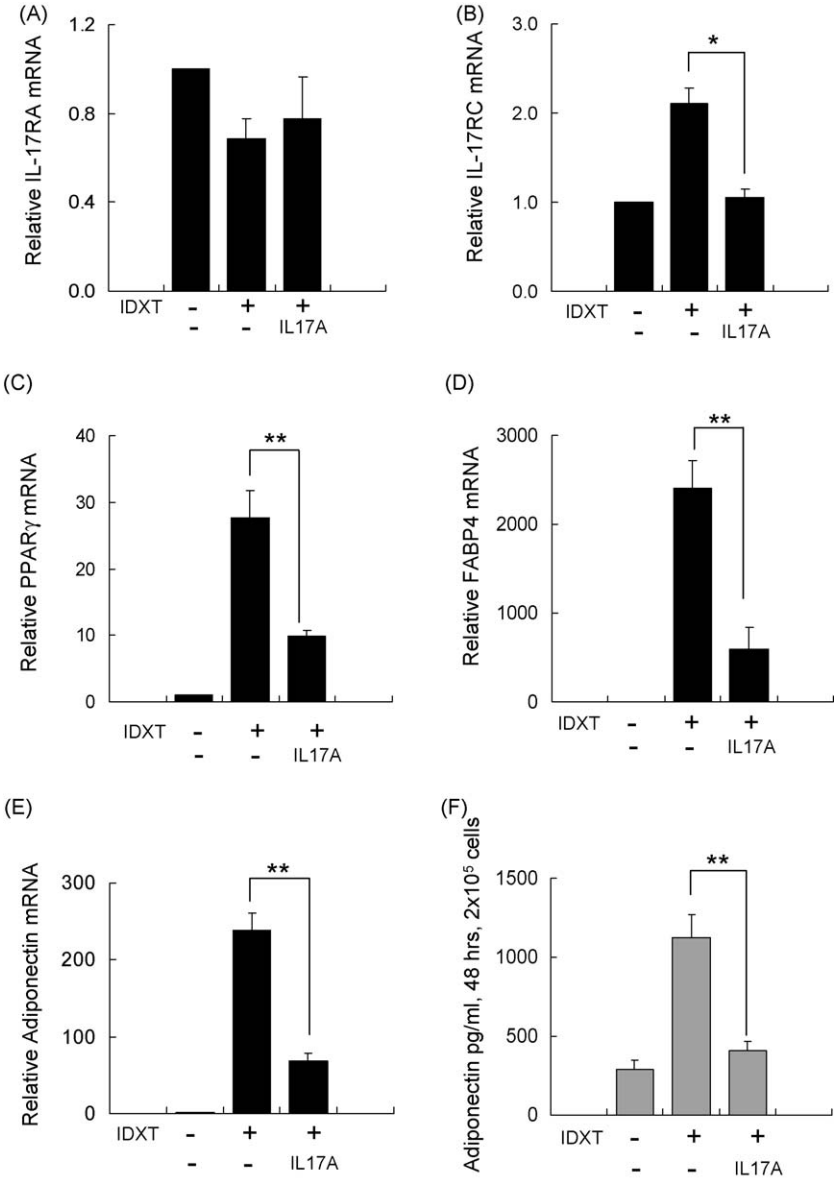

Fig. 2. Effects of IL-17A on expression of IL-17 receptor isoforms and adipocyte specific markers during adipocyte differentiation in hBM-MSCs. hBM-MSCs were grown under IDXT conditions and co-treated with IL-17A (25 ng/mL). On day 5 after incubation of IDXT, mRNA samples were extracted and evaluated by Q-RT-PCR. Relative expressions of IL-17RA (A), IL-17RC (B), PPARγ (C), FABP4 (D), and adiponectin (E) were calculated using GAPDH as an internal standard. Data were normalized by setting IDXT (−) as 1 (mean ± S.D., $n = 3$). For adiponectin ELISA (F), hBM-MSCs were grown under the same conditions. The cell culture supernatant was collected 48 h after the medium exchange at the day 3 of IDXT incubation to measure the concentration of adiponectin (mean ± S.D., $n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with IDXT (+) condition.

the crosstalk between adipose tissue and Th17 cell-associated immune responses.

2. Materials and methods

2.1. Cell culture and differentiation

Human bone marrow mesenchymal stem cells (hBM-MSCs) were purchased from Lonza, Inc. (Walkersville, MD, USA). These cells were grown in low glucose (1 g/L) Dulbecco Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with penicillin–streptomycin and 1% Glutamax™ (Invitrogen, Carlsbad, CA). To induce adipocyte differentiation, the growth medium was replaced by DMEM containing a high concentration of glucose (4.5 g/L) and supplemented with 10% FBS, 1% Glutamax™, 10 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (IDX condition). IL-17A, insulin, IBMX, troglitazone, aspirin, and ibuprofen were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). NS-398 was purchased from the Cayman Chemical Co. (Ann Arbor, MI, USA). IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, and IL-22, as well as anti-IL-17A and anti-IL-6 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). In order to induce adipocyte differentiation, hBM-MSCs were grown under IDX conditions with 1 μM of troglitazone (IDXT condition). After the induction of adipocyte differentiation in hBM-MSCs, cell culture media were exchanged every third days.

2.2. Cell viability assay

Cell viability of hBM-MSCs (48-well plates; 2×10^4 cells/well) under the presence of IL-17A or IL-17F was evaluated using the WST-1 assay according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). hBM-MSCs or

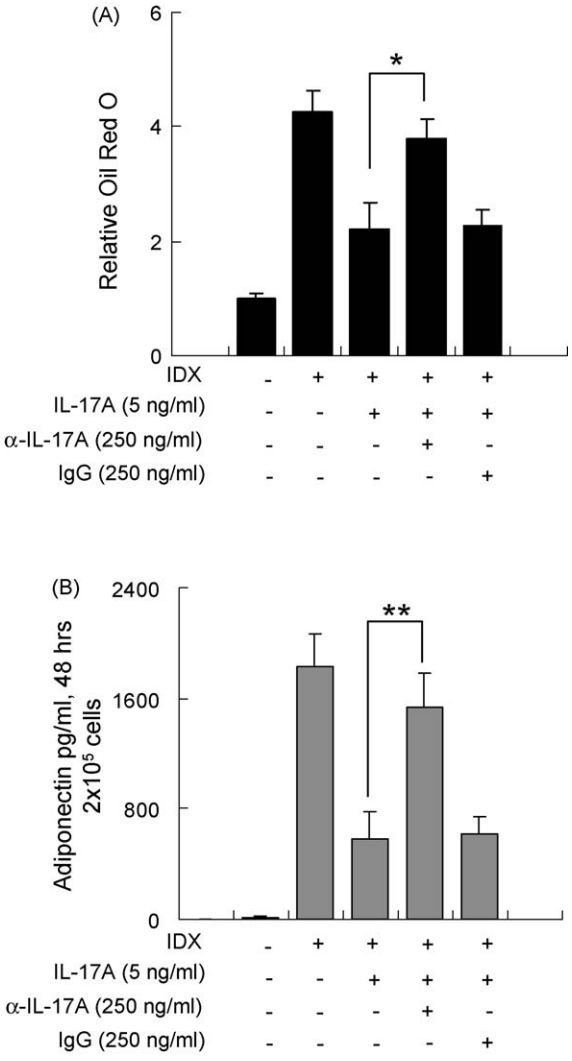

Fig. 3. Anti-IL-17A antibody antagonizes the effects of IL-17A on adipocyte differentiation. hBM-MSCs were grown to confluency in 24 well cell culture plates and the adipocyte differentiation was induced with IDXT. The adipocyte differentiation was performed by the co-treatments with IL-17A (5 ng/mL), as well as with or without anti-IL-17A antibody (250 ng/mL) or control Ig G (250 ng/mL) (A). Lipid droplets in differentiated adipocytes were stained with ORO 15 days after adipogenic stimulation with IDX. After dissolving the ORO in isopropyl alcohol, the level of staining was quantified at 500 nm using a spectrometer. Data were normalized by setting the IDX (−) control as 1. Results are the mean ± S.D. of three independent measurements. To measure the levels of adiponectin in cell culture supernatants, hBM-MSCs were grown under the same conditions. The cell culture supernatant was collected 48 h after the medium exchange at the day 12 of IDXT incubation for adiponectin ELISA (mean ± S.D., $n = 3$) (B). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the IDX and IL-17A co-treatment.

differentiated adipocytes were treated with 50 ng/mL of IL-17A or IL-17F for 15 days. After washing hBM-MSCs with phosphate buffered saline three times, 4-3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate (WST-1; 10 μM pure solution) was added, and cells were incubated for 2 h in a humidified atmosphere. The absorbance at A450 nm was measured and absolute optical density was expressed as a percentage of the control value.

2.3. Oil Red O (ORO) staining and hematoxylin staining

Adipocyte differentiation was assessed using an ORO stain as an indicator of intracellular lipid accumulation. After hBM-MSCs had differentiated to adipocytes, cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS (pH 7.4) for 1 h, and then washed with 60% isopropanol, before being allowed to dry completely. hBM-MSCs were stained with 0.2% ORO reagent for 10 min at room temperature, and then washed with H_2O four times. Following a 10 min elution of each hBM-MSCs sample with 100% isopropanol, absorbance was measured at 500 nm using a spectrophotometer. To visualize the nucleus, hBM-MSCs were counterstained with hematoxylin reagent for 2 min and then washed twice with H_2O. The differentiated adipocytes in hBM-MSC population were observed and photographed using an Olympus IX71 inverted phase-microscope (Olympus Co., Tokyo, Japan).

2.4. Measurement of lipolysis in culture

To determine the glycerol concentration in the cell culture supernatant as a measurement of cellular lipolysis, a glycerol phosphate oxidase (GPO)-Trinder Kit (Sigma, St. Louis, MO, USA) was used according to the manufacturer's instruction. Conditioned medium was removed from each well and incubated with glycerol reagents for 10 min, after which optical density (OD) value was measured at 540 nm using a spectrophotometer. The glycerol concentration of each sample was calculated using the standard curve, obtained with a glycerol standard solution.

2.5. Measurement of cytokines and prostaglandin (PG) E_2

For quantitative determination of adiponectin, IL-6, and IL-8 in cell culture supernatants, a Quantikine™ immunoassay kit was used (R&D Systems, Minneapolis, MN, USA). PGE_2 levels were measured using an enzyme immunoassay (EIA) system from the Cayman Chemical Co. (Ann Arbor, MI, USA). Conditioned media were centrifuged for 5 min at 1000 × *g* and then supernatants were diluted for use in the quantification reaction. Concentrations of adiponectin, IL-6, IL-8, and PGE_2 were determined according to the manufacturer's instructions.

2.6. Real-time quantitative RT-PCR

The hBM-MSCs were differentiated for a 5-day or 15-day exposure to adipogenic conditions and then total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA), followed by purification step with the Qiagen RNeasy Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The RNA concentration of each sample was determined by spectrophotometry at 260 nm. The integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (2 μg) from each sample was reverse transcribed in 100 μL reactions using the Superscript Reverse Transcriptase (RT) II Kit (Invitrogen, Carlsbad, CA, USA) and the reaction was stopped by adding Tris–EDTA buffer (pH 8.0). Quantitative measurements of adipogenic markers in each cDNA sample were carried out using the Assays-on-Demand™ Gene Expression Kits (Applied Biosystems, Foster City, CA, USA). Each kit consists of a FAM™ dye-labeled TaqMans® MGB probe and a primer pair for specific marker genes. The cDNA samples were analyzed for adiponectin (ADIPOQ, Hs00605917_m1), fatty acid binding protein 4 (FABP4, Hs00609791_m1), peroxisome proliferator activated receptor gamma (PPARγ, Hs00233423_m1), cyclooxygenase (COX)-1 (Hs00924803_m1), COX-2 (Hs00153133_m1), IL-17RA (Hs01064648_m1), and IL-17C (Hs00262062_m1), according to the manufacturer's instructions. In order to normalize mRNA expression levels, expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, (GAPDH, 4333764F), was analyzed as a control. The cDNA was amplified by PCR and the FAM fluorescence of each PCR cycle was measured with the Corbett

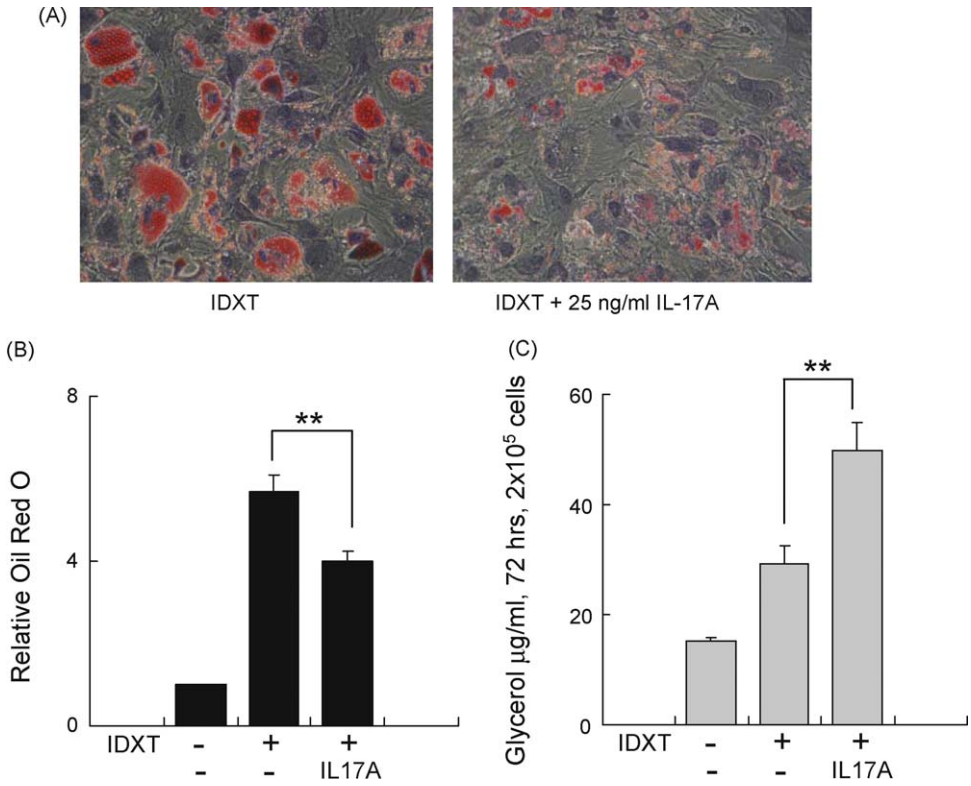

Fig. 4. IL-17A increases lipolysis in fully differentiated adipocytes from hBM-MSCs. hBM-MSCs were differentiated into adipocytes in 24 well cell culture plates under IDXT conditions for 15 days. At the day 15 after incubation of IDXT in hBM-MSCs, the differentiated adipocytes were then treated with IL-17A (25 ng/mL) for 7 days (A, B, C). Lipid droplets in adipocytes were stained with ORO and after dissolving the ORO in isopropyl alcohol, the level of staining was quantified at 500 nm using a spectrometer (A and B). Data were normalized by setting the IDXT (−) control as 1. Results are the mean ± S.D. of three independent measurements. Glycerol levels in the cell culture supernatant accumulated for 48 h after the final IDXT medium exchange were measured using a GPO-Trinder assay (mean ± S.D., $n = 3$) (C). $^{**}p < 0.01$, compared with the IDXT (+) condition.

Research Rotor-Gene 6 Detection System (Corbett Research, Mortlake, NSW, Australia). Quantitative(Q)-RT-PCR reactions were performed in triplicate or in quadruplicate for each sample using the manufacturer's instruction after adjusting the reaction volume to 20 μL. Results of Q-RT-PCR data were represented as C_t values, where C_t is defined as the threshold cycle. Quantification of relative differences among various adipogenic conditions was calculated using equations from a mathematical model developed by Pfaffl [32].

2.7. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and were presented as means ± S.D. of at least three independent experiments. The threshold of significance was set at $p < 0.05$.

3. Results

3.1. IL-17A inhibited adipocyte differentiation in hBM-MSCs in a dose dependent manner

Adipocyte differentiation in hBM-MSCs was induced by treatment with insulin, dexamethasone, 3-isobutyl-1-methylxanthine, and troglitazone (IDXT). After 2 weeks of incubation of IDXT, the fraction of differentiated cells in hBM-MSCs was increased by 50–60% and did not significantly change over 30 days of incubation with IDXT medium [31]. In our study, we defined this equilibrium state of adipocyte differentiation in hBM-MSCs as "differentiated adipocytes." In order to investigate the effects of Th17 cell cytokines on adipocyte differentiation, hBM-MSCs were co-treated with IDXT and 25 ng/mL IL-17A, IL-17F, or IL-22 for 15 days. In comparison to treatment with IDXT alone (control), co-treatment with IDXT and IL-17A or IL-17F inhibited differentiation by 77.6% (±2.8% S.D.) and 45.6% (±12.9% S.D.), respectively (Fig. 1A and B). No statistical significant effects on hBM-MSCs differentiation were observed following co-treatment with 25 ng/mL IL-22. Having confirmed that both IL-17 cytokines affect adipocyte differentiation, we evaluated other members of the IL-17 family of cytokines. In contrast to IL-17A and IL-17F, no significant inhibition of hBM-MSC adipocyte differentiation was observed following co-treatment with IL-17B, IL-17C, IL-17D, or IL-17E, compared with the IDXT control alone (Fig. 1C). We then evaluated whether IL-17A exhibited concentration-dependent inhibition of adipocyte differentiation in hBM-MSCs. Co-treatment of hBM-MSCs with IDXT and 25, 5, or 1 ng/mL of IL-17A suppressed adipocyte differentiation by 81.9, 75.6, or 46.2%, respectively, in comparison to the IDXT control (Fig. 1D). In the parallel study, no significant effect on adipocyte differentiation was observed with 10 ng/mL of IL-17F (data not shown). In addition, the cell viability during adipocyte differentiation in hBM-MSCs was not changed in the presence of IL-17A (up to 50 ng/mL) (Fig. 1E). The cell viability test supports that the anti-adipogenic effect of IL-17A on hBM-MSCs is not due to the apoptotic and/or necrotic cell death generally induced by cytokines. Therefore, these findings suggest that IL-17A exhibit dose-dependent inhibition of adipocyte differentiation in hBM-MSCs.

3.2. IL-17A affects transcription of IL-17RC gene, but not IL-17RA gene during adipocyte differentiation in hBM-MSCs

Two transmembrane receptor proteins, IL-17RA and IL-17RC, are known to play critical roles in IL-17A and IL-17F signaling [24,25]. Although SAGE analysis has indicated that IL-17RA is one

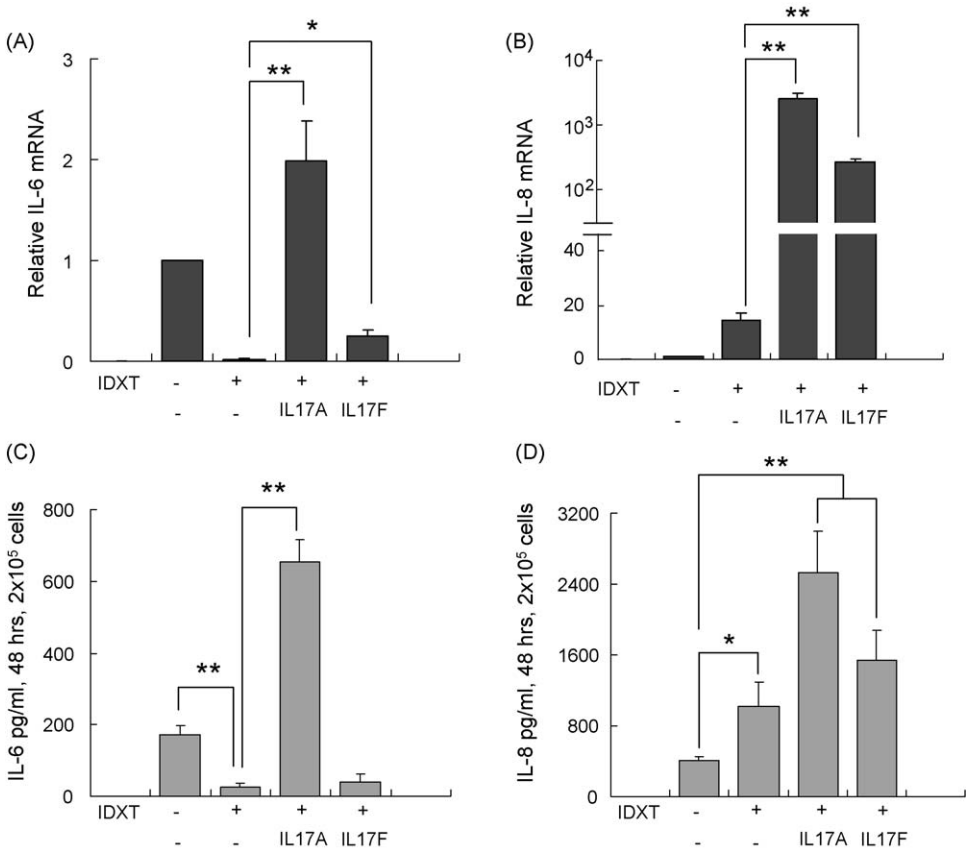

Fig. 5. Effects of IL-17A and IL-17F on production of IL-6 and IL-8 in differentiated adipocytes. hBM-MSCs were grown to confluency and adipocyte differentiation was induced under IDXT conditions for 15 days. In the differentiated adipocytes at the day 15, IL-17A or IL-17F (each 25 ng/mL) were treated and incubated for 2 days with IDXT (A–D). Relative mRNA expression levels of IL-6 (A) and IL-8 (B) were determined for each sample. Data were calculated using GAPDH as an internal standard and were normalized by setting IDXT (−) as 1 (mean ± S.D., $n = 3$). For determination of IL-6 and IL-8 secretion, hBM-MSCs were differentiated under the same conditions in 24-well cell culture plates. ELISA was used to measure the concentrations of IL-6 (C) and IL-8 (D) in the cell culture supernatants harvested 48 h after the final medium exchange (mean ± S.D., $n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with IDXT (+) condition.

of the most highly expressed cytokine receptor genes in hBM-MSCs [33], the expression of these IL-17 receptors during adipocyte differentiation in hBM-MSCs is not known. Since lipid droplets are usually observed around 5 days after adipogenic stimulation of differentiated hBM-MSCs, we measured IL-17A-induced transcriptional changes in IL-17RA and IL-17RC mRNA expression during the early stages of adipocyte differentiation (Fig. 2A and B). Q-RT-PCR analysis detected significant levels of IL-17RA and IL-17RC mRNAs under both non-adipogenic and adipogenic hBM-MSC culture conditions. However, 5 days after induction, we were unable to detect any significant difference between IL-17RA mRNA levels of undifferentiated control, the IDXT stimulated differentiated hBM-MSCs, and the IL-17A treated cells in the presence of IDXT (Fig. 2A). In contrast, we detected a two-fold increase in IL-17RC gene expression in IDXT stimulated cells compared to the undifferentiated control. Moreover, IL-17RC mRNA levels were affected significantly by the presence of IL-17A (Fig. 2B).

To verify the effects of IL-17A on adipocyte differentiation in hBM-MSCs, we evaluated the transcriptional activities of adipocyte specific genes, such as peroxisome proliferator activating receptor γ (PPARγ), fatty acid binding protein 4 (FABP4), and adiponectin. Five days after induction of adipocyte differentiation, the mRNA levels of gene encoding PPARγ, FABP4, and adiponectin were significantly reduced in cells co-treated with IDXT and IL-17A, compared to the IDXT control (Fig. 2C–E). Transcription of PPARγ, FABP4, and adiponectin was also down-regulated 24 h after IL-17A treatment of fully-differentiated adipocytes from hBM-MSCs (data not shown). In addition, we confirmed that IL-17A treatment significantly decreased adiponectin protein secretion in comparison with the IDXT control (Fig. 2F).

3.3. Anti-IL-17A antibody neutralizes the effect of IL-17A treatment on hBM-MSCs

We investigated whether anti-IL-17A antibodies could neutralize the inhibitory effect of IL-17A on adipocyte differentiation in hBM-MSCs. As shown in Fig. 3A, 250 ng/mL anti-IL-17A antibody antagonized IL-17A-mediated inhibition of adipocyte differentiation. In order to confirm this observation, we used ELISA to measure adiponectin levels during adipocyte differentiation in hBM-MSCs (Fig. 3B). As expected, IL-17A inhibited adiponectin secretion and its effect was significantly diminished by co-treatment with the anti-IL-17 antibody. These results support the suggestion that IL-17A plays a specific role in reduction of adipocyte differentiation in hBM-MSCs.

3.4. IL-17A increases lipolysis in differentiated adipocytes from hBM-MSCs

To determine whether IL-17A regulates the metabolic state of adipocytes directly, we evaluated its effect on lipolysis in fully differentiated adipocytes from a hBM-MSC culture. As shown in Fig. 4A and B, IL-17A decreased both the size and number of lipid droplets in differentiated adipocytes after 7-day incubation of IDXT with IL-17A. In order to measure lipolysis levels, we evaluated the glycerol concentration of supernatants from

differentiated adipocyte cultures, 48 h after IL-17A treatment. We observed that IL-17A increased lipolysis of the adipocytes significantly (Fig. 4C). These results suggest that IL-17A modulates the metabolic state of differentiated adipocytes by regulating lipolysis of the differentiated adipocytes.

3.5. IL-17A increases IL-6 and IL-8 production in differentiated adipocytes

IL-17A stimulates pro-inflammatory IL-6 and IL-8 production in non-immune cells such as keratinocytes and synoviocytes, which express functional receptors for IL-17A signaling [21,22]. Since significant levels of IL-17RA and IL-17RC mRNAs were detected in the differentiated adipocytes (Fig. 2), we evaluated whether IL-17A regulates expression of IL-6 and IL-8 genes in these cells. hBM-MSCs are known to produce IL-6 in cell culture [33–35]. We measured the IL-6 mRNA levels 15 days after adipogenic stimulation for 15 days and observed significant down-regulation of IL-6 transcription under IDXT adipogenic conditions, compared with that the untreated hBM-MSC control (Fig. 5A). In contrast, IL-8 transcription levels increased significantly as adipocyte differentiation progressed, in comparison to the IDXT control (Fig. 5B). Interestingly, we observed a dramatic increase in IL-6 mRNA levels in differentiated adipocytes co-treated with IDXT and 25 ng/mL of IL-17A, and these mRNA levels were significantly higher than those of untreated hBM-MSCs (Fig. 5A). Although IL-6 transcript levels were up-regulated significantly by co-treatment with IDXT and 25 ng/mL IL-17F, the increase was not as substantial as that observed with a same concentration of IL-17A (Fig. 5A). On the other hand, treatment of differentiated adipocytes with both IL-17A and IL-17F increased IL-8 transcript levels significantly (Fig. 5B). We also used ELISA to confirm that IL-6 and IL-8 protein production increased during adipocyte differentiation in hBM-MSCs. Consistent with our gene transcription results, 25 ng/mL of IL-17A significantly increased adipocyte expression of both IL-6 and IL-8 expressions at protein level (Fig. 5C). However, in contrast to the up-regulation of IL-6 mRNA level upon the treatment of IL-17F, we observed no statistically significant difference between IL-6 protein level in untreated cells and those treated with IL-17F. IL-8 exhibits similar patterns of expression for both protein secretion and gene transcription upon treatment with IL-17A and IL-17F (Fig. 5D). Therefore, we conclude that IL-17A regulates IL-6 and IL-8 production in differentiated adipocytes derived from hBM-MSCs.

3.6. IL-17A up-regulates COX-2 gene expression and increases PGE_2 levels in differentiated adipocytes

In human synoviocytes and keratinocytes, IL-17A stimulates secretion of PGE_2 by inducing COX-2 expression [36,37]. Since PGE_2 exerts an inhibitory effect on adipocyte differentiation in an autocrine and/or paracrine manner [38], we investigated whether IL-17A affects cyclooxygenases gene expression and PGE_2 production. In differentiated adipocytes, IL-17A significantly increased COX-2 mRNA levels, whereas it did not alter COX-1 expression levels in hBM-MSCs cultures (Fig. 6A and B). In differentiated adipocyte culture supernatants, IL-17A treatment increased PGE_2 levels significantly (Fig. 6C). These IL-17A-induced changes in COX-2 gene expression and PGE_2 production were antagonized by the treatment with an IL-17A neutralizing antibody (Fig. 6).

In order to evaluate the mechanism by which IL-17A induced up-regulation of COX-2/PGE_2 in differentiated adipocytes, we administered two non-selective COX inhibitors (aspirin and ibuprofen) and one selective COX-2 inhibitor (NS398), to IL-17A treated cells (Fig. 7). ORO staining indicated that in the presence of IL-17A, both 500 μM of aspirin and 1 μM of ibuprofen, significantly increased adipocyte differentiation (29.9 and 29.5%,

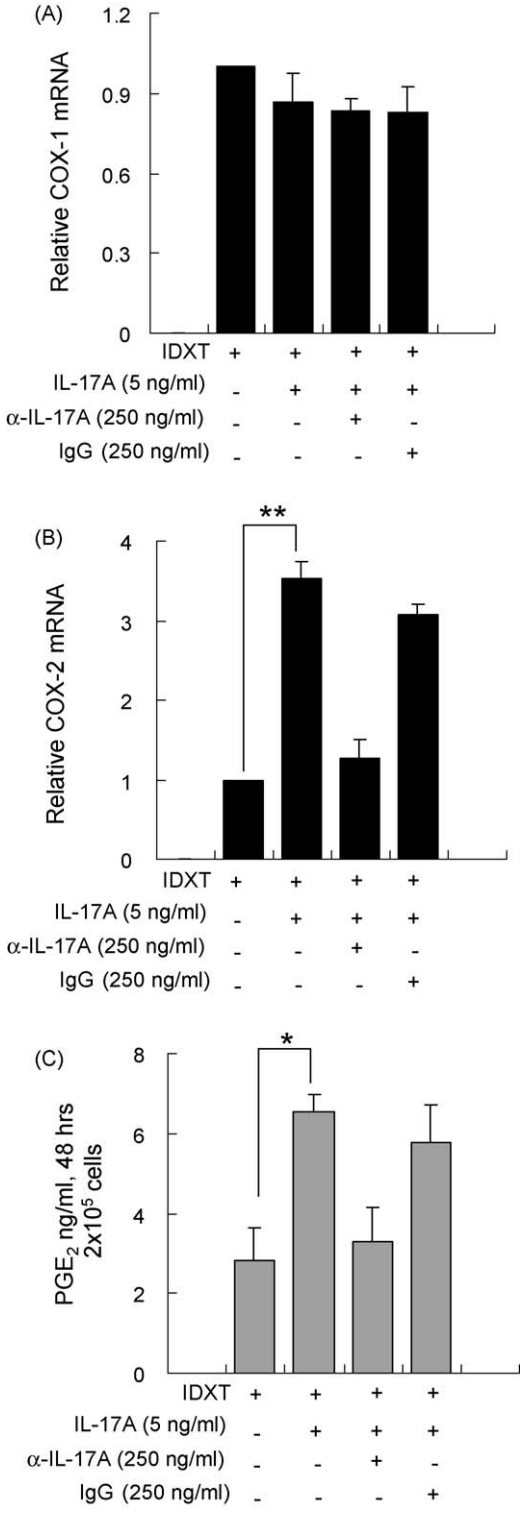

Fig. 6. Effects of IL-17A on COX-1 and COX-2 gene expression in differentiated adipocytes from hBM-MSCs. hBM-MSCs were grown to confluence and adipocyte differentiation was induced with incubation of IDXT for 15 days. In addition, IL-17A (5 ng/mL), as well as with or without anti-IL-17A antibody (a-IL-17A) (250 ng/mL) or control Ig G (250 ng/mL) was co-treated with IDXT during adipocyte differentiation in hBM-MSCs for 15 days (A–C). At the day 15, cells were harvested for total RNA isolation. Relative mRNA expression levels of COX-1 (A) and COX-2 (B) were determined for each sample. Data were calculated using GAPDH as an internal standard and were normalized by setting IDXT (+) conditions without IL-17A treatment as 1 (mean ± S.D., $n = 3$). To determine PGE_2 secretion in the cell culture supernatants, hBM-MSCs were grown to confluency and adipocyte differentiation was induced under the same conditions for 15 days. ELISA was used to measure the concentration of PGE_2 secreted for 48 h after incubation of IL-17A (C) (mean ± S.D., $n = 4$). $^*p < 0.05$, $^{**}p < 0.01$, compared with IDXT (+) condition.

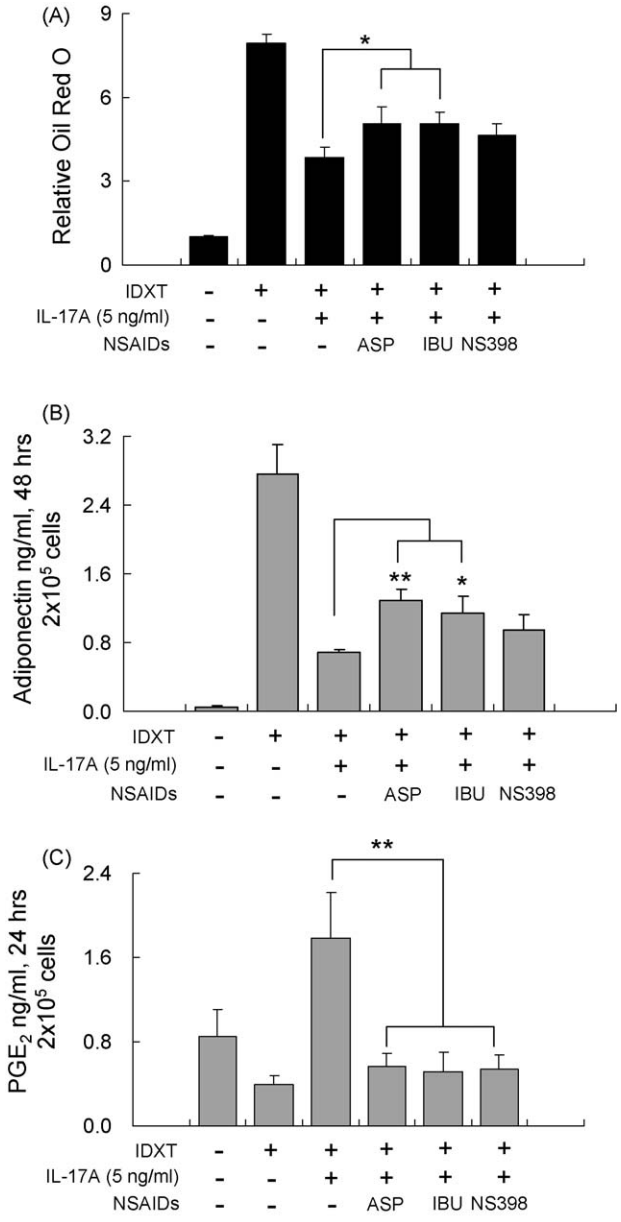

Fig. 7. Treatment with COX-inhibitors partially blocks IL-17A-induced inhibition of adipocyte differentiation in hBM-MSCs. hBM-MSCs were grown to confluency in 24 well cell culture plates. Adipocyte differentiation in hBM-MSCs was induced under IDXT conditions and co-treated with IL-17A (10 ng/mL), as well as with or without COX inhibitors, aspirin (ASP, 500 μM), ibuprofen (IBU, 1 μM), or NS398 (2 μM) (A). Lipid droplets in adipocytes were stained with ORO and then after dissolving the ORO in isopropyl alcohol, the level of staining was quantified at 500 nm using a spectrometer (B). To determine PGE_2 secretion in the cell culture supernatants, adipocyte differentiation was induced under the same conditions for 15 days. ELISA was used to measure the concentration of PGE_2 secreted for 48 h after the final medium exchange (mean ± S.D., $n = 4$) (C). $^*p < 0.05$, $^{**}p < 0.01$, compared with the IDXT (+) condition co-treated with IL-17A.

respectively) relative to the IL-17A treated control (Fig. 7A). NS398 (1 μM) also promoted adipocyte differentiation by 19.3%, although this effect was not statistically significant in comparison to the IL-17A control (Fig. 7A). Consistent with their effects on adipocyte differentiation, cultures that received aspirin or ibuprofen treatments in addition to IDXT and IL-17A, exhibited significantly higher levels of adiponectin in their supernatants, relative to the IL-17A treated control (Fig. 7B). While all three COX inhibitors significantly inhibited IL-17A stimulated PGE_2 release (Fig. 7C), they did not fully block IL-17-induced inhibition of adipocyte differentiation in hBM-MSCs (Fig. 7A). These results suggest that the up-regulation of COX-2 gene expression stimulated by IL-17A, only contributes in part to the inhibitory effect of IL-17A on adipocyte differentiation.

4. Discussion

In the present study, we demonstrated that IL-17A, the signature Th17 cell cytokine, inhibited adipocyte differentiation of hBM-MSCs and increased lipolysis of differentiated adipocytes. Consistent with observations from other somatic tissue cells [25–27], IL-17A induced significant production of IL-6 and IL-8 in the differentiated adipocytes from a hBM-MSC culture. Furthermore, we suggest that IL-17A-stimulated COX-2 up-regulation may partially contribute to its inhibitory effect on adipocyte differentiation. Recently, the links between metabolic diseases and increased inflammatory responses, have been well characterized [1–7]. In these metabolic syndromes, the adipose tissues exhibit low levels of chronic inflammation [13–15]. Considering that T lymphocytes play roles in adaptive immunity during chronic inflammation and related diseases an association between T cells and adipose tissue inflammation may exist [13–15]. A substantial increase in both CD4(+) T cells and interferon (IFN) γ, a Th1 cell cytokine, was detected in the visceral adipose tissues of obese mice raised on a high fat diet [13–15], a finding that implicates Th1 cells in an early phase of obesity progression in an animal model of obesity [13–15]. In a recent clinical study, increased levels of CD4(+) INFγ secreting T cells were observed in obese children, in comparison to lean controls, a finding which suggests that T cells participate in the metabolic syndrome [39]. Whereas the participation of Th1 cells in obesity is now widely accepted, the association between Th17 cells and the adipose tissue inflammation remains poorly explored from the standpoint of progression of obesity and insulin resistance. Considering that human IL-17A producing CD4(+) T cells can also produce IFNγ [21–24], we may speculate that in addition to Th1 cells, other subtypes of IFNγ secreting CD4(+) T cells may be present in obese adipose tissue. Similarly, in psoriatic skin lesions in which IFNγ level is higher than that of normal, both Th1 and Th17 cells play roles in pathologic processes [40]. Since we have observed that IL-17A exerts a significant effect on adipocyte differentiation in hBM-MSCs, as well as performing pro-inflammatory activities in differentiated adipocytes, we postulate that IL-17A-producing Th17 cells may be important in studying adipose tissue inflammation associated with metabolic syndromes.

In the IL-17 cytokine family, IL-17F showed similar effects to IL-17A with respect to adipocyte differentiation in hBM-MSCs, but it was less potent than IL-17A. Recent studies have shown that activated human Th17 cells release IL-17A and IL-17F homodimers as well as IL-17A/IL-17F heterodimer [21–24,41]. The IL-17A/IL-17F heterodimer and two IL-17 homodimers can transmit cellular signaling through dimer-receptor complexes consisted of IL-17RA and IL-17RC, both of which can form homodimer- and heterodimer receptor complex [41–43]. Although these cytokines exhibit different potencies, all three can regulate cellular functions through the three receptor complexes, i.e. IL-17RA homodimer, IL-17RC homodimer, and IL-17RA/IL-17RC heterodimer [41–43]. In our study, IL-17RC mRNA expression increased significantly in differentiated adipocytes compared to the undifferentiated hBM-MSCs control (Fig. 2). Although not statistically significant, IL-17RA mRNA levels in differentiated adipocytes were consistently lower than those in undifferentiated hBM-MSCs in three independent experiments (data not shown). Therefore, the ratio of IL-17RA to IL-17RC varies during adipocyte differentiation in hBM-MSCs, suggesting that the affinity and potency of each IL-17A and IL-17F dimer subtype changes as adipocyte differentiation pro-

gresses. The relative abundances of IL-17RA and IL-17RC may differ among various somatic tissue cells. Since these three IL-17 heterodimer and homodimers exhibit different affinities and potencies for signaling with each of three IL-17 receptor complexes [21–24,41–43], it will be interesting to investigate whether or not the different functional outcomes of stimulations with IL-17A and IL-17F relate to the relative ratio of IL-17RA to IL-17RC among different somatic tissues.

Although IL-17A stimulation of IL-6 production is the most prominent biological response in somatic tissue cells [16,17,21–24], it remains unclear what role of IL-17A plays in the regulation of IL-6 expression in adipocytes. We found significantly lower IL-6 levels in differentiated adipocytes than in undifferentiated hBM-MSCs (Fig. 5A and C). This finding was not unexpected, since hBM-MSCs express elevated levels of IL-6 and other cytokines [16,17,33–35]. Interestingly, when the differentiated adipocytes were challenged with pro-inflammatory stimulation (IL-17A), significant levels of IL-6 expression were detected in the adipocytes in hBM-MSC culture (Fig. 5A and C). As IL-6 has emerged as an important regulator, guiding CD4(+) T cells to differentiate into Th17 cells [16,17], increased IL-6 in obesity and insulin resistance may exert significant impacts on the development of CD4(+) T cells. Therefore, we can postulate a positive feedback regulation for the increase in IL-6 production in the metabolic syndromes. In other words, the early increase in IL-6 favors Th17 cell differentiation, which subsequently leads to the IL-17A triggered IL-6 up-regulation in human adipocytes during obesity and insulin resistance. In addition, we observed that IL-17A treatment of differentiated adipocytes caused a significant increase in the level of IL-8, a CXC chemokine, in comparison to the hBM-MSC control (Fig. 5B and D). Since IL-1 and TNFα were able to promote IL-8 production in the adipocytes [12], our results lead us to a conclusion that IL-17A can promote inflammatory cell infiltration into adipose tissue, in a similar manner to IL-1 and TNFα. Importantly, it was recently reported that IL-17A is important in sustaining rather than inducing inflammation [44]. Therefore, it will be interesting to evaluate in future whether IL-17A is involved in maintaining inflammatory responses in metabolic syndromes.

In this study, we found that IL-17A treatment of differentiated adipocytes increased COX-2 mRNA levels, resulting in up-regulation of PGE_2 levels in the culture (Fig. 6). PGE_2 can inhibit adipocyte differentiation in mammalian pre-adipocytes [38]. Since PGE_2 receptors are present in human adipocytes [45], PGE_2 can regulate adipocyte differentiation in an autocrine or paracrine manner. In relation to the effects of pro-inflammatory cytokines on adipocytes, it has been reported that treatment with COX inhibitors can block lipolysis in cultured fat cells stimulated by IL-1 and TNFα [10]. We observed that COX enzyme inhibition partially blocked the effects of IL-17A during adipocyte differentiation in hBM-MSCs (Fig. 7), suggesting that additional molecular pathways may be involved with the effects of IL-17A on differentiated adipocytes. Further studies will be required to characterize molecular pathways to understand the full effects of IL-17A on adipocyte differentiation and function.

In summary, we found that IL-17A inhibits adipocyte differentiation, promotes lipolysis, and significantly up-regulates the levels of IL-6, IL-8, and PGE_2 in differentiated adipocytes in hBM-MSC culture. Our results imply that the IL-17A-stimulated IL-6 secretion from the adipose tissue is itself regulated by a complex positive feedback system, considering that IL-6 itself regulates Th17 cell development. Moreover, the inhibitory effect of IL-17A on adipocyte differentiation in hBM-MSCs may be partially mediated through IL-17A-induced COX-2 expression. Although our results suggested possible roles of IL-17A and Th17 cells during the progression of obesity and insulin resistance, the role of Th17 cells in the inflammation of adipose tissue should be addressed via *in vivo* animal studies or human studies.

Conflict of interest

Authors, Dong Wook Shin and Minsoo Noh, are employees of AmorePacific Co.

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