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SAIDI, S, et al.

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Abstract

The aim of the present study was to investigate the potential role of the recently discovered IL-1 family member IL-33 in bone remodeling. Our results indicate that IL-33 mRNA is expressed in osteocytes in non-inflammatory human bone. Moreover, IL-33 levels are increased by TNF-α and IL-1β in human bone marrow stromal cells, osteoblasts and adipocytes obtained from three healthy donors. Experiments with the inhibitor GW-9662 suggested that expression of IL-33, in contrast to that of IL-1β, is not repressed by PPARγ, likely explaining why IL-33, but not IL-1β, is expressed in adipocytes. The IL-33 receptor ST2L is not constitutively expressed in human bone marrow stromal cells, osteoblasts or CD14-positive monocytes, and IL-33 has no effect on these cells. In addition, although ST2L mRNA is induced by TNF-α and IL-1β in bone marrow stromal cells, IL-33 has the same effects as TNF-α and IL-1β, and, therefore, the biological activity of IL-33 may be redundant in this system. In agreement with this hypothesis, MC3T3-E1 osteoblast-like cells constitutively express ST2L mRNA, and in these cells IL-33 and TNF-α/IL-1β similarly decrease osteocalcin RNA levels in these cells. In conclusion, our results suggest that IL-33 has no direct effects on normal bone remodeling.

Keywords: IL-33, Bone remodeling, inflammation, IL-1.
Introduction

Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are two major cytokines that lead to bone loss in inflammatory diseases, such as rheumatoid arthritis (RA). In RA, bone loss is indeed reduced by therapies blocking TNF-α or IL-1β (for review see [1]). Interfering with TNF-α and IL-1β action may also be beneficial in preventing bone loss in diseases not usually considered as inflammatory, such as post-menopausal osteoporosis. Indeed, ovariectomized mice do not lose bone in absence of TNF-α [2,3] or IL-1 [3-5]. Moreover, bone resorption due to estrogen deficiency in women can be blocked by etanercept and anakinra, which are inhibitors of TNF-α or IL-1β respectively [6]. The mechanisms used by TNF-α and IL-1β to promote bone loss include activation of osteoclastogenesis, which occurs both directly [7,8] and through the induction of receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) expression by stromal cells [8,9]. It is also generally recognized that both cytokines contribute to decrease bone mineral density by decreasing bone formation. For instance, TNF-α and IL-1β down-regulate collagen secretion through RUNX2 inhibition in human osteoblasts [10].

In addition to IL-1β and TNF-α, other cytokines are likely to play specific important roles in regulating bone remodeling during inflammation. In the present study, we investigated the expression and effects of the recently discovered IL-1 family member IL-33 in bone cells [11]. Indeed, although the role played by IL-33 in bone turnover is unknown, its natural inhibitor, the secreted form of the ST2 receptor (sST2) has been identified as a secreted protein in murine osteoblasts [12] and is abundant in the bone matrix [13]. Moreover, it has been shown that suppression of ST2 expression by antisense RNA abrogates the osteogenic differentiation of human osteosarcoma cells [14].
Since the discovery of the ST2 ligand IL-33 [11], many important studies have provided data on its biological functions. IL-33 is produced as a 30 kDa protein. After binding to the long form of the ST2 receptor (ST2L), IL-33 recruits IL-1RαcP, the common co-receptor for IL-1α and IL-1β, to activate signaling pathways, including NF-κB and MAP kinases [15-17]. This effect is blocked by sST2 [18,19]. IL-33 appears to be predominantly expressed in vivo in endothelial and epithelial cells [20,21], but IL-33 expression may be induced by inflammatory signals in other cell types [11,22]. Cell targets of IL-33 include polarized Th2 cells [11,23,24], mast cells [15,25], basophils [24,26], and dendritic cells [27,28]. Besides these ST2-receptor mediated effects, IL-33 is able to translocate into the nucleus, where it may display transcriptional functions. Indeed, IL-33 has been first localized in the nucleus of endothelial cells, most notably within heterochromatin subdomains and mitotic chromosomes, where it may repress transcription [29]. Subsequently, IL-33 has been identified in the nucleus of epithelial cells [21], synovial fibroblasts [22], and monocytes [30]. Finally, it has been recently suggested that IL-33 might function as an “alarmin”, being secreted during cell necrosis, to initiate immune responses [21,31].

IL-33 has been associated with many different diseases. IL-33 serves as a chemotactic factor for Th2 cells [23], and drives the production of Th2 cytokines in vitro and in vivo, inducing severe pathological changes in mucosal organs upon systemic administration in mice [11]. IL-33 has been involved in atherosclerosis, since in apoE/- mice fed a high fat diet, injection of IL-33 reduced atherosclerotic plaque development. Conversely, administration of sST2 resulted in a significantly higher aortic plaque burden [32]. In addition, IL-33 and ST2 were shown to participate in a paracrine signaling system between cardiac fibroblasts and cardiomyocytes to modulate cardiac hypertrophy and fibrosis [19]. Finally, IL-33 appears to
be involved in joint inflammation. Mice administered intra-articularly with bovine serum albumin exhibited hypernociception which was blocked by sST2 [33]. In addition, IL-33 was recently shown to exacerbate antigen-induced arthritis by activating bone marrow-derived mast cells [34], and administration of a blocking anti-ST2 antibody attenuated the severity of collagen-induced arthritis [22,35].

In the present work, we show that IL-33 RNA is constitutively expressed in human bone, and that osteocytes are a source of IL-33 in vivo. In addition, IL-33 mRNA expression was increased by TNF-α and IL-1β in osteoblasts in vitro. However, human osteoblasts and osteoclasts did not express ST2L in basal conditions and did not respond to IL-33, suggesting that IL-33 does not act directly on bone remodeling.
Materials and methods

Chemicals

Cell culture plastic ware was purchased from D. Dutcher (Brumath, France). Dulbecco’s minimum essential medium (DMEM), α-MEM, fetal calf serum (FCS), L-glutamine, penicillin, streptomycin (P/S), trypsin/EDTA, and Extract-All reagents were from Eurobio (Les Ulis, France). Vitamin D3 (VD3), vitamin C, β-glycerophosphate (β-GP), dexamethasone, isobutylmethylxanthine (IBMX), and indomethacin were obtained from Sigma-Aldrich Corporation (St Quentin Fallavier, France). DNase I, Taq DNA polymerase and SYBR green mix were from Roche Diagnostic (Meylan, France). Random primers were obtained from TibMolBiol (Berlin, Germany). Superscript II reverse transcriptase and dNTPs were purchased from Invitrogen (Cergy Pontoise, France). Human and mouse TNF-α, IL-1β, M-CSF and IL-33 were purchased from R&D Systems (Lille, France). Human RANKL was kindly provided by Amgen Inc (Thousand Oaks, USA). Alizarin red and Oil Red dyes were from Sigma-Aldrich Corporation (St Quentin Fallavier, France). The specific PPARγ inhibitor GW-9662 was obtained from Enzo Life Sciences AG (Lausanne, Switzerland).

Human bone tissue samples

Human bone tissue samples were obtained from heterotopic ossifications around the hip from two head-injured patients and from normal bone pieces obtained perioperatively from the proximal femoral shaft of two other patients who had received prosthesis of the hip joint, as previously published [36]. According to our regional Ethics Committee (‘‘Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lille’’ CCPPRB) surgeons asked for informed consent from patients or from the children’s parents. This procedure was followed for all human bone biopsies used in the present study. Bone fragments were minced
using scalpels and scissors and washed with phosphate-buffered saline (PBS). In some samples, bone marrow and most lining cells were removed from bone surfaces and bone fragments were frozen in liquid nitrogen and stored at −70°C.

**Cell culture**

Human bone marrow stromal cells (BMSCs) were obtained from three healthy donors (Lonza, Verviers, Belgium; certified positive for CD29, CD44, CD105 and CD166, and negative for CD14, CD34 and CD45) and also in bone marrow stromal cells obtained from the iliac crest bone from one patient with Legg-Perthes-Calve disease, as previously published in details [10]. BMSCs were seeded at a density of 5,000 cells per cm² and routinely cultured in a growth medium consisting of DMEM containing 10% FCS, 1% penicillin–streptomycin, and 1% L-glutamine. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in air for 1 or 7 days.

The medium was then replaced with the appropriate differentiation medium, and the cells were cultured for additional 7 or 14 days as described in figure legends. Osteoblastic differentiation was induced by culturing cells in an osteogenic medium, containing 10⁻⁸ M VD3, 50 μM vitamin C and 10 mM β-GP, as previously reported [10]. The adipogenic medium consisted of complete culture medium supplemented with 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 50 μM indomethacin. Media were replaced every 3 or 4 days. Cells were treated with 1 ng/ml of TNF-α and 0.1 ng/ml IL-1β at different time points, as indicated in figure legends. The PPARγ specific inhibitor was added two hours prior to cytokine treatment.
Murine MC3T3-E1 cells were maintained in $\alpha$-MEM supplemented with 10% FCS and 1% penicillin–streptomycin and 1% L-glutamine. All experiments were carried out at a plating density of 15,000 cells/cm$^2$. Cells were maintained at 37°C in a humidified atmosphere with 5% CO$_2$ in air until they reached confluence. Then cells were cultured in $\alpha$-MEM supplemented with 10 mM $\beta$-glycerophosphate for an additional 20 days. Media were replaced every 3 days. To investigate cytokine effects, cells were pretreated with 1 ng/ml TNF-$\alpha$ and 0.1 ng/ml IL-1$\beta$ for two days, and then treated with 20 ng/ml IL-33 for three other days.

**Differentiation of human CD14$^+$ cells into osteoclasts**

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll gradient (Sigma Chemicals Co., St.Louis, MO). CD14$^+$ cells were magnetically labelled with CD14 Microbeads and positively selected by MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14$^+$ monocytes were seeded in 96-well plates (45,000 cells / well) in $\alpha$-MEM containing 10% FCS and 25 ng/ml human M-CSF. After 3 days of culture, medium was changed with fresh medium containing 10% FCS, 25 ng/ml M-CSF with or without 100 ng/ml RANKL and with or without IL-33 (10, 50 or 100 ng/ml). Thereafter, medium was changed every 4 days. The formation of osteoclasts occurred after approximately 15 days of culture and was visualized by TRAP staining (Leucocyte Acid Phosphatase Assay kit, Sigma, France). TRAP positive multinucleated cells were then counted under a light microscope (Leica DM IRB, Camera: Olympus D70, Analysis software: Olympus DP controller/Manager).
RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Extract-All reagent according to the manufacturer’s instructions. Briefly, lysis of the cells in Extract-All was followed by centrifugation at 12,000 g for 15 minutes, at 4°C in the presence of chloroform. The upper aqueous phase was collected, and the RNA was precipitated by addition of isopropanol and centrifugation at 12,000 g for 10 minutes, at 4°C. RNA pellets were washed with 75% ethanol, dried and reconstituted in sterile water. Total RNA was quantified by spectrophotometer at 260 nm and the integrity of RNA verified on agarose gel electrophoresis. Contaminating DNA was removed from RNA samples in a 30 min digestion at 37°C with DNase I. 1 µg of each RNA sample was then used for reverse transcription performed under standard conditions with Superscript II reverse transcriptase and random hexamer primers in a 20 µl final volume. The reaction was carried out at 42°C for 30 min and stopped with incubation at 99°C for 5 min. The RT reactions were then diluted to 100 µl in water. 1 µl of the resulting cDNA template was used in subsequent polymerase chain reactions. The PCR amplifications were performed with 5 min 94°C denaturation followed by 40 cycles of 94°C for 40 sec, 55°C for 30 sec, and 72°C for 20 sec, and with a final extension for 6 min at 72°C. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Primers and PCR conditions are shown in table 1.

Quantitative PCR experiments

Quantitative PCR was performed using a LightCycler system (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Reactions were performed in 10 µl with 0.3 µM primers, 4 mM MgCl₂ and 1 µl of LightCycler-FastStart DNA Master SYBR Green I mix. The protocol consisted of a hot start step (8 min at 95°C) followed by 40 cycles including a 10 s denaturation step (95°C), a 10 s annealing step, and an elongation step at
72°C varying from 15 s to 40 s. The primer sequences and PCR conditions for each cDNA are given in Table 1. Efficiencies of PCR were optimized according to Roche Diagnostic’s recommendations on a standard sample expressing all studied genes. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and subsequent gel electrophoresis. Quantification data represented the mean of duplicate conditions. Relative quantification analyses were performed using the RelQuant 1.01 Software (Roche Diagnostics, Meylan, France).

Alizarin red and Oil Red O staining
Calcium deposition in cell layers was investigated with Alizarin Red staining. Media were discarded and cells were rinsed and stained with 1 ml per well 2% Alizarin Red at pH 4.2 for 2 minutes and thoroughly washed with distilled water. Finally, cells were observed and photographed under phase contrast microscopy. The cellular lipid content was assessed by lipid staining with Oil Red O. Culture media were removed, and cells were washed twice with PBS. Cells were then fixed with 2% paraformaldehyde for 15 minutes. After cells were washed with distilled water and incubated in 60% isopropanol for 5 min. The cells were stained with Oil Red O solution (in 60% isopropanol) for 10 min. After washing three times, the cells were observed and photographed under phase contrast microscopy.

Human osteocalcin ELISA
Secretion of osteocalcin was quantified with Metra Osteocalcin assay from Osteomedical (Paris, France) according to the manufacturer’s instructions, and the amount of osteocalcin was corrected by the cell number in the corresponding well.
**Statistical analysis**

Similar results were obtained with the three MSCs used in the present study. All experiments were performed in triplicates and repeated at least twice with each MSCs. Results are expressed as mean ± the standard error of the mean (SEM). For statistical analysis, a Mann-Whitney test was used. A difference between experimental groups was considered to be significant when $P<0.05$. 
Results

First, we examined IL-33 mRNA expression in human bone. By RT-PCR, we were able to detect IL-33 in non-inflammatory, bone marrow-containing, human bone samples (Fig. 1). This result is consistent with the fact that adipocytes and endothelial cells, which are localized in the bone marrow, express IL-33 [21,37]. To determine whether osteocytes, which are the only resident cells in the bone matrix, express IL-33, we analyzed bone samples from which bone marrow and lining cells had been removed. In these samples, we also observed IL-33 mRNA expression (Fig. 1), indicating that osteocytes constitutively express IL-33 mRNA in vivo.

We then investigated the effects of the inflammatory cytokines TNF-α and IL-1β on the expression of IL-33 mRNA in human bone cells in vitro. Bone marrow stromal cells were either left undifferentiated or differentiated into osteoblasts or into adipocytes, which are abundant cells in the bone marrow. Alizarin red and oil red O staining confirmed that cells differentiated into osteoblasts and adipocytes respectively (Fig. 2A and 2B). Results shown in Figure 2C indicate that in addition to matrix-embedded osteocytes in vivo, osteoblasts also express IL-33 mRNA in vitro. Moreover, in these cells, expression of IL-33 mRNA was significantly up-regulated by TNF-α (1 ng/ml) and IL-1β (0.1 ng/ml). The pattern of IL-33 mRNA expression, at least in vitro, appears to be similar for several mesenchymal cell lineages, as indicated by its low basal expression and upregulation in presence of cytokines in bone marrow stromal cells, osteoblasts and adipocytes (Fig. 2C). In contrast, IL-1β mRNA was not expressed in adipocytes from three different donors, even in the presence of TNF-α and IL-1β (Fig. 2C). This result might reflect inhibition of IL-1β gene transcription by PPARγ, which is strongly activated in adipocytes (reviewed in [38]), and is responsible for the
resolution of inflammation in other cell types, by NF-κB-dependent and independent mechanisms [39,40].

To determine whether IL-33 expression is also controlled by PPARγ, we treated osteoblasts for 48 hours with TNF-α and IL-1β in presence or absence of the specific PPARγ inhibitor GW9662. Indeed, after 48 hours, the effects of TNF-α and IL-1β on IL-1β and IL-33 mRNA levels are mostly resolved (our unpublished observation and [37]). Figure 3A shows that inhibition of PPARγ strongly up-regulated IL-1β mRNA levels, confirming that the IL-1β gene is negatively controlled by PPARγ. In contrast, IL-33 mRNA levels were not affected by GW9662 treatment (Fig. 3B).

We then investigated the expression of the IL-33 receptor ST2L in human bone marrow stromal cells and in stromal cells differentiated into osteoblasts. Bone marrow stromal cells are indeed responsible for most of the effects of TNF-α and IL-1β on bone resorption through RANKL secretion [8]; and osteoblasts are responsible for bone formation. Figure 4 indicates that, while stromal cells and osteoblasts do not constitutively express ST2L mRNA, expression of this transcript is induced by TNF-α and IL-1β (Fig. 4A). Consistently with these results, in absence of cytokines, IL-33 had no effect on osteocalcin secretion in osteoblasts (Fig. 4B). Similarly, IL-33 treatment had also no effect on expression of several osteoblast and inflammatory markers, as assessed by quantitative PCR, on alkaline phosphatase activity or on mineralization (data not shown). Additional experiments indicated that induction of ST2L mRNA occurred already after 2 hours of cytokine treatment in stromal cells (data not shown). To examine a potential effect of IL-33 in the presence of TNF-α and IL-1β, we treated cells with IL-33 after a 48-hour pretreatment with TNF-α and IL-1β. However, even when ST2L mRNA expression was induced by preincubation with TNF-α and IL-1β, IL-33
displayed no significant effect, as shown in Figure 4C on IL-6 secretion for instance. This might reflect the fact that IL-33 activates signaling pathways similar to those activated by IL-1β, and might therefore trigger effects redundant with those of IL-1β. In agreement with this hypothesis, we report that in murine MC3T3-E1 osteoblast-like cells, which constitutively express IL-33 and ST2L (Fig. 4D), IL-33 displays the same inhibitory effect as IL-1β on osteocalcin expression, and has no additional effect when added together with TNF-α and IL-1β (Fig. 4E). These results suggest that IL-33 may act on human stromal cells and osteoblasts only in presence of cytokines such as TNF-α and IL-1β, but that its effects may be redundant.

Finally, we investigated potential effects of IL-33 on osteoclastogenesis, using human CD14-positive monocytes differentiated into osteoclasts in the presence of M-CSF and RANKL. As shown in Figure 5, IL-33 had no effect on osteoclast differentiation in the absence or presence of RANKL. This is consistent with the observation that CD14 monocytes, like mesenchymal cells, do not constitutively express ST2L mRNA (data not shown).
Discussion

Our results suggest that IL-33 mRNA is constitutively expressed in bone. Moreover, IL-33 mRNA appears to be constitutively expressed in human osteocytes in vivo. Consistently, in vitro, IL-33 mRNA was expressed in human osteoblasts from three different donors and in murine MC3T3-E1 osteoblasts. Furthermore, IL-33 expression was increased by TNF-α and IL-1β in human osteoblasts and bone marrow stromal cells. We also report in the present study that IL-33 mRNA is produced by human adipocytes differentiated from bone marrow stromal cells, confirming a previous report demonstrating IL-33 expression in adipocytes from visceral fat [37].

IL-33 is a pro-inflammatory cytokine, belonging to the IL-1 family, although its biology presents some differences with that of IL-1β. For instance, whereas IL-1β is a Th1/Th17 inducing cytokine, IL-33 often elicits Th2 responses [11]. We report in the present study differences in the transcriptional control of IL-1β and IL-33 expression in bone marrow cells. Indeed, in contrast to IL-33 mRNA, IL-1β mRNA was not expressed in non-inflammatory human bone samples, and was barely detectable in adipocytes treated with TNF-α and IL-1β. These differences might be related to the fact that expression of IL-1β, but not IL-33, is repressed by PPARγ [39] (Fig. 3). PPARγ is stimulated during adipocyte differentiation, and is also involved in the resolution of inflammation in many other cell types, in which it down-regulates levels of inflammatory proteins, such as COX-2 and IL-1β. In fact, the expression pattern of IL-33 was more similar to that of IL-1 receptor antagonist, the natural inhibitor of IL-1β, which is also expressed in non-inflammatory bone [41] and in adipocytes (our unpublished data and [42]). It will be interesting to examine whether the expression of IL-33, like that of IL-1Ra, is also controlled by PPARα [43, 44] and PPARβ/δ [45].
Despite expression of IL-33 mRNA in osteocytes and adipocytes derived from bone marrow, it appears from our results that IL-33 is not involved in normal bone turnover. Indeed, in basal conditions, neither stromal cells, nor osteoblasts or osteoclast precursors expressed the membrane form of the ST2 receptor whereas mRNA encoding the secreted, inhibitory ST2 isoform was constitutively expressed (our unpublished data and [12,13]). The observation that inflammatory cytokines increased IL-33 mRNA levels in adipocytes and ST2L mRNA levels in stromal cells and osteoblasts may suggest that IL-33 expression and responsiveness are up-regulated in human post-menopausal and senile osteoporosis. Indeed, adipocytes progressively become the most abundant cell lineage in the bone marrow during aging (reviewed in [38]), and aging is characterized by a low grade pro-inflammatory state [46], which is exacerbated by hormonal deficiency [47]. However, even if during aging IL-33 was secreted and if its receptor on stromal cells was functional, our data suggest that direct effects of IL-33 on bone remodeling are likely to be the same as those of IL-1β and TNF-α. This hypothesis is supported by our observation that in murine MC3T3-E1 osteoblasts, which constitutively express ST2L (Fig. 4E), IL-33 and IL-1β had redundant effects (Fig. 4E). It is therefore quite unlikely that IL-33 impacts bone remodeling through bone marrow stromal cells or osteoblasts. Similarly, IL-33 is also unlikely to directly modulate osteoclastogenesis since ST2L was not expressed in normal osteoclast precursors and IL-33 did not modulate osteoclastogenesis in our experiments (Fig. 5). However, we cannot exclude the possibility that IL-33 might be able to impact bone remodeling by indirect means. For instance, IL-33 has recently been shown to stimulate dendritic cell generation through GM-CSF secretion [27], and thus may be able to inhibit osteoclast differentiation indirectly [48].

Recent important data suggest that IL-33 is not a “classical” cytokine like IL-1β, but behaves like a novel “alarmin”, as do IL-1α and HMGB1 [21]. Several immunohistochemical
experiments showed that IL-33 is located in the nucleus of endothelial, epithelial and fibroblast cells [21,22,29], where, like IL-1α, it may exert a transcriptional activity [29]. Moreover, recent data suggest that IL-33 may not be secreted in physiological conditions, but may be released during cell necrosis [50]. During apoptosis, IL-33 may be inactivated after cleavage by caspase-3 and/or caspase-7 [30,49,50]. Therefore, like IL-1α and HMGB1, IL-33 may only be released after tissue trauma or infection, but in contrast to IL-1α and HMGB1, IL-33 may be specifically able to recruit and activate Th2 cells [11,23]. In this model, although IL-33 may be expressed in bone in endothelial cells [21], adipocytes and osteoblasts (Figs 1 and 2), it may not be released unless bone is seriously damaged. This model may explain why we failed to detect by ELISA significant amounts of IL-33 released by human stromal cells treated or not with TNF-α and IL-1β. In the light of these data, a putative role played by IL-33 during fracture healing and osteonecrosis deserves consideration. Finally, it is also possible that IL-33 has nuclear functions in undamaged bone cells, independently of ST2L receptor. Interestingly, nuclear IL-1α was shown to inhibit cell proliferation and stimulate IL-6 production in human SaOS-2 osteosarcoma cells [51]. This hypothetic nuclear function of IL-33 in normal bone cells also merits investigation.

Conclusions

Our experiments reveal that IL-33 mRNA is expressed in human osteoblasts/osteocytes in vivo and in vitro, and that adipocytes may also be a source of IL-33 in human bone marrow. In addition, we show in this article that IL-33 mRNA expression in bone marrow stromal cells, osteoblasts and adipocytes is increased by TNF-α and IL-1β. However, IL-33 has no effect on these cells, nor on human CD14-positive osteoclast precursors, and none of these cell types expresses the ST2L receptor in basal conditions. Moreover, although TNF-α and IL-1β
induce ST2L mRNA in mesenchymal cells, the effects of IL-33 appear to be redundant in these cells. Finally, the recently proposed “alarmin” role of IL-33, according to which IL-33 might be released only by necrotic cells, suggests that IL-33 may play a role during osteonecrosis.

Acknowledgements

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Figure legends

Figure 1: IL-33 and ACTIN mRNA levels in 4 human non-inflammatory bone samples. “Bone marrow +” indicates samples studied with their bone marrow. “Bone marrow –” indicates bone samples studied after removal of their bone marrow, as detailed in the “Materials and Methods” section (Neg: negative control).

Figure 2: Cytokine mRNA levels in human bone marrow stromal cells (BMSCs), osteoblasts and adipocytes from three different donors, and cultured for 21 days, as detailed in the “Materials and Methods” section. Alizarin red staining (A) and oil red O staining (B) confirms osteoblast and adipocyte differentiation respectively (one representative experiment is shown). IL-1β and IL-33 mRNA levels are shown for human BMSCs, osteoblasts and adipocytes, with or without continuous treatment with 1 ng/ml TNF-α and 0.1 ng/ml IL-1β from day 7 to day 21. (C). In each cell type, the highest level of cytokine expression has been set as 100%. *P<0.05 as compared with cells cultured in the corresponding control condition (in the same differentiation medium, without cytokines).

Figure 3: IL-1β (A) and IL-33 (B) mRNA levels in human BMSCs cultured for 7 days in growth medium, then for 4 additional days in osteogenic medium and treated for 48 hours with 1 ng/ml of TNF-α, in the presence or absence of 100 µM of GW-9662. *P<0.05 as compared with cells cultured in control conditions (without cytokine, without GW-9662); #P<0.05 as compared with cells cultured in presence of TNF-α. (ns: not significant).
**Figure 4:** Expression of ST2L mRNA (A) in human BMSCs cultured for 7 days in growth medium, (BMSCs) or in osteogenic medium (Osteoblasts) in the presence or absence of 1 ng/ml of TNF-α and 0.1 ng/ml of IL-1β. (B) Effect of a 48-hour IL-33 treatment on osteocalcin secretion in human osteoblasts. (C) Effect of a 48-hour IL-33 treatment on IL-6 mRNA levels, in BMSCs cultured for 7 days with or without a 24-hours pretreatment with 1 ng/ml of TNF-α and 0.1 ng/ml of IL-1β. (D) Expression of ST2L and (E) osteocalcin (oc) mRNA in murine MC3T3-E1 osteoblasts cultured for 21 days after a 72-hours IL-33 treatment and with or without a 48-hour pretreatment with 1 ng/ml of TNF-α and 0.1 ng/ml of IL-1β. *P<0.05 as compared with cells cultured in control conditions.

**Figure 5:** Effect of IL-33 on osteoclastogenesis in CD14 positive monocytes cultured as detailed in the “Materials and Methods” section. (A) Photographs after TRAP staining (x 100); (B) Number of TRAP-positive osteoclasts per well.
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<td>55</td>
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<td>198</td>
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<tr>
<td>Human ST2L</td>
<td>NM_016232</td>
<td>55</td>
<td>F: 5'-GGATTGAGGCCACTCTGCT-3'</td>
<td>269</td>
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<tr>
<td>Human IL-1β</td>
<td>NM_000576</td>
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<td>R: 5'-GGAGCCAATGAGTGTTG-3'</td>
<td>146</td>
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<td>Human IL-6</td>
<td>NM_000600</td>
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<td>F: 5'-ATTTGCTGCTGTGTTGACAC-3'</td>
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<tr>
<td>Human OC</td>
<td>NM_199173</td>
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<td>R: 5'-TGAGAGCTAGCGGGAAGTCCGAGGAT-3'</td>
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<td>Mouse IL-33</td>
<td>NM_133775</td>
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<td>F: 5'-TGGAAAGCTGTGGCGTGATG-3'</td>
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<td>Mouse ST2L</td>
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<td>Mouse GAPDH</td>
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</table>

Shown are the primer sequences (F: forward; R: reverse), annealing temperatures (Ta), base pair (bp) lengths of the corresponding PCR products, and GenBank accession numbers (GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin; OC: osteocalcin; RPLP0: acidic ribosomal phosphoprotein P0).
**Figure 1**

<table>
<thead>
<tr>
<th></th>
<th>IL-33</th>
<th>ACTIN</th>
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<tr>
<td>Bone sample</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>Bone marrow</td>
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</tr>
</tbody>
</table>

Bone sample and bone marrow results for IL-33 and ACTIN expression.
Figure 2

A. Osteogenic medium

B. Adipogenic medium

C. IL-1β/GAPDH (%)

IL-33/GAPDH (%)

<table>
<thead>
<tr>
<th>BMSCs</th>
<th>Osteoblasts</th>
<th>Adipocytes</th>
<th>BMSCs</th>
<th>Osteoblasts</th>
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<td>+</td>
</tr>
</tbody>
</table>

TNF-α + IL-1β

BMSCs1

BMSCs2

BMSCs3
Figure 3

A

IL-1β/RPLP0

GW-9662

-  +  -  +

TNF-α

-  +

B

IL-33/RPLP0

GW-9662

-  +  -  +

TNF-α

-  +

*  #  ns
Figure 4

A  

![Image of gel electrophoresis showing ST2L and ACTIN expression under different conditions](image)

B  

![Graph showing osteocalcin (ng/ml/1,000 cells) levels with varying IL-33 concentrations](image)

C  

![Bar graph showing IL-6/GAPDH expression with TNF-α and IL-1β treatment](image)

D  

![Image of gel electrophoresis showing IL-33, ST2L, and ACTIN expression under different conditions](image)

E  

![Graph showing OC/GAPDH expression with TNF-α and IL-1β treatment](image)
Figure 5

A

- RANKL

- IL-33
  10 ng/ml

- IL-33
  50 ng/ml

- IL-33
  100 ng/ml

B

osteoclasts/well

RANKL - + - - - + + +

IL-33 - - 10 50 100 10 50 100