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Osteoclast precursors from different bone sites respond differently to IL-1β

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ABSTRACT
Osteoclasts from different bone sites are heterogeneous with respect to the way they resorb bone. How these subsets of osteoclasts respond to cytokines like IL-1β is not known. Here we investigated whether osteoclast precursors from different bone sites respond differently to the inflammatory cytokine IL-1β. IL-1β was added to in vitro osteoclastogenesis cultures in which bone marrow cells from calvaria and long bones were cultured for 6 days in the presence of M-CSF and RANKL. The number of osteoclasts, resorptive activity and osteoclast size was analyzed. Under the influence of IL-1β larger osteoclasts were formed in both marrow cultures. These cells contained more nuclei and had a higher resorptive activity. A dose-dependent increase in gene expression of the decoy receptor IL-1R2 was found only in the calvaria osteoclast cultures and not in long bone cultures. Calvaria cultures showed a significantly increased expression of IL-1β under the influence of IL-1β. Our findings indicate that the formation of osteoclasts from marrow obtained from different bone sites is stimulated by IL-1β in a comparable way. Yet, marrow cells from calvaria respond differently to IL-1β; these cells revealed an increased gene expression of IL-1R2 and IL-1β.
INTRODUCTION
Inflammatory processes such as periodontitis and rheumatoid arthritis are characterized by increased levels of various pro-inflammatory cytokines. This increased expression of cytokines coincides with a concomitant increase in osteoclast activity resulting in bone loss. One of the highly expressed cytokines under inflammatory conditions is interleukin 1β (IL-1β) [1]. This cytokine is known for its catabolic activity in both soft connective tissues and bone. With respect to bone, the cytokine influences osteoclast differentiation and activity either by stimulation of pre-osteoclast fusion and subsequent resorption or indirectly by enhancing RANKL expression by osteoblasts [2-4]. IL-1β knock-out mice were shown to have denser bone containing fewer osteoclasts, indicating that IL-1β also plays a role in physiological osteoclast formation [5].

The cytokine is synthesized in an inactive pro-form that becomes activated through cleavage by caspase-1 [6]. When activated IL-1β binds to its receptor IL-1R1. This ligand-receptor-complex is rapidly internalized and downstream signaling pathways, such as the transcription factors NFATc1, MitF and c-Fos become activated [7]. A second receptor for IL-1β, IL-1R2 acts as a decoy receptor, not contributing to downstream signaling. Binding of IL-1β to this receptor prevents downstream activation of signaling pathways [8].

Since it is known that osteoclasts from different bone sites exhibit differences in their bone resorbing activities [9-13], we hypothesized that osteoclast precursors from different bone sites respond differently to IL-1β. To investigate this we isolated bone marrow cells from calvaria and long bone and cultured these cells with M-CSF and RANKL, and with different concentrations of IL-1β. After 6 days of culture we assessed osteoclast formation by analyzing their number, size, and activity (resorption). In addition, we analyzed gene expression of RANK, RANKL, IL-1β, IL-1R1, IL-1R2, IL-1Ra, DC-STAMP, syncytin, caspase-1, NFATc1, MitF and c-Fos.
MATERIALS & METHODS

Osteoclast generation

Permission for the animal experiments was obtained from the Animal Welfare committee of the VU University Amsterdam. Osteoclasts were generated as described [14]. Briefly, for each experiment, 6 weeks old C57BL/6 mice were sacrificed with a peritoneal injection of sodium pentobarbital (0.1 ml Euthesate, Ceva Sante Animale, Naaldwijk, The Netherlands). Tibiae and calvariae were dissected and cleaned of soft tissue. Calvariae were cut in small pieces and both bone tissues were subsequently ground in a mortar with culture medium α-MEM (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Antibiotic Antimycotic solution, Sigma, St. Louis, MO, USA), and heparin (170 IE/ml; Leo Pharmaceutical Products B.V., Weesp, The Netherlands). The cell suspension was aspirated through a 21-gauge needle and filtered over a 70 µm pore-size Cell Strainer filter (Falcon/ Becton Dickinson, Franklin Lakes, NJ, USA). Cells were washed twice in culture medium, centrifuged (5 min, 200 g), and plated in 96-well flat-bottomed advanced CT tissue-culture plates (Cellstar, Greiner Bio-One, Monroe, NC, USA) at a density of 1.10^5 cells per well. In addition, cells were seeded on bovine cortical bone slices (650 µm thick) or on Corning® Osteo Assay Surface (calcium phosphate) plates (Corning BV, Tewksbury, MA, USA).

Cells were cultured in 150 µl culture medium containing 30 ng/ml recombinant murine M-CSF (R&D systems, Minneapolis, MI, USA), 20 ng/ml recombinant murine RANKL (R&D systems) and 0, 0.1, 1 and 10 ng/ml IL-1β (Sigma, St. Louis, MO, USA). Ten ng/ml IL-1β was used as highest concentration, since this concentration was shown by others to result in a maximal response [8, 15]. Culture media were refreshed on day 3. After six days of culture, wells were washed with PBS and either fixed in 4% PBS buffered formaldehyde, stored at 4 °C, and used for tartrate-resistant acid phosphatase (TRAcP) staining, or the cells were dissolved in RNA lysis buffer (see paragraph on QPCR) and stored at -80 °C until RNA isolation. The wells with the bone slices were stored in MilliQ water at 4°C for bone resorption visualization or fixed in 4% PBS buffered formaldehyde and used for TRAcP staining.
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**Number of nuclei and cell size**
The cells were stained for TRACP enzyme activity by using the Acid Phosphatase, Leukocyte (TRACP) Kit from Sigma. The procedure was according to the manufacturer’s instructions. The number of positively stained multinucleated cells on bone in standardized fields and on plastic was counted (data combined from 3 independent experiments). The cells were grouped into one of the following categories: (i) cells with 3-5, (ii) cells with 6-10 and (iii) cells with >10 nuclei. Also the size of the cell-body was analyzed using Image Pro Plus (Mediacybernetics, Carlsblad, CA, USA). The osteoclast size was measured in 3 different experiments with respectively 3, 6 and 3 mice. Micrographs were made at standardized positions in the wells. In total 815 osteoclasts were measured (218 in long bone and 197 in the calvaria cultures without IL-1β; 221 in long bone and 179 in the calvaria cultures with IL-1β).

**Resorption**
Resorption was measured on cortical bone slices and on the osteo assay plates. After 3 or 6 days of culture with M-CSF and RANKL and without or with IL-1β in a concentration of 0.1, 1, 10 ng/ml, resorption of the bone slices was visualized with a Coomassie brilliant blue staining as described in [13]. The resorbed area was measured in pre-determined fields. For the osteo assay plates the areas devoid of non-translucent material were visualized according to the manufacturer’s protocol. In short, cells were removed from the plates by adding a 10% bleach solution and incubated for 5 min. at room temperature. Subsequently, the plates were washed two times with MilliQ water and air dried. Areas where osteoclasts had digested the coating were visible as translucent areas. For measurement of the resorbed area micrographs were made at pre-determined positions with x10 magnification. The size of the resorption area was calculated with Image Pro Plus( Media Cybernetics. San Diego, CA, USA).

**Quantitative RT-PCR**
RNA from cultured bone marrow cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After measuring RNA concentration with a multilabel plate reader (Synergy HT BioTek Instruments, Bad Friedrichshall, Germany), 100 ng RNA was reversed transcribed to cDNA for real-time quantitative PCR (qPCR). The RT-PCR reactions were
performed with the ABI PRISM 7000 (Applied Biosystems) using 5 ng cDNA and 300 nM of each primer in a total volume of 15 μl containing SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq Gold DNA polymerase, and dNTPs with dUTP instead of dTTP, Applied Biosystems), following manufacturer’s instructions. Samples were normalized for the expression of hypoxanthine guanine phosphoribosyl transferase (HPRT), expression of which was not altered by the experimental conditions, by calculating the ΔCt (Ct gene of interest – Ct HPRT); expression of the different genes is given as $2^{-\Delta Ct}$. The primers used for the detection of the various genes are indicated in Table I.

**Table I: Primer sequences used in RT-PCR reactions**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
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<tr>
<td>HPRT</td>
<td>Fw: CCTAaAgATgAgCgCAAgTTgAA</td>
</tr>
<tr>
<td></td>
<td>Rv: CCACAggACTAgAACACCTgCTAA</td>
</tr>
<tr>
<td>RANKL</td>
<td>Fw: CTgAggCCAgCCATTTg</td>
</tr>
<tr>
<td></td>
<td>RV: ggAACCggATgggATgCT</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Fw: TgTATCggCTCATCTCCTCCTCA</td>
</tr>
<tr>
<td></td>
<td>Rv: gACTCCTTgggTTCCCTgCTT</td>
</tr>
<tr>
<td>Syncytin</td>
<td>Fw: ggTTAggCATCgCgggTA</td>
</tr>
<tr>
<td></td>
<td>Rv: gCTgATgATggAggTggCTATT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fw: ggACCCATAgCTgAAgCAAAgCT</td>
</tr>
<tr>
<td></td>
<td>RV: TgTgccCTTggTTCTCCTTT</td>
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<tr>
<td>IL-1R1</td>
<td>Fw: AgTTACCggAgTCCAgTggTA</td>
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<td></td>
<td>RV: AgCCACATTTTCACCAACAg</td>
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<tr>
<td>IL-1R2</td>
<td>Fw: AgCCAaAgATgTggAggTAA</td>
</tr>
<tr>
<td></td>
<td>RV: CAgTggAgCTgTTCTTgAA</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Fw: TCACCCCTgCCCTCTCCTCA</td>
</tr>
<tr>
<td></td>
<td>RV: CTgATgCTCTTgACTggCTCC</td>
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<td>NFATc1</td>
<td>Fw: CATgCgAgCCATCATCgA</td>
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<td></td>
<td>RV: TgggATgTgAACCTgAAaAgAC</td>
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<tr>
<td>MitF</td>
<td>Fw: CgTgCATgCAgATgAgATgAT</td>
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<td></td>
<td>RV: TgggCTggACAgAgAgATgCT</td>
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<td>Caspase-1</td>
<td>Fw: TgAAgTTgCTgCTgAgAgATCT</td>
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<td></td>
<td>RV: TgggCAgAgCAAAATTC</td>
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<td>IL-1Ra</td>
<td>Fw: ACCAgCTCATTgCTgAggTACTT</td>
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<tr>
<td></td>
<td>RV: gTggATgCCCAgAAACACT</td>
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Statistics
A paired sample t-test was used for the comparison between control IL-1-treated if a single concentration of IL-1β was used and a one way ANOVA with repeated measurements was used to compare the number of multinucleated cells, resorption and qPCRs in the presence of different concentrations of IL-1β. Data are expressed as mean values of at least 3 measurements and SEM. Differences were considered significant when p<0.05.

RESULTS
IL-1β induces more and larger osteoclasts
In general, long bone as well as calvaria bone marrow cultures supplemented with IL-1β (10 ng/ml) induced a higher number of osteoclasts (Figure 1). Under the influence of IL-1β, osteoclast size was larger than untreated osteoclasts. This was only significant for the long bone osteoclasts (Figure 1B). Especially the number of osteoclasts with 6-10 and more than 10 nuclei was enhanced. This was shown for each substrate used: tissue culture plastic (Figure 1C), bone (Figure 1D) and osteo assay plates (Figure 1E). An increased number of osteoclasts with a higher number of nuclei was significant on plastic and on osteo assay plates, but not significant on bone. In the calvaria cultures seeded on the osteo assay plates (Figure 1D) the number of multinucleated cells with more than 10 nuclei was significantly higher than in long bone cultures.

In long bone the mean number of cells with >10 nuclei was: 91 ±75 (IL-1β), vs. 8 ± 9 (control) and for calvaria cells it was 237± 122 vs 8 ± 14, respectively (n=10, *p<0.05).
Figure 1AB. Osteoclasts generated after culturing for 6 days with M-CSF and RANKL without or with 10 ng/ml IL-1β. (A) Osteoclasts were generated on the three substrates tested. Micrographs are shown of osteoclasts (OC) generated on Corning osteo assay plates. (B) Osteoclasts generated on corning osteo plates from long bone marrow are larger compared to the ones generated from calvaria marrow. (n=12 mice; **p<0.01,*p<0.05).
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Figure 1C-E. Osteoclasts generated after culturing for 6 days with M-CSF and RANKL without or with 10 ng/ml IL-1β. (C-E): number of multinucleated cells, formed on plastic (C), bone (D) and Corning osteo assay plates (E) from long bone and calvaria bone marrow without or with 10 ng/ml IL-1β. Cells were scored as cells containing 3-5 nuclei, 6-10 nuclei and more than 10 nuclei. Osteoclasts generated in the presence of IL-1β were larger and had more nuclei. This was found on plastic (n=9 *p<0.05,**p<0.01) (C) as well as on Corning osteo assay plates (E) (n=10, *p<0.05, **p<0.01). On bone such an effect was not seen.
IL-1β enhances resorption

The resorption of bone slices by calvarial as well as long bone cultures is stimulated by IL-1β (10ng/ml), although being only statistically significant for the calvarial cultures (Figure 2A). Osteoclasts cultured on the osteo assay plate, revealed a dose-dependent increased digestion of the substrate by calvaria as well as long bone cultures (Figure 2B, C, D). Osteoclasts generated from each of the two different bone sites showed a similar IL-1β induced stimulation of substrate digestion. Of interest was the lower level of digestion in the presence of 0.1 ng/ml IL-1β compared to the cultures without IL-1β. This was apparent for both osteoclast populations.

Figure 2AB. Effect of IL-1β on resorption. (A) Resorption of bone by osteoclasts generated from long bone and calvaria marrow without or with 10 ng/ml IL-1β. (B) The resorption area was expressed as a % of the bone surface area (n=5 long bone, n=5 calvaria, only significant for calvaria cultures *p<0.05).
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**Figure 2C-E.** Effect of IL-1β on resorption. (C) Resorption area of bone site specific osteoclasts cultured on osteo assay plates with increasing concentrations of IL-1β. Arrows indicate the dissolved area in the presence of 0, 0.1 and 1 ng/ml IL-1β. In the cultures with 10 ng/ml IL-1β nearly all the substrate was dissolved, therefore the non-dissolved areas are marked with an asterisk (*). The resorption area increased dose-dependently. (D, E) Measurements of the resorption area are presented as a % of the total surface area of the well; in D for long bone cells and E for calvarial cells (n=6, **p<0.005, ***p<0.0005).
**IL-1β differently affects gene expression of IL-1R2**

We next assessed whether IL-1β caused an altered gene expression pattern at 3 and 6 days. At day 3 hardly any multinucleated osteoclast was formed, whereas at day 6 high numbers was apparent. The expression of genes involved in fusion such as DC-STAMP and syncytin were not changed (at day 3 or 6; not shown). Interestingly, at day 6 IL-1β caused an increased expression of IL-1β, IL-1R1 and IL-1R2 (Figure 3A, B, C). The increase of IL-1R2 was only noted in calvarial cultures; expression of this gene was not affected in long bone cultures. In calvarial cultures IL-1R2 was dose-dependently upregulated; an effect apparent at day 6 (Figure 4), but not at day 3 (not shown).

![Figure 3. Gene expression of IL-1β, IL-1R1, IL-1R2 after 6 days of culture.](image)

- **A**
  - Gene expression of IL-1β after 6 days of culture. Increased expression of IL-1β and IL1-R1 in both long bone and calvarial cultures (A and B), whereas IL1-R2 is only upregulated in calvaria cultures (C). Mean ± S.E.M. is shown, n=10 bone marrows were used. *p<0.05
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Figure 4. Effect of IL-1β on gene expression of IL-1R2 at day 6 of culture. Gene expression of IL-1R2 is dose dependently enhanced with IL-1β at day 6 in the calvaria cultures, (n=4 for long bone not significant and n=3 **p<0.01 for 0 compared to 10 ng IL-1β in the calvaria cultures).

Since it is known that binding of IL-1β to the IL-1R2 receptor inhibits signaling via transcription factors such as NFATc1, MitF, c-FOS we analyzed gene expression of these factors. We could not find any difference in expression of these genes (not shown). In addition, we assessed the expression of the IL-1 receptor antagonist IL-1Ra and the protease involved in activation of IL-1β, caspase-1. No effect was found of IL-1β on the expression of these genes and also no difference in expression was found between the two cell populations (not shown).

DISCUSSION
Bone marrow cells isolated from calvaria and tibia, generated larger osteoclasts under the influence of IL-1β. The osteoclasts derived from calvaria as well as long bone precursors contained more nuclei per cell and their resorative activity was higher compared to the osteoclasts generated without IL-1β. The resorption was dose dependently enhanced. Yet, quite remarkable was the lower level of resorption in the presence of 0.1 ng/ml IL-1β, compared to the control cultures without IL-1β. This effect was seen for both populations. Thus, our findings suggest that IL-1β can exert both a stimulatory and an inhibitory effect on osteoclastic activity; an effect depending on the concentration of the cytokine. Such a biphasic effect of the cytokine has been shown before by Lin et al. [16] for osteoblast differentiation and they suggested that this effect is due to binding of IL-1β to one
of the receptors. How the activity of IL-1β can be influenced by the binding to one of the receptors and whether this also counts for osteoclast activity needs further investigation.

We measured the mRNA expression of IL-1β and the two receptors for IL-1β; IL-1R1 and IL-1R2. An upregulation was found of mRNA of IL-1β, IL-1R1 and the decoy receptor IL-1R2. For IL-1R2 this effect was only found in the calvaria cultures. It is known that IL-1β can bind to IL-1R1 and IL-1R2, but will preferably bind to IL-1R2 [17]. This binding is almost irreversible and causes the captured IL-1β to be unavailable for binding to the activating receptor IL-1R1 [8, 18]. Since binding to the decoy receptor IL-1R2 does not result in signal transduction, increased expression of IL-1R2 might be a mechanism to reduce a further upregulation of inflammatory cytokines and chemokines, which will occur when IL-1β binds to the IL-1R1 receptor [19, 20]. Since binding to the decoy receptor IL-1R2 does not result in signal transduction, increased expression of IL-1R2 might be a mechanism to reduce a further upregulation of inflammatory cytokines and chemokines, which will occur when IL-1β binds to the IL-1R1 receptor [19, 20]. A significant upregulation of IL-1R2 was only seen in the calvarial cultures. This suggests that osteoclasts from different bone sites respond differently to IL-1β. If not all IL-1β is captured by IL-1R2, the excess of IL-1β will bind to IL-1R1. Mentioned by Jimi et al. and reviewed by Dinarello [20, 21], binding of IL-1β to the IL-1R1 receptor induces multinucleation, prolonged survival and enhanced resorption. The formation of larger osteoclasts in the calvaria cultures, in our study, might be tempered in the long run by the upregulation of the expression of IL-1R2, since binding to IL-1R2 does not result in signal transduction.

We did not find any difference in mRNA expression of proteins involved in fusion such as DC-STAMP and syncytin, which implies that the formation of larger osteoclasts in the presence of IL-1β was not accompanied by an upregulation of mRNA of these fusion proteins.

In conclusion, the data in this study are the first to show that different osteoclast precursors respond differently to IL-1β. The calvaria as well as long bone osteoclasts respond to IL-1β by increasing the expression of IL-1β, but this is more pronounced in the calvarial cultures. We revealed that only calvaria osteoclast precursors responded to IL-1β by increasing the expression IL-1R2. It is known that binding of IL-1β to the decoy receptor IL-1R2 does not result in signal transduction. Thus increasing expression levels of IL-1R2 might be a mechanism to temper an inflammatory response. This ability of calvarial osteoclasts appears to distinguish them from long bone osteoclasts.
ACKNOWLEDGMENTS
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REFERENCES


8. Trebec-Reynolds DP, Voronov I, Heersche JN, Manolson MF. IL-1alpha and IL-1beta have different effects on formation and activity of large osteoclasts. J.Cell Biochem. 109[5], 975-982. 1-4-2010.


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