Title	IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways in vitro
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1 **Original Article**

2 IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways *in*

3 *vitro*

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24   **Abstract**

25   It has been suggested that interleukin-6 (IL-6) plays a key role in the pathogenesis of rheumatoid arthritis

26   (RA), including osteoporosis not only in inflamed joints but also in the whole body. However, previous *in*

27   *vitro* studies regarding the effects of IL-6 on osteoblast differentiation are inconsistent. The aim of this

28   study was to examine the effects and signal transduction of IL-6 on osteoblast differentiation in

29   MC3T3-E1 cells and primary murine calvarial osteoblasts. IL-6 and its soluble receptor significantly

30   reduced alkaline phosphatase (ALP) activity, the expression of osteoblastic genes (Runx2, osterix, and

31   osteocalcin), and mineralization in a dose-dependent manner, which indicates negative effects of IL-6 on

32   osteoblast differentiation. Signal transduction studies demonstrated that IL-6 activated not only two major

33   signaling pathways, SHP2/MEK/ERK and JAK/STAT3, but also the SHP2/PI3K/Akt2 signaling

34   pathway. The negative effect of IL-6 on osteoblast differentiation was restored by inhibition of MEK as

35   well as PI3K, while it was enhanced by inhibition of STAT3. Knockdown of MEK2 and Akt2 transfected

36   with siRNA enhanced ALP activity and gene expression of Runx2. These results indicate that IL-6

37   negatively regulates osteoblast differentiation through SHP2/MEK2/ERK and SHP2/PI3K/Akt2

38   pathways, while affecting it positively through JAK/STAT3. Inhibition of MEK2 and Akt2 signaling in

39   osteoblasts might be of potential use in the treatment of osteoporosis in RA.

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Introduction

Inflammation-mediated bone loss is a major feature of various bone diseases, including rheumatoid

arthritis (RA). Interleukin-6 (IL-6) contributes to the development of arthritis and is present at high

concentrations in the serum and synovial fluid of patients with RA [1-4]. Soluble IL-6 receptor (sIL-6R)

is also elevated in the serum and synovial fluid of RA patients [5, 6], and IL-6 exerts its action by binding

either to its membrane-bound receptor (mIL-6R) or to sIL-6R. Moreover, IL-6 is closely associated with

the expression of receptor activator of NF-κB ligand (RANKL) in osteoblasts [7]. That is to say, IL-6 acts

indirectly on osteoclastogenesis by stimulating the release of RANKL by cells within bone tissues such as

osteoblasts [8]. It can unquestionably be said that IL-6 plays a major role in the pathogenesis of RA

[9-12], including osteoporosis not only in inflamed joints but also in the whole body.

There have been several studies on the effect of IL-6 on bone turnover in animal models. In IL-6

knock-out mice, microstructure abnormalities in cortical bones and delayed fracture healing were

observed [13, 14], in spite of the evident normal phenotype [15]. Also, bone loss after estrogen depletion

was mitigated in IL-6-deficient mice, while a high level of IL-6 and bone loss are seen in wild-type mice

[13]. Moreover, IL-6-overexpressed-transgenic mice develop osteopenia and defective ossification, in

which the activity of mature osteoblasts is significantly decreased [16]. All these findings, together with

studies on human RA patients [17, 18], indicate that IL-6 plays a major role in bone turnover and is an

important regulator of bone homeostasis.

60   Recently, several biological agents have been introduced for the treatment of RA and have

61   demonstrated not only potent anti-inflammatory effects but also inhibitory effects on joint destruction.

62   Among these biological agents, tocilizumab, an anti- IL-6 receptor antibody, has been reported to increase

63   serum bone formation markers in RA patients [19], suggesting that IL-6 has a negative effect on

64   osteoblast differentiation. However, previous reports regarding the effects of IL-6 on osteoblast

65   differentiation *in vitro* have been inconsistent [20]. IL-6 has been shown to decrease the expression of

66   differentiation markers in osteoblasts [21, 22] and to inhibit bone formation [23], while it has been shown

67   to induce osteoblast differentiation [24, 25].

68   Binding of IL-6 with sIL-6R or mIL-6R leads to subsequent homodimerization of the

69   signal-transducing molecule gp130, followed by activation of two major intracellular signaling pathways,

70   Janus protein tyrosine kinase (JAK)/ signal transducer and activator of transcription factors (STAT) 3, or

71   Src-homology domain 2 containing protein-tyrosine phosphatase (SHP2)/ mitogen-activated protein

72   kinase-extracellular signal–regulated kinase kinase (MEK)/ mitogen-activated protein kinase (MAPK),

73   also called extracellular signal-regulated kinase (ERK) [26]. There have been many reports in which the

74   effects of IL-6 on JAK/STAT3 and SHP2/ERK signal transduction pathways have been studied in

75   osteoblasts, though it is still controversial whether differentiation is enhanced by IL-6 [9, 20]. SHP2 can

76   also form a tertiary complex with the scaffolding proteins Gab1/2 and the p85 subunit of

77   phosphatidylinositol-3-kinase (PI3K) [27], which leads to activation of the Akt pathway. Several papers

78   have so far reported that the PI3K/Akt pathway triggered by IL-6 plays important roles in various cells

79   [28-32], but no reports have been published regarding the effect of IL-6 on this pathway in osteoblasts.

80   The purpose of this study was to clarify the effect of IL-6 on osteoblast differentiation *in vitro*,

81   with consideration of intracellular signaling pathways in murine MC3T3-E1 osteoblastic cells and

82   primary murine calvarial osteoblasts.

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93   **Materials and methods**

94   **Ethics statement**

95   Prior to the study, all experimental protocols were approved by the Ethics Review Committee for Animal

96   Experimentation of Osaka University School of Medicine.

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98   **Cell culture**

99   MC3T3-E1 osteoblastic cells were purchased from Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells

100   were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS;

101   Equitech-Bio, Kerrville, TX, USA) and 1% penicillin and streptomycin at 37°C in a humidified

102   atmosphere of 5% CO_2. All media were purchased from Life Technologies Japan (Tokyo, Japan). Murine

103   primary osteoblasts were isolated from the calvariae of 3-days-old C57BL/6 mice (Charles River

104   Laboratories Japan, Inc, Osaka, Japan) by sequential collagenase digestion as described previously [33].

105   MC3T3-E1 cells and murine calvarial osteoblasts were seeded at 1×10^5 cells per well in 12-well

106   plates. After the cells reached confluence, the medium was replaced to induce osteoblast differentiation.

107   The differentiation medium contained 10% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid

108   in the absence or presence of recombinant mouse (rm) IL-6 (R&D Systems, Inc., Minneapolis, MN,

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109   USA) (10, 50 ng/mL), and rm sIL-6R (R&D Systems) (100 ng/mL). The medium and reagents were

110   renewed every 3 days.

111   To study signal transduction, the following inhibitors or vehicle (DMSO) (Sigma-Aldrich,

112   St.Louis, MO, USA) were added to culture medium at several concentrations; MEK inhibitor (U0126; 1,

113   2.5, 5 μM; Cell Signaling Technology, Danvers, MA, USA), STAT3 inhibitor (V Stattic; 2.5, 5 μM;

114   Calbiochem, La Jolla, CA, USA), PI3K inhibitor (LY294002; 1, 2.5, 5 μM; Cell Signaling Technology),

115   and SHP2 inhibitor (PHPS1; 5, 20, 40 μM; Sigma-Aldrich). These inhibitors were added 1 h before

116   treatment with IL-6/sIL-6R. All inhibitors were maintained until the end of the culture period at the

117   indicated concentrations.

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119   **Alkaline phosphatase (ALP) staining and activity**

120   MC3T3-E1 cells and murine calvarial osteoblasts were treated with or without IL-6/sIL-6R and signal

121   pathway inhibitors after the cells reached confluence and were incubated for 6 days.

122   For ALP staining, after fixation with 10% formalin, cells were washed twice with

123   phosphate-buffered saline (PBS) (pH 7.4) and incubated with ALP substrate solution, 0.1 mg/ml naphthol

124   AS-MX (Sigma-Aldrich), and 0.6 mg/ml fast violet B salt (Sigma-Aldrich) in 0.1 M Tris-HCl (pH 8.5)

125   for 20 min.

126   To measure ALP activity, cells were washed twice with PBS and lysed in Mammalian Protein

127   Extraction Reagent (Pierce, Rockford, IL, USA) according to manufacturer's protocol. ALP activity was

128   assayed using *p*-nitrophenylphosphate as a substrate by an Alkaline Phosphatase Test Wako (Wako Pure

129   Chemicals Industries, Ltd., Osaka, Japan), and the protein content was measured using the Bicinchoninic

130   Acid Protein Assay Kit (Pierce).

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132   **Proliferation assay**

133   MC3T3-E1 cells were cultured in 96-well plates at a concentration of 2.0×10^4 cells/cm^2 in α-MEM

134   containing 10% FBS. Cells were incubated for 1 day, after which the medium was treated with

135   IL-6/sIL-6R for 3 days. Cell proliferation was assessed using the Premix WST-1 Cell Proliferation Assay

136   System (Takara Bio, Inc., Otsu, Japan) according to the manufacturer' s instructions. We performed this

137   assay every 24 h.

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139   **Alizarin red staining**

140   After fixation with 10% formalin, MC3T3-E1 cells and murine calvarial osteoblasts were washed with

141   distilled water, and stained with alizarin red S solution (Sigma-Aldrich) (pH 6.0) for 10 min, followed by

142   incubation in 100 mM cetylpyridinium chloride for 1 h at room temperature to dissolve and release

143   calcium-bound alizarin red. The absorbance of the released alizarin red was then measured at 570 nm

144   [34]. To measure the value of absorbance for alizarin red, the absorbance data were normalized by total

145   DNA content. Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf,

146   Germany).

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148   **Knockdown of MEK1, MEK2, Akt1 and Akt2 using RNA interference**

149   MC3T3-E1 cells were transfected with small interfering RNAs (siRNA) using Lipofectamine RNAiMAX

150   (Life Technologies Japan) according to the reverse transfection method in the manufacturer's protocol.

151   The siRNAs for MEK2, Akt1 and Akt2 and that for MEK1 were purchased from Cell Signaling

152   Technology and Qiagen, respectively, with negative controls for each molecule. MC3T3-E1 cells

153   transfected with siRNA were seeded in 24-well plates at a concentration of 1.0×10^4 cells/cm^2 for 48 h.

154   The medium was then replaced with differentiation medium with vehicle or with 20 ng/ml IL-6 and 100

155   ng/ml sIL-6R and the cells were incubated for 3 days prior to use for further experiments.

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157   **Western blotting**

158 Cells cultured in 6-well plates for 2 days were washed twice with PBS and then homogenized with 100 μl

159 of Kaplan buffer (150 mM NaCl, 50 mM Tris-HCL pH 7.4, 1% NP40, 10% glycerol, and 1 tablet per 50

160 ml buffer of protease inhibitor cocktail and phosphatase inhibitor cocktail). The lysates were centrifuged

161 at 13,000 rpm for 20 min at 4°C, and the supernatants were used for electrophoresis after a protein assay

162 using bovine serum albumin as standard. Western blotting was performed by use of the following

163 antibodies purchased from Cell Signaling Technology, except for phosphate anti-Akt2 antibody from

164 Enogene Biotech (New York, NY, USA): phosphate anti-STAT3 (Tyr705) (1:2000) and anti-STAT3

165 (1:1000); phosphate anti-Akt (Ser473) (1:2000), phosphate anti-Akt2 (Ser474) (1:1000), anti-Akt1,

166 anti-Akt2, and anti-Akt (1:1000); phosphate anti-ERK (Thr202/Tyr204) (1:2000), anti-MEK1,

167 anti-MEK2 and anti-ERK (1:1000); and phosphate anti-SHP2 (Tyr542) (1:1000). To control for protein

168 loading, blots were additionally stained with anti-β actin antibody (1:1000).

169

170 **Reverse transcription polymerase chain reaction (RT–PCR)**

171 Total RNA was extracted from cells with an RNeasy Mini Kit (Qiagen), and first-strand cDNA was

172 synthesized using SuperScript II RNase H-reverse transcriptase (Life Technologies Japan). Then PCR

173 was performed using Ex Taq (Takara Bio) and the following primers:

174   *Osteocalcin* (forward primer 5′-CTCACTCTGCTGGCCCTG-3′; reverse primer

175   5′-CCGTAGATGCGTTTGTAGGC-3′);

176   *Osterix* (forward primer 5′-AGGCACAAAGAAGCCATAC-3′; reverse primer

177   5′-AATGAGTGAGGGAAGGGT-3′);

178   *Runx2* (forward primer 5′-GCTTGATGACTCTAAACCTA-3′; reverse primer

179   5′-AAAAAGGGCCCAGTTCTGAA-3′);

180   *GAPDH* (forward primer 5′-TGAACGGGAAGCTCACTGG-3′; reverse primer

181   5′-TCCACCACCCTGTTGCTGTA-3′).

182

183   **Quantitative real-time PCR analysis**

184   We obtained cDNA by reverse transcription as mentioned above, and proceeded with real-time PCR

185   using a Light Cycler system (Roche Applied Science, Basel, Switzerland). The SYBR Green assay using

186   a Quantitect SYBR Green PCR Kit (Qiagen), in which each cDNA sample was evaluated in triplicate 20

187   µl reactions, was used for all target transcripts. Expression values were normalized to GAPDH.

188

189   **Statistical analysis**

190   The results are expressed as the mean ± standard error (SE). Between-group differences were assessed

191   using the ANOVA test. A probability value of <0.05 was considered to indicate statistical significance.

192   **Results**

193   **IL-6/sIL-6R does not affect proliferation, but significantly reduces ALP activity and expression of**

194   **osteoblastic genes in MC3T3-E1 cells**

195   We first measured the proliferation of MC3T3-E1 cells with IL-6. Cell proliferation did not show

196   significant difference in any culture condition (Fig. 1a).

197   To investigate the influence of IL-6 treatment on osteoblast differentiation, we examined ALP

198   activity in MC3T3-E1 cells. As shown in Figs. 1b and 1c, IL-6/sIL-6R significantly reduced ALP activity

199   in a dose-dependent manner. The single addition of sIL-6R did not show a significant difference as

200   compared to the negative control with vehicle. As shown in Figs. 1d and 1e, gene expression of Runx2,

201   osterix and osteocalcin was significantly down-regulated by IL-6/sIL-6R in a dose-dependent manner.

202   Again, the single addition of sIL-6R did not show significant difference as compared to the negative

203   control with vehicle.

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205   **IL-6/sIL-6R significantly inhibits mineralization of extracellular matrix (ECM) in MC3T3-E1 cells**

206   As shown in Fig. 2a, IL-6/sIL-6R significantly inhibited the mineralized area in a dose-dependent

207   manner. The single addition of sIL-6R did not show a significant difference as compared to the negative

208   control with vehicle (Fig. 2a). Quantitative analysis of mineralization by measuring the absorbance of

209   alizarin red revealed significant decrease by IL-6/sIL-6R in a dose-dependent manner (Fig. 2b).

210

211   **IL-6/sIL-6R activates ERK, STAT3 and Akt2 signal transduction pathways in MC3T3-E1 cells**

212   When MC3T3-E1 cells were incubated in the presence of IL-6/sIL-6R, phosphorylation of ERK, STAT3

213   and Akt was clearly observed at 15 min, and their activation became weaker at 30 min. When only

214   sIL-6R was added, there was no apparent activation of ERK, STAT3, or Akt as compared to the negative

215   control (Fig. 3a). As for Akt, the phosphorylation by IL-6/sIL-6R was recognized more strikingly as early

216   as 5 min in a dose-dependent manner, both for whole and for Akt2 only, one of its three isoforms (Fig.

217   3b).

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219   **IL-6-induced activation of ERK is enhanced by blocking the STAT3 signaling pathway, and**

220   **IL-6-induced ERK and Akt signaling pathways negatively regulate each other reciprocally.**

221   The SHP2 inhibitor PHPS1 [35] inhibited IL-6-induced phosphorylation of ERK and Akt to the

222   constitutive level, but did not inhibit STAT3 (Fig. 4a and Supplementary Fig. S1a), suggesting that the

223   downstream pathways of SHP2 are ERK and Akt, not STAT3. The STAT3 inhibitor V Stattic inhibited

224   the phosphorylation of STAT3 but enhanced ERK significantly (Fig. 4a and Supplementary Fig. S1a),

225   suggesting that STAT3 could negatively regulate ERK, which is consistent with previous reports [36].

226   The MEK/ERK inhibitor U0126 completely inhibited both constitutive and IL-6-induced phosphorylation

227   of ERK but enhanced those of Akt. Moreover, the PI3K/Akt inhibitor LY294002 completely inhibited

228   both constitutive and IL-6-induced phosphorylation of Akt but enhanced those of ERK (Fig. 4b and

229   Supplementary Fig. S1b). From these findings, we concluded that IL-6-induced ERK and Akt signaling

230   pathways, both of which are downstream of SHP2, can negatively regulate each other reciprocally.

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232   **The negative effects of IL-6 on osteoblast differentiation are restored by inhibition of MEK, PI3K**

233   **and SHP2, while they are enhanced by inhibition of STAT3.**

234   To identify the intracellular signaling pathways associated with the down-regulation of osteoblast

235   differentiation, the effects of various signal transduction inhibitors, consisting of a MEK inhibitor

236   (U0126), PI3K inhibitor (LY294002), SHP2 inhibitor (PHPS1), and STAT3 inhibitor (V Stattic), were

237   assessed for ALP activity, the expression of osteoblastic genes (Runx2, osterix and osteocalcin), and the

238   mineralization of ECM.

239   The negative effect of IL-6/sIL-6R on ALP activity was restored by treatment with either U0126,

240   LY294002, or PHPS1 in a dose-dependent manner. On the other hand, the negative effect of IL-6/sIL-6R

241   on ALP activity was enhanced by treatment with V Stattic (Fig. 5a). These results indicate that the

242   SHP2-associated signal transduction molecules MEK/ERK and PI3K/Akt have a negative effect on

243   osteoblast differentiation, whereas the JAK-associated molecule STAT3 has a positive effect.

244   The negative effect of IL-6/sIL-6R on the expression of osteoblastic genes (Runx2, osterix and

245   osteocalcin) was also restored by treatment with either U0126, LY294002, or PHPS1 in a dose-dependent

246   manner, while it was enhanced by treatment with V Stattic (Fig. 5b). Moreover, a high dose of PHPS1, 20

247   μM, caused significantly up-regulated expression of osteocalcin.

248   For mineralization of ECM, the negative effect of IL-6/sIL-6R was restored by treatment with

249   either U0126, LY294002, or PHPS1. As with ALP activity and osteoblastic gene expression, the negative

250   effect of IL-6/sIL-6R on mineralization was enhanced by treatment with V Stattic (Figs. 6a and 6b). ALP

251   activity, osteoblastic gene expression, and mineralization of ECM in cells treated only with each inhibitor

252   demonstrated the same behavior (Figs. 5 and 6).

253   Furthermore, the negative effects of ALP activity, osteoblastic gene expression and mineralization

254   of ECM by stimulation with IL-6/sIL-6R were compared between in the presence and in the absence of

255   each inhibitor. The negative effects on osteoblast differentiation by IL-6/sIL-6R showed a tendency to

256   decrease in the presence of each inhibitor, as compared to the absence of inhibitors (Figs. 5 and 6). The

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257　negative effects were decreased by 15-44%, 20-61%, 7-140%, and 21-80% in the presence of U0126,

258　LY294002, PHPS1 and V Stattic, respectively, as compared to the absence of inhibitors. These results

259　indicate that the effects of IL-6/sIL-6R on osteoblast differentiation might be mediated either by

260　MEK/ERK, PI3K/Akt, or JAK/STAT3 pathways.

261

262　**Knockdown of MEK2 and Akt2 via siRNA transfection restores ALP activity and Runx2 gene**

263　**expression**

264　To further confirm the effects of MEK and Akt inhibition on osteoblast differentiation in MC3T3-E1 cells,

265　we studied cell differentiation after knockdown of MEK and Akt. For each protein, RNAs of two

266　isoforms were separately blocked: MEK1 and MEK2 for MEK, and Akt1 and Akt2 for Akt.

267　　　The protein expression level of each molecule was found to be diminished selectively at 48 h after

268　transfection of the respective siRNAs (Fig. 7a). The ALP activity in MC3T3-E1 cells treated with

269　IL-6/sIL-6R was restored by knockdown of MEK2 and Akt2 as compared to that in cells transfected with

270　negative control siRNA. On the other hand, knockdown of MEK1 and Akt1 enhanced the negative effects

271　of IL-6/sIL-6R on ALP activity (Fig. 7b). (ALP activity after transfection with each siRNA without

272　IL-6/sIL-6R demonstrated the same behavior; Fig. 7b.) Quantitative real-time PCR analysis revealed that

273　the gene expressions of Runx2, osterix, and osteocalcin were restored by knockdown of MEK2. On the

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274   other hand, knockdown of Akt2 also restored Runx2, but decreased osteocalcin expression (Fig. 7c),

275   while knockdown of Akt2 without IL-6/sIL-6R caused no significant difference in Runx2 expression (Fig.

276   7b). As was recognized for ALP activity, knockdown of MEK1 and Akt1 enhanced the down-regulation

277   of osteocalcin expression (Figs. 7b and 7c). Also, the negative effects of IL-6/sIL-6R on osteoblast

278   differentiation showed some tendency to decrease with each knockdown compared to those without

279   knockdown. The negative effects were decreased by 2-24%, 4-27%, 7-43%, and 21-26% with knockdown

280   of MEK1, MEK2, Akt1, and Akt2, respectively, as compared to those without knockdown. These results

281   indicate that IL-6 may suppress osteoblast differentiation through MEK2 and Akt2.

282

283   **IL-6/sIL-6R inhibits the differentiation of primary murine calvarial osteoblasts by activating**

284   **phosphorylation of ERK, Akt2, and STAT3**

285   Experiments were repeated with murine calvarial osteoblasts isolated from the calvariae of 3-day-old

286   C57BL/6 mice. As was recognized in MC3T3-E1 cells, IL-6 inhibited ALP activity (Fig. 8a), the

287   expression of osteoblastic genes (Fig. 8b), and mineralization (Figs. 8c and 8d) in a dose-dependent

288   manner. Furthermore, IL-6 induced phosphorylation of ERK, Akt2, and STAT3 (Fig. 8e), which was

289   exactly the same as with MC3T3-E1 cells.

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Discussion

294　We examined the effects of IL-6 and its soluble receptor on the proliferation and differentiation of murine

295　MC3T3-E1 osteoblastic cells and primary murine calvarial osteoblasts. Our results showed that they

296　significantly reduced ALP activity, bone mineralization, and expression of the osteoblastic genes Runx2

297　osterix and osteocalcin, in a dose-dependent manner. From these experiments, we clearly demonstrated

298　that IL-6 inhibited osteoblast differentiation of MC3T3-E1 cells and primary murine calvarial osteoblasts.

299　　　It has been demonstrated that the JAK/STAT3 signaling pathway has important roles both *in vivo*

300　and *in vitro* in the differentiation of osteoblasts [37, 38]. Our results are consistent with previous reports

301　and imply that the activation of STAT3 induced by IL-6 may induce osteoblast differentiation.

302　　　IL-6 activates another major intracellular signaling pathway, SHP2/ERK, and can also lead to the

303　activation of an additional signaling cascade involving SHP2/PI3K/Akt. IL-6-induced activation of PI3K

304　and downstream protein kinase Akt/PKB has been reported to play important roles in the proliferation of

305　prostate cancer cells [30, 31], hepatoma cells [32], and multiple myeloma cells [29]. They were also

306　reported to associate with neuroendocrine differentiation of prostate cancer cells induced by IL-6 [32]. In

307　this study, we focused on the PI3K/Akt pathway triggered by IL-6, because no reports have demonstrated

19

308   the role of IL-6 in the activation of PI3K/Akt signaling pathway in osteoblasts. We have demonstrated for

309   the first time that IL-6-induced activation of Akt2, one of the downstream pathways of SHP2, may be a

310   key player in the negative regulation of osteoblast differentiation induced by IL-6. Among the three

311   isoforms of Akt, Akt1 and Akt2 are highly expressed in osteoblasts [39]. Mice lacking Akt1, the major

312   isoform in bone tissue, exhibit osteopenia [40, 41], and the impact of Akt1 deficiency in osteoblast

313   differentiation and bone development have also been published [39, 42-44], all of which are consistent

314   with our results showing that knockdown of Akt1 signaling by siRNA inhibited osteoblast differentiation.

315   In contrast, Mukherjee et al. reported enhanced osteogenic differentiation in the absence of Akt1 in cell

316   lines [44]. Moreover, they reported that Akt2 was required for BMP2-initiated osteoblast differentiation

317   of cultured murine mesenchymal stem cells but that Akt1 was dispensable in this assay [45], which is

318   inconsistent with our results showing that knockdown of Akt2 signaling by siRNA promoted osteoblast

319   differentiation. These discrepancies might be due to the difference between cell types, i.e.

320   intramembranous (calvariae) cells and endochondral (long bones) cells.

321   In this study, gene expression of osteocalcin, a late osteoblastic differentiation marker, was

322   up-regulated by treatment with a PI3K/Akt inhibitor, but was down-regulated by knockdown of both Akt1

323   and Akt2. Moreover, complete blockade with a high dose (more than 10μM) of the PI3K/Akt inhibitor

324   conversely down-regulated the expression of osteocalcin (data not shown). This discrepancy may be due

325   to the difference between the temporary or partial blockade by the inhibitor and constitutive knockdown

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326   by siRNA. Since bone formation has been reported to increase without impairment of mineralization and

327   resorption even in osteocalcin-deficient mice [46], the expression of osteocalcin may not directly affect

328   bone formation.

329   We have previously reported that osteoblast differentiation was significantly promoted by MEK

330   inhibitor in BMP-2-treated C2C12 cells and MC3T3-E1 cells [47]. Our findings in the present study are

331   consistent with our previous report and others [47-49] at the point that IL-6-induced activation of ERK

332   significantly down-regulated osteoblast differentiation. In addition, our results suggest that there might be

333   different roles in osteoblast differentiation between MEK1 and MEK2. Constitutively active expression of

334   MEK1 has been reported to accelerate bone development both *in vitro* [50] and *in vivo* [51], which is

335   consistent with the results showing that knockdown of MEK1 inhibited osteoblast differentiation in the

336   present study. As for MEK2, there are no reports concerning its roles in osteoblast differentiation, and we

337   are the first to demonstrate that MEK2 may also be a key player in the negative regulation of osteoblast

338   differentiation induced by IL-6. The effects of a MEK inhibitor that inhibits both MEK1 and MEK2 on

339   bone formation are still controversial [52].These controversies might be due to different roles played

340   between MEK1 and MEK2 in osteoblast differentiation, and the effects of MEK inhibitors could depend

341   on which pathway is predominantly inhibited in each study.

342   With respect to intracellular signaling pathways, our results showed that IL-6 triggers three

343   signaling pathways, one of which has a conflicting function with the others. SHP2/ERK and SHP2/Akt2

344 negatively affects osteoblast differentiation, whereas JAK/STAT3 positively affects it (Fig. 9). In other

345 cells, it is often that simultaneous activation of the SHP2/ERK and JAK/STAT3 cascades generate

346 opposing, or at least different signals. In osteoclasts, for example, SHP2/ERK activation inhibits

347 osteoclastogenesis [53], whereas STAT3 is a pro-osteoclastic molecule after phosphorylation on

348 serine727 [54]. In myeloid leukemic M1 cells, STAT3 induces differentiation *in vitro* [55], whereas the

349 SHP2/ERK pathway promotes their proliferation [56]. These examples suggest that the integration of

350 opposing activities transduced by more than one pathway could provide a biologically balanced state in

351 the end, remaining availability to respond to another physiological situation. Indeed, Hirano and

352 colleagues have proposed a "signaling orchestration" model in a single cell, where the balance or

353 interplay of simultaneously generated contradictory signals eventually determines the biological outcome

354 [57]. Thus, the inconsistent results regarding the effects of IL-6 on osteoblast differentiation in previous

355 reports could be explained by which intracellular signaling pathway was predominantly activated in each

356 study. The balance of three signaling pathways could be influenced by such conditions as the variety of

357 cultured cells, the stage of cell differentiation, and the employed culture conditions.

358   To the best of our knowledge, this is the first report of signal crosstalk in which IL-6-induced ERK

359 and Akt signaling pathways negatively regulated each other in cultured osteoblastic cells. In this study,

360 however, cancellation of the negative effects of IL-6/sIL-6R on osteoblast differentiation by inhibitors

361 was incomplete as compared to the absence of inhibitor (Figs. 5 and 6). This might be because ERK, Akt

362 and STAT3 are all critical pathways in osteoblast differentiation even in the absence of IL-6/sIL-6R, and

22

363 even though one pathway is blocked, another pathway is enhanced by reciprocal regulation in the

364 crosstalk between IL-6-activated signaling pathways (Fig. 9). Our results demonstrated that a STAT3

365 inhibitor significantly enhanced IL-6-induced activation of ERK and SHP2, but not of Akt (Fig. 4a).

366 SHP2 could predominantly lead to the activation of the ERK signaling pathway as compared to Akt, and

367 the enhanced signaling of ERK may restrain the enhancement of the Akt signaling pathway in a negative

368 feedback manner.

369   The results obtained from the present study show that SHP2, MEK and PI3K inhibitors would be

370 of potential use for the treatment of osteoporotic changes in RA patients. In particular, SHP2 inhibitors

371 not only could inhibit the negative effect of IL-6-induced MEK/ERK and PI3K/Akt2 signaling, but also

372 enhance the positive effect of IL-6-induced STAT3 signaling on osteoblast differentiation [37]. However,

373 since a pro-inflammatory effect of STAT3 on synovitis has been reported [36, 58], selective inhibition of

374 MEK2 and Akt2 signaling in osteoblasts may be more promising in order to avoid the enhancement of

375 synovitis and consequent joint destruction.

376   In conclusion, our study provides new insights in the pathophysiology as well as potential

377 treatment options for bone loss in RA, focusing on osteoblast differentiation *in vitro*. Our results

378 demonstrated that IL-6 could inhibit osteoblast differentiation through MEK2/ERK and PI3K/Akt2

379 signaling pathways, both of which are SHP2-dependent downstream signaling pathways.

380

381 **Conflict of interest**

382 All authors have no conflicts of interest.

383

384 References

385 1. Hashizume M,Mihara M (2011) The roles of interleukin-6 in the pathogenesis of rheumatoid arthritis.

386 Arthritis 2011:765624

387 2. Ito A, Itoh Y, Sasaguri Y, Morimatsu M,Mori Y (1992) Effects of interleukin-6 on the metabolism of

388 connective tissue components in rheumatoid synovial fibroblasts. Arthritis Rheum 35:1197-1201

389 3. Nishimoto N,Kishimoto T (2004) Inhibition of IL-6 for the treatment of inflammatory diseases. Curr

390 Opin Pharmacol 4:386-391

391 4. De Benedetti F, Robbioni P, Massa M, Viola S, Albani S,Martini A (1992) Serum interleukin-6 levels

392 and joint involvement in polyarticular and pauciarticular juvenile chronic arthritis. Clin Exp Rheumatol

393 10:493-498

394 5. De Benedetti F, Massa M, Pignatti P, Albani S, Novick D,Martini A (1994) Serum soluble interleukin

395 6 (IL-6) receptor and IL-6/soluble IL-6 receptor complex in systemic juvenile rheumatoid arthritis. J Clin

396 Invest 93:2114-2119

24

397 6. Kotake S, Sato K, Kim K J, Takahashi N, Udagawa N, Nakamura I, Yamaguchi A, Kishimoto T, Suda

398 T,Kashiwazaki S (1996) Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from

399 rheumatoid arthritis patients are responsible for osteoclast-like cell formation. J Bone Miner Res 11:88-95

400 7. Kwan Tat S, Padrines M, Theoleyre S, Heymann D,Fortun Y (2004) IL-6, RANKL, TNF-alpha/IL-1:

401 interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 15:49-60

402 8. Palmqvist P, Persson E, Conaway H H,Lerner U H (2002) IL-6, leukemia inhibitory factor, and

403 oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B

404 ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J Immunol

405 169:3353-3362

406 9. Le Goff B, Blanchard F, Berthelot J M, Heymann D,Maugars Y (2010) Role for interleukin-6 in

407 structural joint damage and systemic bone loss in rheumatoid arthritis. Joint Bone Spine 77:201-205

408 10. Hirano T, Matsuda T, Turner M, Miyasaka N, Buchan G, Tang B, Sato K, Shimizu M, Maini R,

409 Feldmann M,et al. (1988) Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid

410 arthritis. Eur J Immunol 18:1797-1801

411 11. Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S, Kopf M, Katada Y, Tanaka T,

412 Suemura M,Kishimoto T (1998) Interleukin 6 plays a key role in the development of antigen-induced

413 arthritis. Proc Natl Acad Sci U S A 95:8222-8226

414 12. Dasgupta B, Corkill M, Kirkham B, Gibson T,Panayi G (1992) Serial estimation of interleukin 6 as a

415 measure of systemic disease in rheumatoid arthritis. J Rheumatol 19:22-25

416   13. Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, Ciliberto G, Rodan G A,Costantini

417   F (1994) Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. EMBO J

418   13:1189-1196

419   14. Yang X, Ricciardi B F, Hernandez-Soria A, Shi Y, Pleshko Camacho N,Bostrom M P (2007) Callus

420   mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice. Bone

421   41:928-936

422   15. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann

423   H,Kohler G (1994) Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature

424   368:339-342

425   16. De Benedetti F, Rucci N, Del Fattore A, Peruzzi B, Paro R, Longo M, Vivarelli M, Muratori F, Berni

426   S, Ballanti P, Ferrari S,Teti A (2006) Impaired skeletal development in interleukin-6-transgenic mice: a

427   model for the impact of chronic inflammation on the growing skeletal system. Arthritis Rheum

428   54:3551-3563

429   17. Naka T, Nishimoto N,Kishimoto T (2002) The paradigm of IL-6: from basic science to medicine.

430   Arthritis Res 4 Suppl 3:S233-242

431   18. Wong P K, Campbell I K, Egan P J, Ernst M,Wicks I P (2003) The role of the interleukin-6 family of

432   cytokines in inflammatory arthritis and bone turnover. Arthritis Rheum 48:1177-1189

433   19. Garnero P, Thompson E, Woodworth T,Smolen J S (2010) Rapid and sustained improvement in bone

434   and cartilage turnover markers with the anti-interleukin-6 receptor inhibitor tocilizumab plus

435 methotrexate in rheumatoid arthritis patients with an inadequate response to methotrexate: results from a

436 substudy of the multicenter double-blind, placebo-controlled trial of tocilizumab in inadequate responders

437 to methotrexate alone. Arthritis Rheum 62:33-43

438 20. Franchimont N, Wertz S,Malaise M (2005) Interleukin-6: An osteotropic factor influencing bone

439 formation? Bone 37:601-606

440 21. Li Y P,Stashenko P (1992) Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not

441 IL-1, down-regulate the osteocalcin gene promoter. J Immunol 148:788-794

442 22. Peruzzi B, Cappariello A, Del Fattore A, Rucci N, De Benedetti F,Teti A (2012) c-Src and IL-6

443 inhibit osteoblast differentiation and integrate IGFBP5 signalling. Nat Commun 3:630

444 23. Hughes F J,Howells G L (1993) Interleukin-6 inhibits bone formation *in vitro*. Bone Miner 21:21-28

445 24. Nishimura R, Moriyama K, Yasukawa K, Mundy G R,Yoneda T (1998) Combination of interleukin-6

446 and soluble interleukin-6 receptors induces differentiation and activation of JAK-STAT and MAP kinase

447 pathways in MG-63 human osteoblastic cells. J Bone Miner Res 13:777-785

448 25. Taguchi Y, Yamamoto M, Yamate T, Lin S C, Mocharla H, DeTogni P, Nakayama N, Boyce B F,

449 Abe E,Manolagas S C (1998) Interleukin-6-type cytokines stimulate mesenchymal progenitor

450 differentiation toward the osteoblastic lineage. Proc Assoc Am Physicians 110:559-574

451 26. Ishihara K,Hirano T (2002) Molecular basis of the cell specificity of cytokine action. Biochim

452 Biophys Acta 1592:281-296

27

453   27. Takahashi-Tezuka M, Yoshida Y, Fukada T, Ohtani T, Yamanaka Y, Nishida K, Nakajima K, Hibi

454   M,Hirano T (1998) Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK

455   mitogen-activated protein kinase. Mol Cell Biol 18:4109-4117

456   28. Hideshima T, Nakamura N, Chauhan D,Anderson K C (2001) Biologic sequelae of interleukin-6

457   induced PI3-K/Akt signaling in multiple myeloma. Oncogene 20:5991-6000

458   29. Tu Y, Gardner A,Lichtenstein A (2000) The phosphatidylinositol 3-kinase/AKT kinase pathway in

459   multiple myeloma plasma cells: roles in cytokine-dependent survival and proliferative responses. Cancer

460   Res 60:6763-6770

461   30. Chung T D, Yu J J, Kong T A, Spiotto M T,Lin J M (2000) Interleukin-6 activates

462   phosphatidylinositol-3 kinase, which inhibits apoptosis in human prostate cancer cell lines. Prostate

463   42:1-7

464   31. Qiu Y, Robinson D, Pretlow T G,Kung H J (1998) Etk/Bmx, a tyrosine kinase with a

465   pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in

466   interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc Natl Acad Sci U S A

467   95:3644-3649

468   32. Chen R H, Chang M C, Su Y H, Tsai Y T,Kuo M L (1999) Interleukin-6 inhibits transforming growth

469   factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and

470   activators of transcription 3 pathways. J Biol Chem 274:23013-23019

471   33. Schmidt K, Schinke T, Haberland M, Priemel M, Schilling A F, Mueldner C, Rueger J M, Sock E,

472   Wegner M,Amling M (2005) The high mobility group transcription factor Sox8 is a negative regulator of

473   osteoblast differentiation. J Cell Biol 168:899-910

474   34. Ratisoontorn C, Seto M L, Broughton K M,Cunningham M L (2005) *In vitro* differentiation profile of

475   osteoblasts derived from patients with Saethre-Chotzen syndrome. Bone 36:627-634

476   35. Hellmuth K, Grosskopf S, Lum C T, Wurtele M, Roder N, von Kries J P, Rosario M, Rademann

477   J,Birchmeier W (2008) Specific inhibitors of the protein tyrosine phosphatase Shp2 identified by

478   high-throughput docking. Proc Natl Acad Sci U S A 105:7275-7280

479   36. Ernst M, Inglese M, Waring P, Campbell I K, Bao S, Clay F J, Alexander W S, Wicks I P, Tarlinton

480   D M, Novak U, Heath J K,Dunn A R (2001) Defective gp130-mediated signal transducer and activator of

481   transcription (STAT) signaling results in degenerative joint disease, gastrointestinal ulceration, and failure

482   of uterine implantation. J Exp Med 194:189-203

483   37. Itoh S, Udagawa N, Takahashi N, Yoshitake F, Narita H, Ebisu S,Ishihara K (2006) A critical role for

484   interleukin-6 family-mediated Stat3 activation in osteoblast differentiation and bone formation. Bone

485   39:505-512

486   38. Sims N A (2004) Glycoprotein 130 regulates bone turnover and bone size by distinct downstream

487   signaling pathways. Journal of Clinical Investigation 113:379-389

488   39. Kawamura N, Kugimiya F, Oshima Y, Ohba S, Ikeda T, et al. (2007) Akt1 in osteoblasts and

489   osteoclasts controls bone remodeling. PLoS One 2:e1058

490   40. Chen W S, Xu P Z, Gottlob K, Chen M L, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R,

491   Tobe K, Kadowaki T,Hay N (2001) Growth retardation and increased apoptosis in mice with

492   homozygous disruption of the Akt1 gene. Genes Dev 15:2203-2208

493   41. Yang Z Z, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E,Hemmings B A

494   (2003) Protein kinase B alpha/Akt1 regulates placental development and fetal growth. J Biol Chem

495   278:32124-32131

496   42. Vandoorne K, Magland J, Plaks V, Sharir A, Zelzer E, Wehrli F, Hemmings B A, Harmelin

497   A,Neeman M (2010) Bone vascularization and trabecular bone formation are mediated by PKB

498   alpha/Akt1 in a gene-dosage-dependent manner: *in vivo* and *ex vivo* MRI. Magn Reson Med 64:54-64

499   43. Choi Y H, Choi H J, Lee K Y,Oh J W (2012) Akt1 regulates phosphorylation and osteogenic activity

500   of Dlx3. Biochem Biophys Res Commun 425:800-805

501   44. Mukherjee A,Rotwein P (2012) Selective signaling by Akt1 controls osteoblast differentiation and

502   osteoblast-mediated osteoclast development. Mol Cell Biol 32:490-500

503   45. Mukherjee A, Wilson E M,Rotwein P (2010) Selective signaling by Akt2 promotes bone

504   morphogenetic protein 2-mediated osteoblast differentiation. Mol Cell Biol 30:1018-1027

505   46. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S,

506   Gundberg C, Bradley A,Karsenty G (1996) Increased bone formation in osteocalcin-deficient mice.

507   Nature 382:448-452

508   47. Higuchi C, Myoui A, Hashimoto N, Kuriyama K, Yoshioka K, Yoshikawa H,Itoh K (2002)

509   Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and

510   mineralization of the extracellular matrix. J Bone Miner Res 17:1785-1794

511   48. Chaudhary L R,Avioli L V (2000) Extracellular-signal regulated kinase signaling pathway mediates

512   downregulation of type I procollagen gene expression by FGF-2, PDGF-BB, and okadaic acid in

513   osteoblastic cells. J Cell Biochem 76:354-359

514   49. Lin F H, Chang J B,Brigman B E (2011) Role of mitogen-activated protein kinase in osteoblast

515   differentiation. J Orthop Res 29:204-210

516   50. Ge C, Xiao G, Jiang D,Franceschi R T (2007) Critical role of the extracellular signal-regulated

517   kinase-MAPK pathway in osteoblast differentiation and skeletal development. J Cell Biol 176:709-718

518   51. Matsushita T, Chan Y Y, Kawanami A, Balmes G, Landreth G E,Murakami S (2009) Extracellular

519   signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in

520   supporting osteoclastogenesis. Mol Cell Biol 29:5843-5857

521   52. Schindeler A,Little D G (2006) Ras-MAPK signaling in osteogenic differentiation: friend or foe? J

522   Bone Miner Res 21:1331-1338

523   53. Sims N A, Jenkins B J, Quinn J M, Nakamura A, Glatt M, Gillespie M T, Ernst M,Martin T J (2004)

524   Glycoprotein 130 regulates bone turnover and bone size by distinct downstream signaling pathways. J

525   Clin Invest 113:379-389

526   54. Duplomb L, Baud'huin M, Charrier C, Berreur M, Trichet V, Blanchard F,Heymann D (2008)

527   Interleukin-6 inhibits receptor activator of nuclear factor kappaB ligand-induced osteoclastogenesis by

528   diverting cells into the macrophage lineage: key role of Serine727 phosphorylation of signal transducer

529   and activator of transcription 3. Endocrinology 149:3688-3697

530   55. Yamanaka Y, Nakajima K, Fukada T, Hibi M,Hirano T (1996) Differentiation and growth arrest

531   signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation.

532   EMBO J 15:1557-1565

533   56. Nakajima K, Yamanaka Y, Nakae K, Kojima H, Ichiba M, Kiuchi N, Kitaoka T, Fukada T, Hibi

534   M,Hirano T (1996) A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1

535   leukemia cells. EMBO J 15:3651-3658

536   57. Hirano T, Matsuda T,Nakajima K (1994) Signal transduction through gp130 that is shared among the

537   receptors for the interleukin 6 related cytokine subfamily. Stem Cells 12:262-277

538   58. Krause A, Scaletta N, Ji J D,Ivashkiv L B (2002) Rheumatoid arthritis synoviocyte survival is

539   dependent on Stat3. J Immunol 169:6610-6616

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546 Figure legends

547 **Fig. 1**

548 IL-6/sIL-6R significantly reduced ALP activity and expression of osteoblastic genes in MC3T3E1 cells,

549 but did not affect proliferation.

550 (a) Proliferation of MC3T3-E1 cells with IL-6/sIL-6R was examined. Cells were pre-incubated for 1 day

551 and then the medium was treated with or without IL-6/sIL-6R for 3 days. Cell proliferation assay was

552 performed daily throughout the 4 days of incubation. Cell proliferation did not show significant

553 differences in any culture condition. (b) ALP staining was performed in MC3T3-E1 cells treated with or

554 without IL-6/sIL-6R for 6 days. Apparently significant reduction of ALP staining was recognized in cells

555 treated with either 10 ng/ml or 50 ng/ml IL-6. (c) ALP activity of the lysates of MC3T3-E1 cells treated

556 with or without IL-6/sIL-6R for 6 days was measured using p-nitrophenylphosphate as a substrate.

557 IL-6/sIL-6R significantly reduced ALP activity in a dose-dependent manner. (d) Total RNA was

558 extracted from MC3T3-E1 cells treated with or without IL-6/sIL-6R for 6 days and subjected to RT-PCR

559 for osteoblastic genes Runx2, osterix, and osteocalcin. Apparently significant reduction of osteoblastic

33

560   gene expression was recognized in cells treated with either 10 ng/ml or 50 ng/ml IL-6. (e) Real-time PCR

561   for Runx2, osterix, and osteocalcin was performed for quantitative analysis. Data were normalized to

562   GAPDH expression and are shown as the ratio of expression compared to control cells treated with

563   vehicle. The expression of osteoblastic genes was significantly down-regulated by IL-6/sIL-6R in a

564   dose-dependent manner. Representative data from at least 3 independent experiments are shown. Data are

565   shown as means ± SE. *n.s.* not significant; *$P < 0.05$; **$P < 0.001$; ***$P < 0.001$

566   **Fig. 2**

567   IL-6/sIL-6R significantly inhibited mineralization of ECM in MC3T3E1 cells.

568   MC3T3-E1 cells were treated with or without IL-6/sIL-6R and were incubated for 21 days. (a) After

569   fixation, the cells were stained with alizarin red solution. Apparently significant reduction of alizarin red

570   staining was recognized in the cells treated with either 10 ng/ml, 25 ng/ml, or 50 ng/ml IL-6. (b) Matrix

571   mineralization was quantified by the measurement of absorbance of alizarin red and normalized by total

572   DNA content. Matrix mineralization was significantly reduced by IL-6/sIL-6R in a dose-dependent

573   manner. Representative data from at least 3 independent experiments are shown. Data are shown as

574   means ± SE. *n.s.* not significant; *$P < 0.05$; **$P < 0.001$; ***$P < 0.001$.

575   **Fig. 3**

576   IL-6/sIL-6R activated ERK, STAT3, and Akt2 signal transduction pathways in MC3T3-E1 cells.

577   (a) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml or 50 ng/ml IL-6 and 100 ng/ml sIL-6R in

578   a time-course experiment (0, 15, and 30 min). Western blot analysis was performed using cell lysates for

579   the detection of ERK, STAT3, and Akt, either phosphorylated or not. IL-6/sIL-6R significantly induced

580   the phosphorylation of ERK, STAT3, and Akt in a dose-dependent manner. (b) MC3T3-E1 cells were

581   incubated with increasing concentrations of IL-6 and 100 ng/ml sIL-6R for 5 min. Western blotting was

582   performed using cell lysates for the detection of ERK, STAT3, as well as Akt, either non-phosphorylated,

583   phosphorylated, or the phosphorylated isoform Akt2. The phosphorylation of both whole Akt and Akt2

584   by IL-6/sIL-6R was recognized more strikingly in a dose-dependent manner. Representative data from at

585   least 3 independent experiments are shown.

586   **Fig. 4**

587   IL-6-induced activation of ERK was enhanced by blocking the STAT3 signaling pathway, and

588   IL-6-induced ERK and Akt signaling pathways negatively regulated each other reciprocally.

589   (a) MC3T3-E1 cells were stimulated with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (15 min) after

590   pre-treatment either with PHPS1 (5, 20, 40 μM; 1 h), with U0126 (5 μM; 1 h), or with V Stattic (5 μM; 1

591   h), and the cell lysates were subjected to Western blotting. PHPS1 inhibited IL-6-induced

592   phosphorylation of ERK and Akt to the constitutive level, but not of STAT3. IL-6-induced activation of

593   ERK was enhanced by V Stattic. (b) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml IL-6 and

594   100 ng/ml sIL-6R (15 min) after pre-treatment either with U0126 (5 μM; 1 h) or with LY294002 (10 μM;

595   1 h), and the cell lysates were subjected to Western blotting. Both constitutive and IL-6-induced

596   phosphorylation of Akt and ERK were enhanced by treatment with U0126 and LY294002, respectively.

597   Representative data from at least 3 independent experiments are shown.

598   **Fig. 5**

599   The negative effects of IL-6 on ALP activity and the expression of osteoblastic genes were restored by

600   inhibition of MEK, PI3K, and SHP2, while they were enhanced by inhibition of STAT3.

601   MC3T3-E1 cells were pre-treated either with U0126 (1, 2.5, 5 μM; 1 h), LY294002 (1, 2.5, 5 μM; 1 h),

602   PHPS1 (5, 20 μM; 1 h), or V Stattic (5 μM; 1 h), then stimulated either with 10 ng/ml IL-6 and 100 ng/ml

603   sIL-6R or with vehicle and incubated for 6 days. (a) ALP activity of the cell lysates was measured using

604   p-nitrophenylphosphate as a substrate. The negative effect of IL-6 on ALP activity was restored by

605   treatment with either U0126, LY294002, or PHPS1 in a dose-dependent manner, while it was enhanced

606   by treatment with V Stattic. (b) Total RNA was extracted and real-time PCR for Runx2, osterix, and

607   osteocalcin was performed. Data were normalized to GAPDH expression and are shown as the ratio of

608   gene expression compared to control cells treated with vehicle. The negative effect of IL-6 on expression

609   of osteoblastic genes was restored by treatment either with U0126, LY294002, or PHPS1 in a

610   dose-dependent manner, while it was enhanced by treatment with V Stattic. Representative data from at

611   least 3 independent experiments are shown. Data are shown as means ± SE. *n.s.* not significant; [#]$P < 0.05$;

612 $^{##}P < 0.001$; $^{###}P < 0.001$, compared to the group treated with vehicle. $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P <$

613 0.001, compared to group treated with IL-6/sIL-6R.

614 **Fig. 6**

615 The negative effect of IL-6 on mineralization of ECM was restored by inhibition of MEK, PI3K, and

616 SHP2, while it was enhanced by inhibition of STAT3.

617 MC3T3-E1 cell were pre-treated either with U0126 (1 µM; 1 h), LY294002 (1 µM; 1 h), PHPS1 (20 µM;

618 1 h), or V Stattic (2.5 µM; 1 h), then stimulated with either 10 ng/ml IL-6 and 100 ng/ml sIL-6R or with

619 vehicle and incubated for 21 days. (a) After fixation, the cells were stained with alizarin red solution. The

620 reduction of alizarin red staining by IL-6/sIL-6R was restored in cells treated with either U0126,

621 LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. (b) Quantification of matrix

622 mineralization was by measurement of absorbance for alizarin red normalized by total DNA content. The

623 reduction of matrix mineralization by IL-6/sIL-6R was restored in cells treated with either U0126,

624 LY294002, or PHPS1, while it was enhanced in those treated with V Stattic. Representative data from at

625 least 3 independent experiments are shown. Data are shown as means ± SE. *n.s.* not significant; $^{#}P < 0.05$;

626 $^{##}P < 0.001$; $^{###}P < 0.001$, compared to the group treated with vehicle. $^*P < 0.05$; $^{**}P < 0.001$; $^{***}P <$

627 0.001, compared to group treated with IL-6/sIL-6R.

628 **Fig. 7**

37

629   Knockdown of MEK2 and Akt2 in cells transfected with siRNA restored ALP activity and Runx2 gene

630   expression.

631   (a) MC3T3-E1 cells transfected with respective siRNAs were cultured for 48 h. Western blotting was

632   performed using cell lysates stimulated with vehicle or with 20 ng/ml IL-6 and 100 ng/ml sIL-6R (15

633   min). Expression levels of each protein, MEK1, MEK2, Akt1, and Akt2, were selectively diminished at

634   48 h after transfection with respective siRNAs. (b) MC3T3-E1 cells transfected with respective siRNAs

635   were incubated for 48 h after which the medium was changed to differentiation medium with vehicle or

636   with 20 ng/ml IL-6 and 100 ng/ml sIL-6R. The cells were then incubated for 3 days to evaluate osteoblast

637   differentiation. ALP activity in MC3T3-E1 cells treated with IL-6/sIL-6R was restored by knockdown of

638   MEK2 and Akt2 as compared to that in cells transfected with negative control siRNA. (c) Expression of

639   osteoblastic genes in MC3T3-E1 cells transfected with respective siRNAs was assessed by real-time PCR.

640   The expression of each gene was normalized against GAPDH expression. The gene expressions of Runx2,

641   osterix, and osteocalcin were restored by knockdown of MEK2. Knockdown of Akt2 also restored Runx2,

642   but decreased osteocalcin. Representative data from at least 3 independent experiments are shown. Data

643   are shown as means ± SE. *n.s.* not significant; $^{\#}P < 0.05$; $^{\#\#}P < 0.001$; $^{\#\#\#}P < 0.001$, compared to negative

644   control group treated with vehicle. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.001$, compared to negative control

645   group treated with IL-6/sIL-6R.

646   **Fig. 8**

647   IL-6/sIL-6R inhibited the differentiation of primary murine calvarial osteoblasts with the activated

648   phosphorylation of ERK, Akt2, and STAT3.

649   (a) ALP activity of lysates of murine calvarial osteoblasts treated with or without IL-6/sIL-6R for 6 days

650   was measured using p-nitrophenylphosphate as a substrate. IL-6/sIL-6R significantly reduced ALP

651   activity in a dose-dependent manner. (b) Total RNA was extracted from murine calvarial osteoblasts

652   treated with or without IL-6/sIL-6R for 6 days, and real-time PCR for Runx2, osterix, and osteocalcin was

653   performed. Data were normalized to GAPDH expression and are shown as the ratio of gene expression as

654   compared to control cells treated with vehicle. The expression of osteoblastic genes was significantly

655   down-regulated by IL-6/sIL-6R in a dose-dependent manner. (c) Murine calvarial osteoblasts were treated

656   with or without IL-6/sIL-6R and were cultured for 21 days. After fixation, the cells were stained with

657   alizarin red solution. Apparently significant reduction of alizarin red staining was recognized in cells

658   treated with either 10 ng/ml or 50 ng/ml IL-6. (d) Matrix mineralization was quantified by measurement

659   of absorbance for alizarin red normalized by total DNA content. IL-6/sIL-6R significantly inhibited

660   mineralization of ECM in a dose-dependent manner. (e) Primary murine calvarial osteoblasts were treated

661   with vehicle or 10 ng/ml or 50 ng/ml IL-6 and 100 ng/ml sIL-6R in a time-course experiment (5, 15, and

662   30 min). Western blotting was performed using cell lysates. IL-6 significantly induced the

663   phosphorylation of ERK, Akt2, and STAT3 in a dose-dependent manner. Representative data from at

664   least 3 independent experiments are shown. Data are shown as means ± SE. *n.s.* not significant; *P <

665   0.05; **P < 0.001; ***P < 0.001.

666

667   **Fig. 9**

668   Schematic presentation of signaling pathways involved in osteoblast differentiation induced by IL-6.

669   IL-6-induced novel SHP2/MEK2/ERK and SHP2/PI3K/Akt2 signal crosstalk in osteoblastic cells; ERK

670   and Akt signaling pathways, both of which are downstream of SHP2, negatively regulate each other

671   reciprocally. On the other hand, the STAT3 signaling pathway negatively regulates the ERK signaling

672   pathway. MEK2/ERK and PI3K/Akt2 have negative effects on osteoblast differentiation, whereas STAT3

673   has a positive effect. Overall, IL-6 inhibits osteoblast differentiation through MEK2 and Akt2 signaling

674   pathways.

675

676   **Supplementary Fig. S1**

677   IL-6-induced activation of ERK was enhanced by blocking the STAT3 signaling pathway, and

678   IL-6-induced ERK and Akt signaling pathways negatively regulated each other reciprocally.

679   (a) MC3T3-E1 cells were stimulated with 10 ng/ml IL-6 and 100 ng/ml sIL-6R (30 min) after

680   pre-treatment either with PHPS1 (5, 20, 40 μM; 1 h), U0126 (5 μM; 1 h), or V Stattic (5 μM; 1 h), and the

681   cell lysates were subjected to Western blotting. PHPS1 inhibited IL-6-induced phosphorylation of ERK

682   and Akt to the constitutive level, but not phosphorylation of STAT3. IL-6-induced activation of ERK was

683   enhanced by V Stattic. (b) MC3T3-E1 cells were treated with vehicle or with 10 ng/ml IL-6 and 100

684   ng/ml sIL-6R (30 min) after pre-treatment with either U0126 (5 μM; 1 h) or LY294002 (10 μM; 1 h), and

685   and the cell lysates were subjected to Western blotting. Both constitutive and IL-6-induced

686   phosphorylation of Akt and ERK were enhanced by treatment with U0126 and LY294002, respectively.

687   Representative data from at least 3 independent experiments are shown.

Figure1

Figure2

a

b

Figure3

a

IL-6 treating time
(minutes)

0   15   30

P-ERK

P-STAT3

P-Akt

ERK

STAT3

Akt

β actin

Control | sIL-6R only | sIL-6R + IL-6 10ng | sIL-6R + IL-6 50ng | Control | sIL-6R only | sIL-6R + IL-6 10ng | sIL-6R + IL-6 50ng

b

IL-6 treating time
(minutes)

5

P-Akt(Ser473)

P-Akt2(Ser474)

Akt

P-STAT3

P-ERK

β actin

Control | sIL-6R + IL-6 10ng | sIL-6R + IL-6 50ng | sIL-6R + IL-6 100ng

Figure4

a IL-6 treating time (minutes)   15

P-STAT3
P-SHP2
P-ERK
P-Akt
STAT3
ERK
Akt
β actin

PHPS1(μM)	—	—	5	20	40	—	—
U0126(μM)	—	—	—	—	—	5	—
V Stattic(μM)	—	—	—	—	—	—	5
sIL-6R+IL-6	—	+	+	+	+	+	+

b IL-6 treating time (minutes)   15
    vehicle   IL-6 10ng

P-STAT3
P-Akt
P-ERK
Akt
ERK
β actin

| U0126(μM) | — | 5 | — | — | 5 | — |
| LY294002(μM) | — | — | 10 | — | — | 10 |

Figure5

a

ALP/protein units/μg protein

sIL-6R+IL-6	−	+		−	+	−	+		−	+	−	+		−	+	−	+		−	+
U0126(μM)	−	−		1	1	5	5		−	−	−	−		−	−	−	−		−	−
LY294002(μM)	−	−		−	−	−	−		1	1	5	5		−	−	−	−		−	−
PHPS1(μM)	−	−		−	−	−	−		−	−	−	−		5	5	20	20		−	−
V Stattic(μM)	−	−		−	−	−	−		−	−	−	−		−	−	−	−		5	5

b

Runx2/GAPDH

Relative mRNA expression

sIL-6R+IL-6	−	+		−	+	−	+		−	+	−	+		−	+	−	+		−	+
U0126(μM)	−	−		1	1	2.5	2.5		−	−	−	−		−	−	−	−		−	−
LY294002(μM)	−	−		−	−	−	−		1	1	2.5	2.5		−	−	−	−		−	−
PHPS1(μM)	−	−		−	−	−	−		−	−	−	−		5	5	20	20		−	−
V stattic(μM)	−	−		−	−	−	−		−	−	−	−		−	−	−	−		5	5

osterix/GAPDH

Relative mRNA expression

sIL-6R+IL-6	−	+		−	+	−	+		−	+	−	+		−	+	−	+		−	+
U0126(μM)	−	−		1	1	2.5	2.5		−	−	−	−		−	−	−	−		−	−
LY294002(μM)	−	−		−	−	−	−		1	1	2.5	2.5		−	−	−	−		−	−
PHPS1(μM)	−	−		−	−	−	−		−	−	−	−		5	5	20	20		−	−
V stattic(μM)	−	−		−	−	−	−		−	−	−	−		−	−	−	−		5	5

osteocalcin/GAPDH

Relative mRNA expression

sIL-6R+IL-6	−	+		−	+	−	+		−	+	−	+		−	+	−	+		−	+
U0126(μM)	−	−		1	1	2.5	2.5		−	−	−	−		−	−	−	−		−	−
LY294002(μM)	−	−		−	−	−	−		1	1	2.5	2.5		−	−	−	−		−	−
PHPS1(μM)	−	−		−	−	−	−		−	−	−	−		5	5	20	20		−	−
V stattic(μM)	−	−		−	−	−	−		−	−	−	−		−	−	−	−		5	5

Figure6

a

b

Figure7

Figure8

Figure9

a   IL-6 treating time (minutes)   30

P-STAT3
P-SHP2
P-ERK
P-Akt
STAT3
ERK
Akt
β actin

PHPS1(μM)	—	—	5	20	40	—	—
U0126(μM)	—	—	—	—	—	5	—
V Stattic(μM)	—	—	—	—	—	—	5
sIL-6R+IL-6	—	+	+	+	+	+	+

b   IL-6 treating time (minutes)   30   vehicle   IL-6 10ng

P-STAT3
P-Akt
P-ERK
Akt
ERK
β actin

| U0126(μM) | — | 5 | — | — | 5 | — |
| LY294002(μM) | — | — | 10 | — | — | 10 |