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Interleukin 6 and/or Interleukin 17A Modulate the OPG/RANKL System of MC3T3-E1 Murine Osteoblast Cell Line

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Abstract Cytokines such as interleukin-6 (IL-6) and IL-17 which act as key regulators of the immune response have been identified to have a potential role in the bone remodeling mechanism. Receptor activator of NF-κB ligand (RANKL) has been shown to regulate osteoclast differentiation and function while the osteoprotegerin (OPG) blocks the binding of RANKL and inhibits the differentiation of osteoclasts, thus favoring osteogenesis. Alkaline phosphatase (ALP) on the other hand works as early mineralization indicator in bone regulation. The current study aims to determine the potential role of IL-6 and IL-17A in regulating the OPG/RANKL system of the murine osteoblast cell line (MC3T3-E1). Gene expression analysis showed significant up-regulation of OPG and ALP by all the treated groups (rIL-6, rIL-17A and rIL-6 + rIL-17A). In contrast, treatment of cells with rIL-6 and/or rIL-17A showed down-regulation of RANKL expression. Interestingly, the osteoblast cells treated with combinations of rIL-6 + rIL17A showed marked increased in OPG/RANKL ratio. Similar pattern of protein expression was observed in the osteoblasts treated with rIL-6 and/or rIL-17A as detected by western blotting and ELISA. These findings suggest a new mechanism of regulation by these cytokines on the expression of OPG and RANKL, which could promote osteogenesis and diminish osteoclastogenesis.

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Introduction

Numerous proteins and growth factors have been widely explored to understand the biological fixation and activity to overcome bone complications and strengthen bone immunology (Hadi et al. 2011). The bone remodeling cycle is highly regulated by a variety of factors such as hormones, cytokines and growth factors. Recent studies have demonstrated interactions between bone and immune cells via cytokines which are mainly involved in bone metabolism (Oishi et al. 2012; Raggatt and Partridge 2010; Riggs 2000). It is reported that the cytokines produced in bone defines the response of bone cells to the bone mechanism and developments of bone remodeling (Horowitz and Lorenzo 2008). Cytokines which acts in bone formation might also be involved in bone resorption under certain circumstances (Manolagas and Jilka 1995). Identifying such functional cytokines involved in these mechanisms have been widely studied (Horowitz and Lorenzo 2008; Lorenzo 2011; Murakami et al. 2011). Interleukins such as IL-6 and IL-17 for instance, acts as key regulators in the immune response and bone mechanism (Murakami et al. 2011; Tokuda et al. 2004; Tokuda et al. 2002; Won et al. 2011).

IL-6 is a multifunctional cytokine which plays a major role in bone homeostasis by balancing the osteoblast and osteoclast production. It regulates immune cell function and is involved in osteoblastic cells proliferation and differentiation (Shaama 2005). Murine and human osteoblast cells produce IL-6 and IL-6 receptors, naturally present in the bone microenvironment (Horowitz and Lorenzo 2002).

Although, IL-6 is a pro-inflammatory cytokine, it has been reported to display regenerative or anti-inflammatory activity (Scheller et al. 2011). IL-6 has been shown to stimulate both RANKL and OPG production in bone via RANKL dependent (Lorenzo 2011; Yoshitake et al. 2008) and RANKL independent (Kudo et al. 2003) mechanisms. This suggests that IL-6 may target osteoblastic cells under varying conditions (Frost et al. 2001). Osteoblasts naturally does secrete IL-6, therefore IL-6 may have distinct role on bone metabolism, although the pathway is still unclear (Horowitz and Lorenzo 2002).

Meanwhile, IL-17 also has various contributions in bone regulation (Witowski et al. 2004). It is mainly synthesized by activated T cells and its receptors present in osteoblast cells (Van bezooijen et al. 1999). Several clinical studies have indicated a pivotal role for IL-17 involvement in bone remodeling, either in enhancing or aggravating osteogenesis, depending on the microenvironment involved (Goswami et al. 2009; Huang et al. 2009; Kwan Tat et al. 2008; Moon et al. 2012). It has been recently reported that, rheumatoid arthritis disease cause high level of IL-17 production which points out osteoclastogenesis subjugated condition (Moon et al. 2012). Although IL-17 is primarily associated with the induction of tissue inflammation, other findings stated strong implications for bone remodeling which favors bone formation (Goswami et al. 2009). However, the exact mechanism of IL-17 in bone cells is yet to be clarified.

In-depth understanding of bone metabolism implies the involvement of osteoprotegerin (OPG) and receptor activator of RANK ligand (RANKL) that balances bone metabolism (Alkady et al. 2011). RANKL is present in osteoblasts and is responsible for the initiation of bone resorption. In addition, RANKL stimulates osteoclastogenesis and osteoclast activity by binding to the cell surface receptor RANK, which is located on osteoclast precursors and mature osteoclasts (Kawashima et al. 2007). This binding leads to the activation of specific signaling pathways that is involved in the formation and survival of osteoclasts in bone resorption. Meanwhile, OPG which is secreted by osteoblasts also acts as a soluble decoy receptor for RANKL (Saidak and Marie 2012). Thus, RANKL and OPG appear to be the key regulators in controlling the bone remodeling process (Whyte 2013).

Understanding the role of cytokines in bone metabolism shall provide insights into the mechanisms that regulate the development of bone related diseases and could lead to new therapies for bone healing. Our preliminary study showed that treatments with rIL-6 or rIL-17A respectively enhanced the proliferation and alkaline phosphatase activities in dose dependent manner (Sritharan et al., 2014). Therefore, further studies on the regulatory properties of IL-6 and IL-17 are crucial to understand their roles on osteoblasts formation and bone metabolism. The aim of this study is to evaluate the role of IL-6 and IL-17A in the modulation of the OPG/RANKL system of murine osteoblast cell line (MC3T3-E1).

Methodology

Cell Culture

MC3T3-E1 cell line was cultured in Alpha minimum essential medium (α-MEM) (Nacalai Tesque, Japan) under sterile conditions in a controlled 37 °C temperature and 5 % CO_2 humidified atmosphere. The cells were supplemented with 10 % heat-inactivated fetal bovine serum (GIBCO, UK) and 1 % penicillin/streptomycin antibiotics. After seeding cells, treatment was carried out in 24 h. The cells were treated with rIL6 ± rIL17A (eBiosciences, USA) at 10 ng/ml based on our previous study (Sritharan et al. 2014). The cells were divided into 4 groups i.e. MC3T3-E1 alone, MC3T3-E1 + rIL6, MC3T3-E1 + rIL17A and MC3T3-E1 + rIL6 + rIL17A.

Intracellular Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was measured by quantifying the release of p-nitrophenol (pNP). The ALP assay was carried out using alkaline phosphatase activity kit (Biovision, USA). The cells were seeded into a 24-well plate at a density of 1×10^5 cells per well. The cells were incubated for 1, 3, 7, 10 and 14 days. At the harvest day, the supernatant was removed and the cells were homogenized in assay buffer. The assay was carried out according to the manufacturer's instructions (Biovision, USA). Finally, the optical density (OD) was read at 405 nm. ALP activity was calculated as follows: ALP activity (U/ml) = amount of pNP generated by samples/volume/time.

Validation of Bone Regulatory Genes Using Real-Time PCR

Real-time PCR was performed on ALP, RANKL and OPG genes. Total RNA was isolated from three biological replicates of the treated and untreated samples and converted to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). Primers and probes for qRT-PCR were purchased from the Applied Biosystems, USA and the assay was performed as described by the manufacturer. The assay was conducted using the ABI 7500 Real-Time PCR System (Applied Biosystems, USA) with the following conditions: 50 °C for 2 min, 95 °C for 20 s for enzyme activation, 40 cycles of denaturation at 95 °C

for 3 s, 60 °C for 33 s for annealing and extension. The expression level was estimated by the Applied Biosystems 7500 Software v2.0.

OPG and RANKL Expression by Western Blotting

MC3T3-E1 cells were washed and lysed using RIPA buffer. The lysates were centrifuged at 13,000 rpm for 20 min and the supernatants were denatured using Laemmli buffer. Lysates were fractionated on 12 % (w/v) polyacrylamide gels using electrophoresis method before transferring to polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 5 % skimmed milk solution for an hour before incubation with OPG mouse anti-goat antibody and RANKL mouse anti-mouse antibody for 2 h at 37 °C followed by incubation with the secondary HRP-antibody for 1 h at 37 °C. Chemiluminescence was generated by an ECL western blot detection reagent. An antibody specific to the house keeping gene, GAPDH was used as a control. Integrated density of the band was measured using Image J software where the integrated OPG and RANKL density was normalized with integrated density of GAPDH.

Detection of OPG and RANKL Secretion by ELISA

The OPG and RANKL expressions were determined from the culture supernatants of the treated and untreated cells by using Raybio mouse ELISA (Enzyme-Linked Immunosorbent Assay) kits, according to the manufacturer's instructions. This assay employs an antibody specific for mouse OPG and RANKL coated on a 96-well plate. The optical density (OD) of 450 nm was measured using spectrophotometer (Bio-rad, USA) and the concentration (pg/ml) was determined by comparison with the standard curve. Untreated cells were used as a control.

Statistical Analyses

Statistical analyses were performed using the Statistical Package of Social Sciences (SPSS) software, version 20. The data obtained from independent experiments (n = 3) were presented as the mean ± standard deviation (SD). The data obtained from ALP, real-time, western blot and ELISA were analyzed using one way ANOVA followed by Post Hoc multiple comparison test, Bonferroni procedures. The assumptions of normality, homogeneity of variance and compound symmetry were checked and fulfilled. For all analyses, p value of <0.05 was considered statistically significant.

Results

ALP Activity

ALP activity was measured on day 1, 3, 7, 10 and 14 as shown in Fig. 1. The ALP assay showed that all the treated cells secreted significantly higher levels of ALP compared to the untreated group from day 1 onwards and peaked at day 14 ($p < 0.05$). Remarkably, the ALP activity was significantly increased at day 10 of the group treated with combination cytokines (MC3T3-E1 + rIL6 + rIL17A) ($p < 0.05$) compared to the other treated groups. At day 14, similar significant ALP activity was observed in MC3T3-E1 + rIL6 ($p < 0.05$) group. Extensive increase of ALP activity was detected at day 14 compared to day 1, 3, 7 and 10 in all the treated groups.

Validation Analysis of Specific Genes Using qRT-PCR

The expression of specific bone regulatory genes was examined using qRT-PCR. Values of log fold-change for the up-regulated and down-regulated genes are shown in Fig. 2. GAPDH was used as an internal control. ALP gradually increased and peaks at the phases of mineralization at day 14. Significant up-regulation of the relative fold changes was shown by OPG gene by all the treated groups when compared to the untreated group ($p < 0.05$). OPG showed the highest ($p < 0.05$) relative fold changes

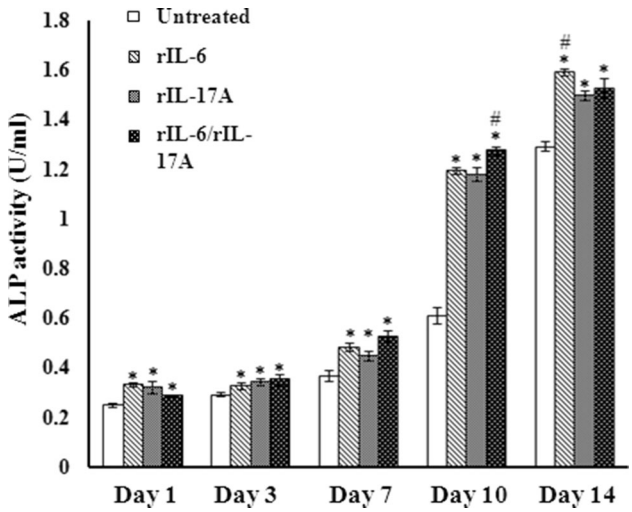

Fig. 1 ALP activity of MC3T3-E1 cells treated with IL-6 and/or IL-17A. ALP activity was measured during the early proliferation period on day 1, 3, 7 and during osteoblastic differentiation period on day 10 and 14. Cells with media alone were used as control. The data presented as mean ± S.D. of quadruplicate cultures. $^{*}p < 0.05$, significantly different from the untreated group. $^{\#}p < 0.05$, significantly different within the treated groups

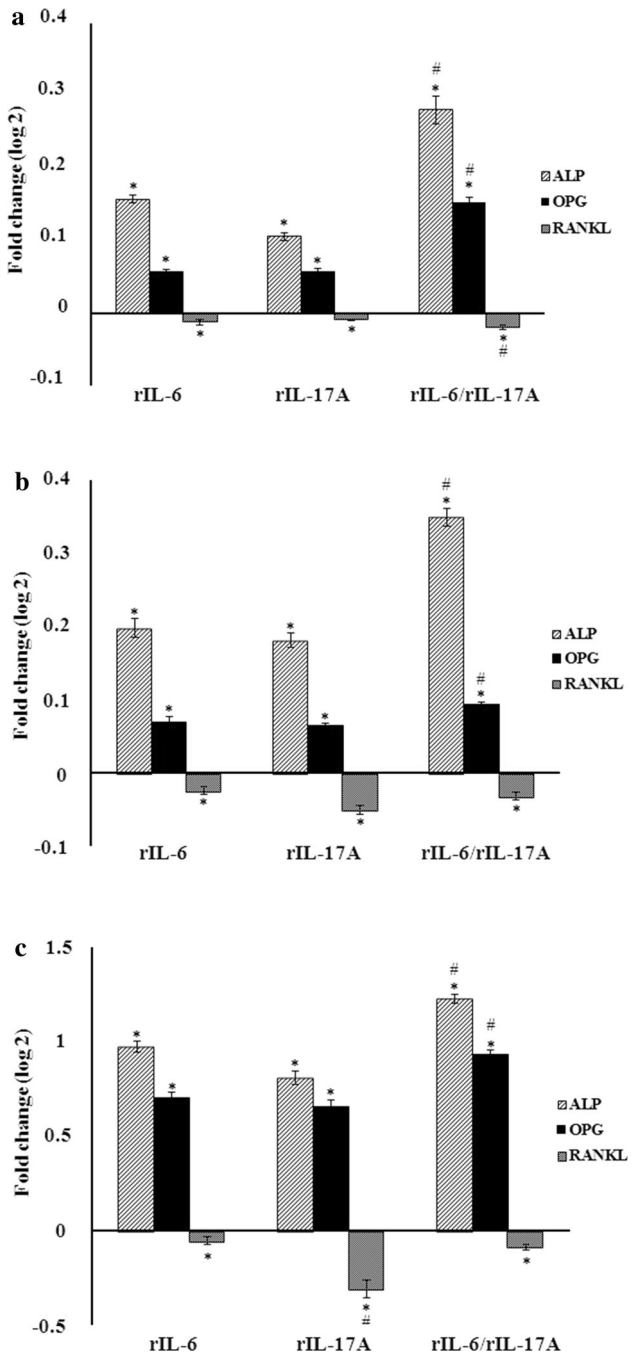

Fig. 2 Validation of gene expression (log fold-change) of selected genes (OPG, RANKL, and ALP genes) in the treated group (n = 3) relative to the untreated group (n = 3) using qRT-PCR. The log fold-change on day **a** 3, **b** 7 and **c** 14. $^{*}p < 0.05$, significantly different from the untreated group. $^{\#}p < 0.05$, significantly different within the treated groups

in MC3T3-E1 + rIL-6 + rIL-17A-treated sample in all treatment days (day 3, 7 and 14) compared to the other treated samples. RANKL, a specific osteoclast marker, gradually decreases in the relative fold changes within the treated groups at all the treated days ($p < 0.05$). Day 14 demonstrated a drastic increase of the OPG and decrease in RANKL expression in all the treated groups. Overall, the cells treated with combination of both cytokines showed significant increase in the OPG expression.

The Expression of OPG and RANKL Proteins in MC3T3-E1 Cells in Response to rIL-6 ± rIL-17A

Immunoblots demonstrated the appearance of a band at approximately 35 and 60 kDa respectively indicating the presence of OPG and RANKL proteins in both treated and untreated cells (Fig. 3). Overall, significant increase in the expression of OPG could be detected at day 7 and 14; while significant reduction of RANKL could be observed at day 3, 7 and 14 in the cells treated with rIL-6 + rIL-17A and rIL-17A (Fig. 3b). In addition, MC3T3-E1 cells treated with combination cytokines demonstrated higher OPG protein expression compared to the untreated and the other treated samples. In contrast, RANKL was expressed higher in the cells treated with rIL-6 alone compared to the other treated groups. Interestingly, at day 7 and 14, OPG expression was significantly elevated in the presence of combination cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) and MCT3T3-E1 + rIL-17A group ($p < 0.05$), while significant decreased in RANKL expression could be also detected in both samples. On the other hand, MCT3T3-E1 + IL-6 has no significant difference in OPG and RANKL at day 3 and 7, however at day 14, OPG marked a significant decrease. The combination cytokines showed significant expression of OPG among all the treated samples on day 7 and 14, hence RANKL was significantly reduced on day 14.

OPG and RANKL Production by MC3T3-E1 Cells in Response to rIL-6 ± rIL-17A

The OPG and RANKL productions were detected in all culture supernatants of the treated and untreated cells. In the treated cells, the OPG production was significantly higher compared to the untreated cells ($p < 0.05$). In the presence of both combination cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) the OPG production was markedly increased ($p < 0.05$) than the other treated samples. All treatment days showed similar OPG production patterns and it was noted that day 14 showed the highest production of OPG (Fig. 4a). In contrast, RANKL production in the treated samples showed no significant difference compared to the untreated cells. Meanwhile, day 3 expressed the lowest RANKL production compared to day 7 and 14 (Fig. 4b).

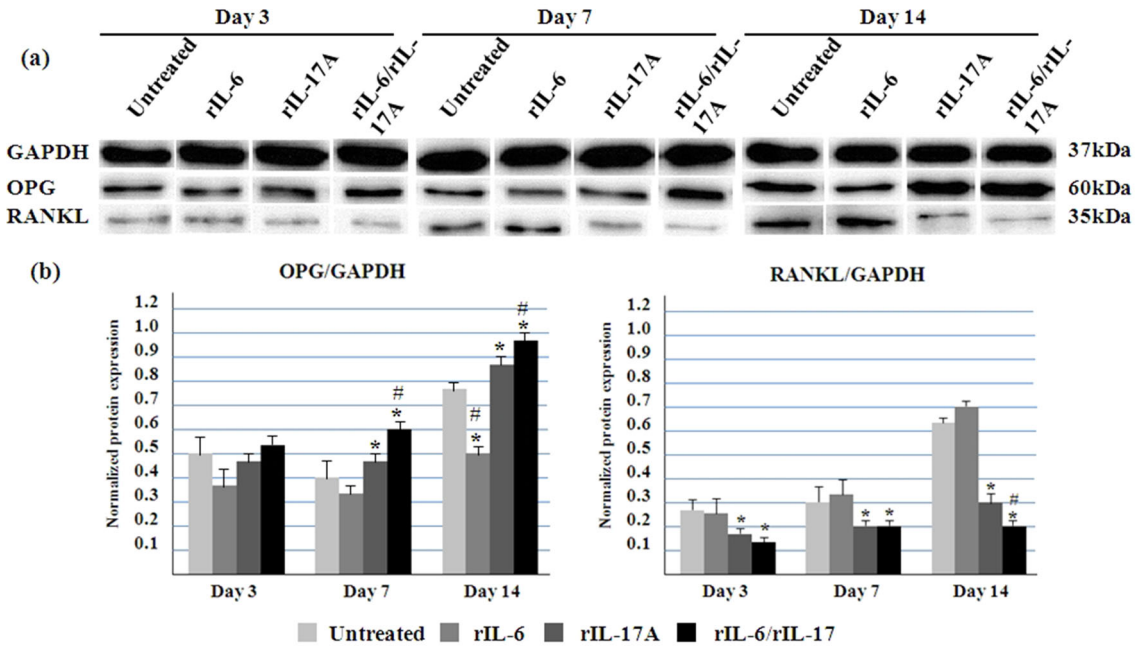

Fig. 3 Expression of OPG and RANKL proteins in MC3T3-E1 cells treated with rIL-6 ± rIL-17A at day 3, 7 and 14. **a** Representative Western blot results of OPG, RANKL and GAPDH proteins **b** Integrated density of OPG and RANKL bands for each sample were normalized to GAPDH. Data are represented as mean intensity of protein expression ± SD for three independent experiments. $^{*}p < 0.05$ is considered significantly different compared to the untreated group. $^{\#}p < 0.05$, significantly different within treated the groups

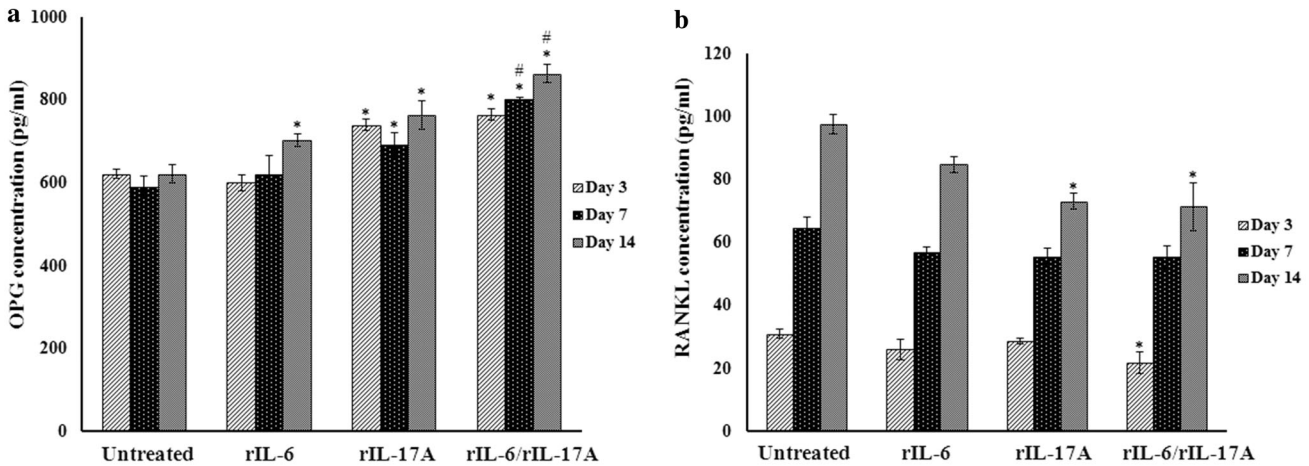

Fig. 4 Production of **a** OPG and **b** RANKL in MC3T3-E1 cells treated with rIL-6 ± rIL-17A and incubated for 3, 7 and 14 days. Data are represented as mean concentration of protein ± SD for three independent experiments. $^{*}p < 0.05$ is considered significantly different compared to the untreated group, $^{\#}p < 0.05$, significantly different within the treated groups

Discussion

Bone is a specialized connective tissue that is continually remodeled according to physiologic events. This remodeling results from involvement of many cell types, primarily osteoblasts and osteoclasts (Bellido et al. 2014). Several precursors have been known to play an important role in bone formation, activation and modulating bone remodeling. This includes OPG and RANKL mechanism in balancing osteoblast and osteoclast activity in bone regulation (Giner et al. 2009a). The OPG-RANKL pathway is an essential signaling pathway involved in cell interaction as well as in osteogenic mechanism where modification of this pathway has major effects on bone remodeling and bone related diseases (Giner et al. 2009b; Pilichou et al. 2008; Pivonka et al. 2010). OPG encodes for osteoblastic

development, which favors bone formation and thus OPG deficiency boosts osteoclastic bone resorption or osteoporosis. OPG is recognized as a decoy receptor for RANKL, the osteoclastic differentiation factor (Udagawa et al. 2000). A study indicated that, increased level of OPG has the tendency to inhibit excessive RANKL production and leads to improved bone metastasis in vivo (Haynes et al. 2003). Various findings have reported a profound connection linking RANK/RANKL/OPG system with regulation on bone development (Carda et al. 2005; Kuhn et al. 2012). Research related to bone tissue tested the distribution of joint development with the OPG and RANKL regulators and a successful coupled mechanism of bone remodeling were observed (Carda et al. 2005). A previous report suggested OPG/RANKL ratio was used to evaluate the activity of adipocyte-secreted factors on bone formation (Kuhn et al. 2012). Another study utilized RANK/RANKL/OPG system to measure the oxidative and anti-oxidative properties of metals in bone activity (Brzoska and Rogalska 2013). It has been also specified that bone diseases such as rheumatoid arthritis, cancerous bone metastases (osteolytic metastases) and osteoporosis are caused by alteration and imbalanced in the network of RANK/RANKL/OPG (Hofbauer et al. 2004; Jones et al. 2002; Terpos et al. 2005; Wada et al. 2006). An experiment was conducted to study the bone metastases in breast cancer patient by assessing the OPG/RANK/RANKL markers (Ibrahim et al. 2011). Therefore, modulation of OPG/RANKL/RANK pathway is expected to hold more promising roles on understanding the bone regulation and preventing bone diseases which are yet to be discovered.

ALP is a multifunctional enzyme that often used as an important marker for osteoblast activity (Sun et al. 2009). During osteogenic differentiation, ALP is involved in the differentiation pathway by expressing matrix-associated proteins which is released during bone mineralization (Fauran-Clavel and Oustrin 1986). In this study, high levels of ALP were released in the treated MC3T3-E1 cell line suggesting that these cytokines were effective regulators for bone formation (Fig. 1). This could indicate a pattern resembling the bone mineralization activity by both rIL-6 and rIL-17A cytokines, where ALP secretion gradually increases in the treated groups. Similarly, IL-6 has also been proven to have steady effects on osteoblast differentiation directly (Itoh et al. 2006). Many studies have proven that IL-6 induces osteoblast differentiation in vitro (Li et al. 2008; Nishimura et al. 1998; Taguchi et al. 1998). IL-6 also modulates osteoblastic differentiation by increasing ALP activity in periodontal ligament cells (Iwasaki et al. 2008). On the other hand, IL-6 had been proven to have negative effects on osteoblast differentiation (Kaneshiro et al. 2013). It is also believed that IL-6 might be involved indirectly on osteoblast differentiation by activating two distinct signaling pathways, namely JAK-STAT and MAP kinase. Nonetheless, previous reports regarding the effects of IL-6 on osteoblast differentiation have been inconsistent on its exact roles (Franchimont et al. 2005). Meanwhile, IL-17 is known to signal the proliferation and differentiation of human mesenchymal stem cells and capable of enhancing bone formation (Huang et al. 2009). A study indicated that IL-17 alone is able to increase ALP activity (Osta et al. 2014), while its inhibitory roles have also been proven in osteoblast differentiation in vivo (Kim et al. 2014).

Therefore, overall this could explain a steady differentiation pattern of cells induced by rIL-6 or rIL-17A or combination of both in the present study. Apparently, day 10 and day 14 showed remarkable increase in ALP activity than day 1, 3 and 7, as it is expected to be high during peak of differentiation period. It was noted at day 10, the combination treatment showed significant increased of ALP level compared to the other treated groups. This might explain the synergistic effect of both cytokines which enhancing higher ALP activity. However, at day 14, treatment of MCT3T3-E1 cells with IL-6 alone showed significant increase of ALP activity compared to treatments with rIL17 alone and the combination cytokines. As proven in the previous study, IL-6 might induce mineralization in the bone microenvironment thus increasing high ALP activity at day 14 (Guihard et al. 2012). Overall day 14 expressed a relatively higher ALP activity; the peak in ALP activity generally denotes the initiation stage of bone mineralization.

Following treatment with rIL-6 and rIL-17A, the expressions of *OPG* and *ALP* genes were increased in time dependent pattern, in contrast to the *RANKL* expression that was decreased in time dependently (Fig. 2a–c). *ALP* gene was expressed at higher levels in all the treated samples. Interestingly, treatment of osteoblast cells with rIL-6 or rIL-I7A or combination of both cytokines showed down-regulation of *RANKL* gene expression and up-regulation of *OPG* gene expression. The significant increase of *ALP* and *OPG* expressions were identified in the samples treated with combination of rIL-6 + rIL-17A compared to the samples treated with the individual cytokines. In addition, treatment of cells with rIL-6 or rIL-17A or combination of both cytokines had improved the OPG/RANKL ratio thus indicating enhancement of osteogenesis mechanism. The results indicated that combination of rIL-6 + rIL-17A has synergistic role on the OPG/RANKL system and might have regulatory role on bone remodeling. These cytokines were reported to influence osteoblast and osteoclast differentiation via OPG/RANKL/RANK signaling pathway as indicated in the previous studies (Axmann et al. 2009; Huang et al. 2009; Lorenzo 2011; Palmqvist

et al. 2002; Weitzmann et al. 2002; Yoshitake et al. 2008). In this study, we demonstrated that the combination treatment of rIL-6 and rIL-17A has promoted osteogenesis as indicated by the gene expression study. Previous reports have highlighted the direct role of IL-6 on osteoclasts and indirect role on osteoblasts that leads to osteoclastogenesis through RANKL signaling pathway (Axmann et al. 2009; Hashizume and Mihara 2011; O'Brien et al. 1999). However, limited findings are available on the direct involvements of IL-6 on osteoblast (Duplomb et al. 2008). A study has indicated that IL-6 has been proven to have steady effects on osteoblast differentiation directly (Itoh et al. 2006). Yoshitake et al. (2008) demonstrated the suppression role of IL-6 on osteoclast progenitor via an inhibition of RANK signaling pathway. In addition, the study also suggested that IL-6 might play an important role in preventing excessive bone resorption during steady-state bone remodeling. In contrast, it has been reported that IL-6 when treated independently leads to osteoclastogenesis (Axmann et al. 2009; O'Brien et al. 1999). According to Shaama (2005), IL-6 triggers osteoclastogenesis in the presence of other precursors, as it is secreted by osteoblasts when stimulated by other cytokines which initiates osteoclast formation (Frost et al. 2001). Our study indicated that at the gene level, the direct involvement of IL-6 has no negative feedback towards osteoblast cells, instead it could contribute to a promising outcome towards osteogenesis.

On the other hand, IL-17 is predicted to be more favorable for bone disruption and also been proven to act as a protective precursor in bone loss (Goswami et al. 2009). However, IL-17 could act as a retroactive mechanism which could modify bone resorption into bone formation phases (Kwan Tat et al. 2008). A previous study has proven that osteoblast regulation were heightened by IL-17 during the initial stage of fracture repair and promoted osteogenesis (Nam et al. 2012). IL-17 has also significantly suppressed bone resorption activity in osteoblast (Kwan Tat et al. 2008). In addition, IL-17 has been proven to have a steady interaction and modulates bone mechanisms when treated in combination with other inflammatory cytokines (Shen et al. 2005). In this study, we demonstrated that the direct involvement of IL-17 in osteoblast could enhance the bone regulation mechanism. Moreover, to date no study has been conducted on the synergic, antagonistic or additive effects of these IL-6 and IL-17 when treated in combination. We believe that both cytokines which has retroactive mechanism independently has a synergic effect when combine together in natural bone and thus favors bone regulation rather than bone disruptions. Overall, the results demonstrated that expression patterns of bone-related genes favors bone formation (ALP and OPG) rather than bone resorption (RANKL). The genes evaluated in the study were important parameters for osteoblast activity in bone formation and the data contributed to further understanding towards molecular mechanism of bone regulation in bone remodeling.

Protein expression study demonstrated no significant difference among all the treated samples at day 3 (Fig. 3). This might be due to early phase of protein regulation and initiation of osteogenic mechanism. Previous studies have stated that the bone regulation mechanisms were usually undetectable at day 3 (Ai-Aql et al. 2008; Cho et al. 2002; Dimitriou et al. 2005). It has been reported that mRNA and protein expressions varies at their optimal peak of production (Schwanhausser et al. 2011; Sullivan et al. 2000). Interestingly, at day 7 and 14, the OPG expression was significantly elevated and the RANKL expression was significantly decrease in the presence of combination cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) and MCT3T3-E1 + rIL-17A (Fig. 3b). This explains the increase of osteogenic property in the samples, as day 7 and 14 portray the peak of proliferation and the most active osteogenesis phase (Ai-Aql et al. 2008; Dimitriou et al. 2005; Kon et al. 2001). Protein expression indicated that treatment of MC3T3-E1 + rIL-6 + rIL-17A demonstrated the highest OPG expression on day 7 and 14 compared to treatments with rIL-6 or rIL-17A respectively. This confirmed that combination of rIL-6 + rIL-17A has direct evidence for the differential expression of proteins which favor osteogenic activity. Findings highlights OPG/RANKL pathway as an important aspect to study local immune response and bone remodeling (Poubelle et al. 2007). In addition, Sood et al. (2011) speculated the essential roles of OPG/RANKL pathway in bone metastases, where the disruption of the OPG protein expression results in osteoporosis and the high RANKL expression leads to tumour stimulation (Sood et al. 2011).

The biochemistry assay further supports that rIL-6 and rIL-17A play a major role in bone regeneration, where it elevates the OPG production significantly higher in combination of cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) and MCT3T3-E1 + rIL-17A compared to the untreated cells (Fig. 4a). In consistent with the previous assays, the combination treatment has a major significant pattern from day 7 to day 14 compared to the other treated samples. This is likely proposing that the combination of cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) has a functional role towards bone remodeling. On the other hand, RANKL production showed less functional effect in ELISA analysis although it showed a significant decrease in combination of cytokines (MCT3T3-E1 + rIL-6 + rIL-17A) and MCT3T3-E1 + rIL-17A-treated samples at day 14 (Fig. 4b).

In contrast with gene expression where mRNA level of *OPG* was elevated when treated with rIL-6 and rIL-17 independently, no significant increase in OPG protein

expression was observed when treated with the rIL-6 respectively. Nonetheless, IL-6 showed a significant decrease of OPG expression at day 14, which is a negative response towards osteogenesis. The similar pattern was noted in a study by Grandjean-Laquerriere et al. (2004) where osteoblasts showed significant elevation in mRNA level of IL-18 but no protein expression was reported (Grandjean-Laquerriere et al. 2004). These might be due to cytokines ability to emit strong signals for transcription but inadequate signals for translation (Vogel and Marcotte 2012). Besides, we postulate that other precursors might have influence to decrease the OPG/RANKL expressions via stimulating different pathways and not favoring bone regulation, due to the retroactive process. However, it is proposed that the regulatory role of IL-6 is depending on the microenvironment conditions (Hidalgo et al. 2011; Yoshitake et al. 2008). In accordance to our results, gene and protein analyses indicate positive effects on MC3T3-E1 when treated in combination with rIL-6 + rIL-17A or rIL-17A alone. Remarkably the combination cytokines (rIL-6 + rIL-17A) shows excellent regulation on osteogenesis. This may contribute to a promising outcome towards bone regeneration at the molecular level.

Conclusion

Our study demonstrated that the synergistic effects of rIL-6 and rIL-17A significantly promoted the differentiation and mineralization of MC3T3-E1 murine osteoblast cells. The findings suggest that the combination treatment of rIL-6 and rIL-17A could serve as a potent osteoblastogenesis enhancing regulator despite expressing contrasting regulatory mechanism when treated independently with cells. However, further studies are needed to understand the regulatory role of rIL-6/rIL-17A on RANK or other signaling pathways.

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Compliance with Ethical Standards

Conflict of Interest No conflict exists: Author A declares that he/she has no conflict of interest. Author B declares that he/she has no conflict of interest. Author C declares that he/she has no conflict of interest. Author D declares that he/she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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