Chapter 2

IL-1β differently stimulates proliferation and multinucleation of distinct mouse bone marrow osteoclast precursor subsets

Yixuan Cao, Ineke D.C. Jansen, Sara Sprangers, Jan Stap, Pieter J.M. Leenen, Vincent Everts, Teun J. de Vries

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ABSTRACT

Osteoclasts are bone-resorbing cells and targets for treating bone diseases. Previously, we reported that distinct murine osteoclast precursor subsets such as early blasts (CD31^hi Ly-6C^-), myeloid blasts (CD31^+ Ly-6C^+), and monocytes (CD31^- Ly-6C^hi) respond differently to the osteoclastogenesis-inducing cytokines M-CSF and RANKL. It is unknown, however, how these cell types respond to the osteoclast-stimulating inflammatory cytokine IL-1β. This study aims to investigate the effect of IL-1β on osteoclastogenesis derived from different mouse bone marrow precursors. Early blasts, myeloid blasts, and monocytes were sorted from mouse bone marrow cells using flow cytometry. Cells were cultured on plastic or on bone slices in the presence of M-CSF and RANKL, without or with IL-1β (0.1-10 ng/ml). We found that IL-1β stimulated multinucleation and bone resorption of osteoclasts derived from the three precursors at a different rate. Highest number of large osteoclasts (>20 nuclei) and highest level of bone resorption (16.3%) was by myeloid blast-derived osteoclasts. IL-1β particularly accelerated proliferation of early blasts and a high number of small osteoclasts (3-5 nuclei) was formed on plastic. Life span varied between osteoclasts derived from different precursors: large osteoclasts (>2400 µm²) formed most rapidly (75 hours) from myeloid blasts but had a short life span (30 hours). Monocytes needed the longest time (95 hours) for the generation of such large osteoclasts, but these cells had a longer life span (50 hours). Our results indicate that the different bone marrow osteoclast precursors are differently stimulated by IL-1β with respect to proliferation, multinucleation, life span and bone resorption.

Key words: early blasts, myeloid blasts, monocytes, osteoclastogenesis, bone resorption.
INTRODUCTION

Inflammatory bone diseases are closely associated with bone loss and bone-structural changes. Typically, these diseases are accompanied by an enhanced osteoclast activity and thus resulting in an imbalanced bone turnover. Rheumatoid arthritis is the most common inflammatory bone disease and is characterized by high osteoclast generation and excessive bone and cartilage resorption in the joints [1]. Another example of such inflammation-induced bone disease is periodontitis, where bacteria of the oral cavity directly or indirectly stimulate the formation and activity of osteoclasts, thus resulting in a progressive loss of alveolar bone around the teeth [2].

Osteoclasts, multinucleated-bone-resorbing cells, control the bone remodeling by balancing formation of bone by osteoblasts [3–5]. Osteoclasts are generated from various progenitors like bone marrow precursors, peripheral blood-borne monocytes and spleen cells [6–10]. Two cytokines have been found to be necessary for osteoclastogenesis: macrophage colony-stimulating factor (M-CSF), which can stimulate osteoclast precursors by regulating their survival and proliferation [11], and receptor activator of nuclear factor Kappa-B ligand (RANKL), which promotes osteoclast differentiation and activity [12–14]. Some studies have shown that osteoclasts at different bone sites are not always identical [15–20]. Everts and colleagues found that both MMPs and Cathepsin K (Cath K) are involved in bone degradation by calvaria osteoclasts, while resorption by long bone osteoclasts primarily depends on the activity of Cath K [17]. Jansen and coworkers showed Ae2_{ab} (Na^{+}-independent chloride/bicarbonate anion exchanger 2) to be relevant for the activity of long bone osteoclasts but not for those in calvaria [18]. Thus, the long bones of Ae2_{ab}^{−/−} mice are osteopetrotic whereas the skull has no altered phenotype [18].

Different investigations have shown that specific mouse osteoclast precursor populations have a distinct osteoclastogenic capacity. Long bone marrow precursors need only 4 days for osteoclastogenesis in vitro instead of 6 days needed by the jaw bone marrow precursors [19]. Charles and coworkers found a particular type of osteoclast precursor being expanded in inflammatory arthritis models: the CD11b^{−lo}Ly6C^{hi} bone marrow subset [21]. Jacquin and coworkers described that specific cells from murine bone marrow, for example CD11b^{−low}CD45R^{−}CD3^{−}CD115^{high}CD117^{+} bone marrow cells, have an enhanced osteoclastogenesis capacity [22].

Osteoclasts can be generated from different differentiation stages of myeloid
lineage progression. Three successive stages in the maturation pathway of this lineage have been recognized [23]. They develop from early blasts (CD31\textsuperscript{hi} Ly-6C\textsuperscript{−}) through myeloid blasts (CD31\textsuperscript{+} Ly-6C\textsuperscript{+}) to monocytes (CD31\textsuperscript{−} Ly-6C\textsuperscript{hi}). Each subset has the potential to differentiate into osteoclasts [6], however they vary in their proliferation capacity [23]. The cellular composition and osteoclastogenic potential of these three subsets also varies at different bone sites, for example jaw and long bone marrow [19]. In long bone marrow, these three subsets responded differently to M-CSF and RANKL: myeloid blasts need less time to differentiate into multinucleated cells than the other two subsets [6]. Moreover, priming with M-CSF alone abolished osteoclast formation by myeloid blasts [24], but not of the other subsets, again showing that the various subsets respond differently to these signaling molecules. Most of these studies were performed in conditions that may resemble physiological conditions. However it is not known how these different subsets respond to compounds known to be present under inflammatory conditions.

Here, we set out to mimic the response of precursors to inflammatory conditions by stimulating cells with Interleukin-1β (IL-1β), one of the highly expressed and driving cytokines in inflammation. Bone cells, especially osteoclasts, are highly sensitive to IL-1 [25,26], in particular to IL-1β which plays an important role in bone resorption [27]. This cytokine enhances multinucleation and bone resorption [25]. Although the stimulatory role for IL-1β has been widely documented [28–30], it is not known whether the different bone marrow precursors respond differently to this cytokine.

The present study aims to evaluate whether IL-1β affects the osteoclastogenic potential of different mouse myeloid subsets: early blasts, myeloid blasts and monocytes. The formation of osteoclasts is analyzed by means of TRAcP staining, bone resorption, live cell imaging and gene expression.
METHODS AND MATERIALS

Bone marrow isolation and cell sorting

Bone marrow was isolated from 6-week-old male C57BL/6J mice (VU Animal Facility, The Netherlands) (Animal experiments were approved by the Animal Welfare committee of the VU University). Long bones from both hind legs were removed, cleaned from soft tissue and mashed with a pestle in a mortar in 10 ml culture medium. Culture medium was α-Minimal Essential Medium (α-MEM; GIBCO, Paisley, UK) supplemented with 5% Fetal Calf Serum (FCS; HyClone, Logan, UT) and 1% Penicillin-Streptomycin-Fungizone (Sigma-Aldrich St. Louis, MO). The released bone marrow was filtered by a 40 µm filter. $4 \times 10^7$ cells/mouse were used and 1 ml FACS buffer (1% albumin from bovine serum (BSA) (Sigma-Aldrich) with 10 µl biotinylated CD-31 antibody (AbD Serotec, Kidlington, UK) was added for cell sorting. After a 30 min incubation, 800 µl Streptavidin PE (Becton Dickinson, San Jose, CA), 200 µl FACS buffer and 10 µl Alexa 488 labeled anti-Ly-6C (AbD Serotec) were added and incubated for a subsequent 30 min. Samples were sorted with FACSAria (Becton Dickinson). Three osteoclast precursor subsets were recognized by the expression level of CD31 and Ly-6C: early blasts (CD31$^{hi}$ Ly-6C$^{-}$), myeloid blasts (CD31$^{+}$ Ly-6C$^{+}$) and monocytes (CD31$^{-}$ Ly-6C$^{hi}$) (Fig. 1 and Table 1). All labeling and centrifuge steps were performed in FACS buffer and samples were kept on ice.

Osteoclastogenesis

The three sorted subsets were cultured in 96-well plates (Cellstar, Greiner Bio-one, Monroe, NC) at $1.5 \times 10^4$ cells in 150 µl culture medium per well, containing 30 ng/ml M-CSF (R&D systems, Minneapolis, MI), 20 ng/ml RANKL (RANKL-TEC, R&D systems), and 0, 0.1, 1, 10 ng/ml IL-1β (Sigma-Aldrich), both on plastic and on 650 µm thick bone slices. Culture media were refreshed every 3 days. After 4 or 6 days of culture, wells were washed with PBS and either stored in water for bone resorption analysis, or fixed in 4% PBS buffered formaldehyde and stored at 4°C for TRAcP analysis, or dissolved in RNA lysis buffer (Qiagen, Hilden, Germany) and stored at -80°C for RNA isolation, or dissolved in 100 µl Cyquant lysis buffer (Molecular Probes, Leiden, Netherlands) and stored at -20°C for DNA concentration measurement.

TRAcP analysis
The fixed cells were stained by using a TRAcP Kit (Sigma-Aldrich) and the staining procedure was conducted following the manufacturer’s instruction. 150 µl TRAcP solution was added to each well. After 10 min incubation, cells were washed with water and nuclei were counterstained with DAPI (4’6-diamidino-2-phenylindole dihydrochloride) for 5 min. After washing with PBS, cells were stored in PBS at 4°C. The number of TRAcP+ multinucleated cells was assessed using a combination of light and fluorescence microscopy (Leica DFC320) and cells were categorized into one of the following four groups: 3-5 nuclei, 6-10 nuclei, 11-20 nuclei and >20 nuclei.

Figure 1. Flow cytometry gating of mouse bone marrow. Following CD31 and Ly-6C labeling, 6 distinct subsets can be discerned. Early blasts, myeloid blasts, and monocytes are gated in P3, P6, and P5, respectively.

Bone resorption

For bone resorption analysis, cells were cultured for 6 days. The bone slices were sonicated with 10% NH₄OH (Merck, Germany) on ice for 30 min. After washing with water, bone slices were incubated in saturated alum for 10 min. Followed by strong water current washing, the bone slices were stained with Coomassie Brilliant Blue. Resorption pits were visualized by light microscopy (Leica DFC320). The surface area of the pits was analyzed using Image-Pro.
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Plus software (MediaCybernetics, Silver Spring, MD, USA).

Table 1. Percentage of each cell subset (early blasts, myeloid blasts, and monocytes) gated after CD31 and Ly-6C labeling

<table>
<thead>
<tr>
<th>Gating</th>
<th>Cell type</th>
<th>Mean ± SD</th>
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<tr>
<td>P2</td>
<td>Lymphocytes</td>
<td>23.0±4.5</td>
</tr>
<tr>
<td>P3</td>
<td>Early Blasts</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>P4</td>
<td>Granulocytes</td>
<td>20.1±3.9</td>
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<tr>
<td>P5</td>
<td>Monocytes</td>
<td>3.8±0.8</td>
</tr>
<tr>
<td>P6</td>
<td>Myeloid Blasts</td>
<td>3.0±0.8</td>
</tr>
<tr>
<td>P7</td>
<td>Erythroid blasts</td>
<td>31.1±9.5</td>
</tr>
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Early blasts, myeloid blasts, and monocytes were gated in P3, P6, and P5, respectively. A previous study [6] showed that no osteoclasts could be cultured from P2 (lymphocytes), P4 (granulocytes), and P7 (erythroid blasts). Means ± SD (n = 6).

Quantitative RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and the isolation procedure was conducted following the manufacturer’s instruction. RNA was reverse transcribed to cDNA and cDNA was diluted to 0.5 ng/µl by adding different volumes of water. Each reaction was prepared with 5 µl cDNA, 7.5 µl SYBR Green Mastermix (Invitrogen Life technologies), 0.75 µl of 6 µM Fw primer, 0.75 µl of 6 µM Rv primer and 1 µl water. qPCR was performed on ABI PRISM 7000 (Applied Biosystems). Porphobilinogen deaminase (PBGD) was used as housekeeping gene. Samples were normalized by calculating the ΔCt (Ct_{gene of interest} - Ct_{PBGD}) and gene expression was calculated as 2^{-(ΔCt)}

Primers we used are shown in Table 2.

Live cell imaging

The three myeloid precursor subsets were seeded at a concentration of 1.6×10^5 cells/well in 12-well culture plates (Cellstar, Greiner Bio-one) in 1 ml culture medium containing 30 ng/ml M-CSF and 20 ng/ml RANKL per well, with or without 10 ng/ml IL-1β. After a 44 hours pre-culture at 37°C, 5%
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\[ \text{CO}_2 \text{, the culture medium was refreshed and layered with mineral oil (Sigma M3516; Sigma St. Louis, MO, USA) to prevent evaporation of the medium. The 12-well plate was placed under an inverted fluorescence Leica DM IRBE microscope (Leica Microsystems, Wetzlar, Germany), equipped with a 10x objective and a cooled CCD camera (PCO type 2000s; PCO AG, Kelheim, Germany). Cells were imaged for 4 days at 37ºC in an atmosphere containing 5% CO}_2. Phase contrast images were acquired at time-intervals of 10 min. Time lapse movies were analyzed using custom-made software.}

**Statistical analysis**

Bone marrow cells were isolated and sorted per mouse, and seeded in duplicate wells. In total 6 mice were sacrificed (n=6) for each experiment. Data were analyzed using GraphPad Prism (Version 5.00) and presented as mean ± SD. One-way ANOVA followed by Tukey-Kramer’s multiple comparison test was used for more than three comparisons. The level of significance was set at P<0.05.
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RESULTS

Osteoclast- size increases in the presence of IL-1β

According to different expression levels of CD31 and Ly-6C, early blasts, myeloid blasts and monocytes were isolated using flow cytometry (Fig. 1). After 4 and 6 days of culturing of these different subsets, osteoclasts were recognized as multinucleated (>2 nuclei) TRAcP+ cells. IL-1β showed a stimulatory effect with each of the three subsets. Yet, with respect to the formation of large osteoclasts the three subsets responded in a subset-specific way (Fig. 2).

Table 2. Primers used for qPCR

<table>
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<th>Gene</th>
<th>Sequence ((5’-&gt;3’)</th>
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<td>PBGD</td>
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<td></td>
<td>Rv: TCTGGACCATCTTCTTGCTGA</td>
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<td>TRAcP</td>
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<td>ENSMUSG0000001348</td>
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<td></td>
<td>Rv: GGGCTGGGGAAGTTCCAG</td>
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</tr>
<tr>
<td>Cathepsin K</td>
<td>Fw: ACAGCAGGATGTGGGTGTCTCA</td>
<td>ENSMUSG00000028111</td>
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<td></td>
<td>Rv: GCAGGAGATTTCCATCCACCT</td>
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<td>DC-STAMP</td>
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<tr>
<td></td>
<td>Rv: GACTCTTTGGGCTTCTTCTTT</td>
<td></td>
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<tr>
<td>SHIP</td>
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<td></td>
<td>Rv: GGGCAGAATCTGTGAAGGTAACA</td>
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<td>Rv: TTGTTCCTGCTCAGATCAGTAG</td>
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<td>CyclinD1</td>
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<td>Bcl2</td>
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<td></td>
<td>Rv: AGACAGCCAGGAGAATCAAACA</td>
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To analyze this stimulatory effect on multinucleation in more detail, osteoclasts were categorized by the number of nuclei, from small to large: 3-5, 6-10, 11-20 and >20 nuclei (Fig. 3). IL-1β dose-dependently stimulated multinucleation,
as shown for myeloid blasts and monocytes (Fig. 3A). Titration of IL-1β for early blasts was not performed due to the low number of retrieved cells per mouse. For subsequent experiments, we chose 4 days (on plastic) and 6 days (on bone) for the analysis, when the optimal number was seen.

**Figure 2. The three osteoclast precursor subsets respond to IL-1β in a distinct way.** A. Micrographs of cultures after 4 days in the presence of M-CSF and RANKL. B. Micrographs of cultures after 4 days in the presence of M-CSF and RANKL, with 10 ng/ml IL-1β. The cells were stained for TRAcP activity. TRAcP⁺ multinucleated cells (arrow) are purple and the nuclei are blue. Scale bars = 100 µm.

On plastic, there was a statistically significant increase caused by IL-1β in the formation of large osteoclasts (>10 nuclei) by all three subsets (Fig. 2 and Fig. 3B). The highest number of large osteoclasts was generated by myeloid blasts (Fig. 2 and Fig. 3B middle) and this was particularly apparent in the presence of IL-1β (Fig. 2B and Fig. 3B middle). In early blast cultures, IL-1β increased the number of all groups of osteoclasts, but this was most pronounced for the smaller sized osteoclasts (3-5 and 6-10 nuclei) (Fig. 2B and Fig. 3B left). Of the three subsets of precursors, lowest number of osteoclasts were generated from monocytes (Fig. 2 and Fig. 3B right). This was found both in the absence and presence of IL-1β. Culturing the different subsets on bone resulted in an enhanced multinucleation induced by IL-1β for all subsets (Fig. 3C). Statistically significant differences were found between the number of cells with 11-20 nuclei and those with >20 nuclei groups in all three subsets.
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Figure 3. IL-1β stimulates multinucleation by the three osteoclast precursor subsets in a different way. A. The graphs show the number of large osteoclasts (>10 nuclei) generated by the three subsets of precursors after 4 days on plastic, and the effect of IL-1β here upon. Due to relative paucity of the cell population only the highest IL-1β concentration was tested for early blasts. B-C. Graphs of the number of TRAcP⁺ multinucleated cells cultured without (white column) or with 10ng/ml (black column) IL-1β in the presence of M-CSF and RANKL, on plastic after 4 days (B) or on bone after 6 days (C). Number of TRAcP⁺ multinucleated cells (Mean ± SD) are presented (n=6, *p<0.05, **p<0.01, ***p<0.001).
IL-1β significantly enhances bone resorption by all subsets, being highest for myeloid blasts

After 6 days, bone resorption pits were observed with all three subsets, both when cultured with only M-CSF and RANKL (Fig. 4A) and when IL-1β was added (Fig. 4B). This indicates that the TRAcP⁺ multinucleated cells were functional osteoclasts. IL-1β stimulated bone resorption dose-dependently.
Figure 4. Bone resorption by early blasts, myeloid blasts and monocytes. A-B. Pit formation (arrows) by the three populations cultured for 6 days without IL-1β (A) and with this cytokine (B). C. Graphs showing bone resorption (expressed in %) by the three subsets with different concentrations of IL-1β. D. Bone resorption per osteoclast by the three subsets without (white column) or with 10 ng/ml IL-1β (black column). (n=6, *p<0.05, **p<0.01, ***p<0.001). Scale bars = 100 µm.

(Fig. 4C). Most resorption was found with myeloid blast-derived osteoclasts cultured with 10 ng/ml IL-1β. Under this condition, 16.3% of the bone surface was resorbed (Fig. 4B and C middle). The resorption results were in line with those on numbers of TRAcP⁺ multinucleated cells, suggesting myeloid blast was the most active osteoclast precursor subset in the IL-1β environment. When comparing the osteoclast activity per cell (bone resorption/TRAcP⁺ cell) for each subset with or without IL-1β, they were all comparable, indicating that IL-1β stimulates osteoclast formation but not function per cell (Fig. 4D).

IL-1β increases and accelerates proliferation in early blast cultures

To investigate the cause of the increased osteoclast formation induced by IL-1β, proliferation assay and live cell imaging were performed. DNA concentration analysis as a measurement of proliferation level, revealed a significantly increased proliferation only in early blast cultures. This effect was seen at both 4 and 5 days in the presence of IL-1β (Fig. 5A). Such stimulatory effect by IL-1β was not found in myeloid blast and monocyte cultures (Fig. 5B and C). From the 5th day of culturing, DNA concentration declined in both early blast and myeloid blast cultures. This indicates a decrease in number of cells probably due to apoptosis in these two groups. In contrast herewith the monocyte-derived osteoclasts appeared to survive.

Using live cell imaging and assessing the total number of nuclei per microscopic field at different time points, we found for the early blasts that under the influence of IL-1β the maximal number of nuclei was reached after 98 hours whereas in control cultures this took longer, 110 hours (Table 3). In the myeloid blast and monocyte cultures, IL-1β did not affect the proliferation rate. These findings suggest that IL-1β significantly accelerated proliferation of early blasts (p=0.0021), but not by myeloid blasts (p=0.1890) and monocytes (p=0.9404).

IL-1β stimulates cell fusion of the three subsets at a different rate, being most pronounced in myeloid blasts

IL-1β enhanced fusion of all three subsets, as assessed by both TRAcP$^+$ multinucleated cell counting (Fig. 3) and live cell imaging. Myeloid blasts were the precursors prone to fuse to generate more large osteoclasts (>10 nuclei) (Fig 3 A-C middle). To obtain a better insight into the formation of the large osteoclasts, live cell imaging micrographs were analyzed and the size of osteoclasts formed by the three subsets were assessed at different time points between 75 to 145 hours.

Figure 5. DNA analysis of the cells differentiated from three subsets of precursors after a culture period of 0, 3, 4, and 5 days on plastic. A. Early blasts. B. Myeloid blasts. C. Monocytes. Data are expressed as mean ± SD ng/ml DNA. White columns are controls and the black column are the cells cultured with IL-1β. (n=6, **p<0.01).
We first compared the total surface area of osteoclast-like cells generated by the three subsets and the effect of the cytokine hereupon (Fig 6. A-C). IL-1β increased the area covered by osteoclasts in all three subsets. With early blasts, we found IL-1β speeded up the osteoclast formation and reached the peak earlier than the control. Myeloid blasts achieved the largest surface area of osteoclasts at the earliest time point, at 86 hours in both control and IL-1β group. Monocytes reached the largest surface area at the latest time point at 118 hours in both control and IL-1β group.

Figure 6. Osteoclast size analysis by time-lapse microscopy. A–C. Percentage of osteoclasts generated by early blasts (A), myeloid blasts (B) and monocytes (C). Dashed line indicates
the controls cultured with M-CSF and RANKL only; solid line indicates cells cultured with 
10 ng/ml IL-1β on plastic. **D.** Number of large osteoclasts (>2400 µm², corresponding to >10 
nuclei) formed per field in the presence of IL-1β on plastic. **E.** Correlation analysis of the size 
of osteoclasts and the number of nuclei of these cells. Data were obtained and combined of all 
three subsets. The size and nuclei number show a positive significant correlation (P<0.0001) 
with R²=0.8862. (n=3, *p<0.05, **p<0.01, ***p<0.001).

We also had a special look at the formation of large osteoclasts (Fig. 6D) (>2400 µm², corresponding to >10 nuclei, Fig. 6E) by the three subsets. Since 
in the absence of IL-1β hardly any large osteoclasts were formed, we only 
analyzed their formation in the presence of IL-1β. Myeloid blasts needed the 
shortest time (75 hours) to form large osteoclasts and they formed within 
this time frame the highest number of such large osteoclasts. These large 
osteoclasts did, however not survive very long: they stayed alive for only 
about 30 hours. The generation of large osteoclasts by early blasts took more 
time, at least 85 hours and their number was much lower than those formed 
by myeloid blasts. The large osteoclasts formed by early blasts survived 
for about 40 hours. Monocytes, formed large osteoclasts at the latest time 
point (95 hours) and they generated the lowest number of such osteoclasts. 
However, these monocyte-derived large osteoclasts survived for the longest 
period, 60 hours.

**Expression of osteoclast-related genes**

In an attempt to explain our findings on the different stimulatory effects of 
IL-1β, osteoclast-related genes were analyzed by qPCR on bone.

**Osteoclast-related genes: TRAcP, Cath K, and DC-STAMP**

We compared the expression of a series of osteoclast-related genes by the 
different subsets of precursors: **TRAcP**, (an enzyme highly expressed by 
osteoclasts); **Cathepsin K**, (a cysteine proteinase involved in the digestion 
of bone matrix); and **DC-STAMP**, (a membrane protein essential for cell-
cell fusion by osteoclasts [31]. For all of these genes, myeloid blasts showed 
the highest expression (Fig. 7 A-C) and IL-1β significantly stimulated the 
expression of these three genes in myeloid blasts (Fig. 7 D-F). This expression 
profile appears to be in line with the formation of higher numbers of large 
osteoclasts by myeloid blasts. However there were no significant differences 
in early blast and monocyte cultures stimulated by IL-1β in these genes (data 
not shown).
IL-1β effects on different osteoclast precursors

Table 3. Time needed for each subset to reach the highest level of proliferation

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<tr>
<th></th>
<th>Control (h)</th>
<th>With IL-1β (h)</th>
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<tr>
<td>Early blasts</td>
<td>110 ± 3</td>
<td>98 ± 2, **</td>
</tr>
<tr>
<td>Myeloid blasts</td>
<td>84 ± 2</td>
<td>82 ± 1, ns</td>
</tr>
<tr>
<td>Monocytes</td>
<td>116 ± 7</td>
<td>117 ± 3, ns</td>
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</table>

Data are shown in Mean± SD, “Control” indicates cells were cultured with solely M-CSF and RANKL on plastic, “With IL-1β” indicates cells were cultured with M-CSF, RANKL and 10 ng/ml IL-1β on plastic. Early blasts Control VS IL-1β: p=0.0021 (**), myeloid blasts control VS IL-1β: p=0.1890 (ns), monocytes control VS IL-1β: p=0.9404 (ns).

Proliferation gene: Cyclin D1

To explain the difference in proliferation, we analyzed the expression of Cyclin D1 (Fig. 7G-I), a gene that positively regulates proliferation of precursors [32]. In early blast cultures, IL-1β significantly increased the Cyclin D1 expression at the 2 days’ time point. IL-1β also shortened the time to reach the peak of proliferation only in early blast cultures. However, no significant differences in the expression of Cyclin D1 were found with myeloid blasts and monocytes with or without IL-1β. Myeloid blasts achieved the peak of Cyclin D1 expression at 2 days, consistent with live cell imaging that myeloid blast responded the fastest. Monocytes achieved the peak after 4 days both with and without IL-1β.

IL-1β-related genes: IL-1RI, IL-1RII and IL-1Ra

For each IL-1β-related gene, such as IL-1RI (Fig. 7J), IL-1RII (Fig. 7K) and IL-1Ra (Fig. 7L), early blasts showed a different pattern compared to the other two subsets. IL-1RI had a high expression level in early blasts immediately after isolation (day 0) while expression of the non-functional receptor IL-1RII was relatively low. These results suggest that especially early blasts are sensitive to IL-1β stimulation early after culture initiation and they respond by proliferating and forming high numbers of small osteoclasts. At a later stage, the opposite was shown: IL-1RI expression was low and IL-1RII expression increased in these cells suggesting a decreased responsiveness to
Figure 7. Gene expression of osteoclasts generated from early blasts, myeloid blasts and monocytes, and the effect of IL-1β hereupon. Cells were cultured with M-CSF and RANKL, without (control groups) or with 10 ng/ml IL-1β (IL-1β groups) on bone. Different colors indicate: early blasts (red), myeloid blasts (green), monocytes (blue), control (dashed line), IL-1β (solid line). A-F. Expression of osteoclast-related genes: Expression of TRAcP (A), Cath K (B) and DC-STAMP (C) by the three precursor subsets in the presence of IL-1β,
and the level of these genes by myeloid blasts in the absence and presence of IL-1β (D-F).

**G-I.** Expression of proliferation-related gene: *Cyclin D1*. J-M. Expression of IL-1β-related genes: *IL-1RI* (J), *IL-1RII* (K) *IL-1RA* (L) expressed by the three subsets in the presence of IL-1β; *IL-1RI* (M) expressed by myeloid blasts in the absence and presence of IL-1β.

**N-P.** Other genes related to osteoclast activity and survival expressed by the three subsets in the presence with IL-1β: *IL-6* (N), *SHIP* (O), and *Bcl2* (P). (n=6, *p<0.05, **p<0.01, ***p<0.001).

IL-1β at a later stage. However, monocytes showed an opposite trend to early blasts: expression of *IL-1RI* was increasing while *IL-1RII* was decreasing with the culture time. Remarkably, the expression of *IL-1Ra* (IL-1 receptor antagonist) remained low in the early blast cultures compared to myeloid blasts and monocytes. Myeloid blasts proved to respond to IL-1β by expressing significantly higher levels of *IL-1RI* mRNA (Fig. 7M). Such a difference was not found in early blast and monocyte cultures (data not shown).

*Other genes: IL-6, SHIP, and Bcl2*

*IL-6*, an inflammatory cytokine expressed by cells from the monocytic lineage, has been shown to stimulate osteoclast differentiation [33–35]. Its expression decreased during osteoclastogenesis in all three subsets. The highest expression was found in early blasts at the onset of the culture period (Fig. 7N). *SHIP* (src homology-2-containing inositol-5-phosphatase), a negative regulator of osteoclast precursor proliferation [36], was expressed at lowest levels in early blast cultures (Fig. 7O). The expression of anti-apoptosis gene *Bcl2* [32], decreased during culturing in early blasts and myeloid blasts (Fig. 7P), suggesting these precursor cells or their derived osteoclasts became apoptotic at a later stage of culturing.
DISCUSSION

In this study, we investigated the response of different murine bone marrow osteoclast precursor subsets, early blasts, myeloid blasts and monocytes, to the inflammatory cytokine IL-1β. We found that IL-1β stimulated osteoclastogenesis by these three subsets in distinct ways: IL-1β induced proliferation by early blasts, and stimulated large osteoclast formation by all three subsets, however at a different rate. The number of osteoclasts formed by monocytes with and without IL-1β was much lower compared to the other two subsets.

The proliferation analysis showed that IL-1β significantly increased the DNA concentration in early blast cultures; an effect not found with the other precursor subsets (Fig. 5). IL-1β also accelerated the time needed to reach the highest level of proliferation by these cells (Table 3). In an attempt to explain this stimulatory effect, we analyzed the expression of two IL-1β receptors, IL-1RI and IL-1RII (Fig. 7J-K), and found that early blasts showed a significant difference compared to myeloid blasts and monocytes. According to our results, IL-1RI was expressed at high levels by early blasts at the beginning of the culture period, whereas the decoy receptor IL-1RII was expressed very low at this time point. This expression profile may give early blasts the opportunity to respond optimally to the cytokine. In line with this finding was the expression of Cyclin D1. Of the three subsets of precursors tested, only early blasts showed a significantly increased expression of Cyclin D1 in the presence of IL-1β. Finally, the negative proliferation regulator, SHIP, which can down-regulate the expression of Cyclin D1 [36], was lowest expressed by early blasts. These findings are in line with the previous findings that early blasts represent myeloid precursors with the highest proliferative capacity, as indicated by their colony-forming potential [37]. Moreover, IL-1 is known to mediate its proliferation-stimulating effects especially on the most immature stages of hematopoietic development [38]. However, since we analyzed DNA concentration of the whole population of cells, mononuclear cells could not be excluded. Using DNA analysis, we established that IL-1β caused an increase in DNA exclusively in early blast cultures. Since most cells in the well, especially at early time points until day four are mononuclear, we can conclude that the IL-1β induced proliferation in early blast cultures is likely due to an expansion of the progenitor pool. Taken together, our findings indicate that of the three precursor subsets only early blast cultures responded to IL-1β with increased proliferation.
IL-1β effects on different osteoclast precursors

Myeloid blasts proved to generate the highest number of large osteoclasts within a relatively short time frame, an effect found with and without IL-1β. This fast response in the absence of the cytokine is in line with a previous study from our group [6]. Myeloid blasts responded to IL-1β by increasing the gene expression of DC-STAMP. This increased expression of this fusion protein coincided with the formation of numerous very large osteoclasts. IL-1β not only induced a higher expression of DC-STAMP, but it also caused significantly higher expression of other osteoclast-related markers, such as TRAcP, Cathepsin K and of IL-1RI, the signaling receptor of IL-1β. Besides, the expression of the decoy receptor IL-1RII remained low in myeloid blast cultures (Fig. 7K), while IL-1RI has an increasing trend (Fig. 7J), which also gives this subset advantage in its osteoclastogenic capacity. Thus, myeloid blasts appear to have the capacity to rapidly generate large osteoclasts in the presence of the cytokine IL-1β.

Our findings have shown that monocytes generated the lowest number of osteoclasts. This is somewhat surprising since this cell type is generally taken to be one of the most prominent precursors of osteoclasts in human [39–41]. Few studies also considered monocytes but also earlier stages to be the most prominent osteoclast precursors in mice [39,42]. However the phenotypic definition of these monocytes varies and may contain also their immediate predecessors resembling myeloid blasts in this study. The monocytes in our study, which do not proliferate very actively are more likely to be the cell cycle-arrested quiescent osteoclast precursors (QuOP), which hardly proliferate and have a longer life span [43]. A possible explanation of the low osteoclastogenesis capacity of monocytes could be due to the fact that monocytes are more mature cells [23] with a relatively low proliferation capacity [6], evidenced by a low amount of DNA after 3 days of culture (Fig. 5C) and a low Cyclin D1 expression (Fig. 7I) in this study. Lari and his colleagues claimed that two types of monocytes are isolated from human blood: proliferative monocytes and non-proliferative ones. The precursors for the osteoclasts are considered to be proliferative monocytes [44]. The monocytes we isolated are likely to have included both subtypes. In addition, the time-dependent increase in expression of IL-1Ra (Fig. 7L) may have resulted in a decreased signaling response to IL-1β. Also the initial low expression of IL-1RI (Fig. 7J) and high expression of IL-1RII (Fig. 7K) would delay the response by monocytes.

Live cell imaging provided intriguing information on the life span of osteoclasts generated by the different precursors. Myeloid blasts, the fastest responding
subset, started to form large osteoclasts (>2400µm², corresponding to >10 nuclei) after a culture period of 75 hours. These large osteoclasts, however, had a short life span: the cells disintegrated within the next 24 hours. The formation of such large osteoclasts by monocytes took place at a much later time point, 95 hours, but these cells survived much longer, up to 3 days. Early blast-derived osteoclasts proved to stay alive for two days. Whether this difference in life span of the different subsets of osteoclasts has any meaning for the *in vivo* situation is not known. One could speculate, though, that depending on the need for a certain type of osteoclastic activity, the different subsets may provide the osteoclast best equipped for that particular need. For instance, as it takes different time spans to digest cortical bone and trabecular bone matrix, they may need osteoclasts with a different life span.

We have shown a significantly enhanced bone resorption in the presence of IL-1β. This coincided with an increase in the number and size of osteoclasts on bone slices. Since there was hardly any difference in the expression between the cells cultured with or without the cytokine of most genes, for example *c-fms*, *RANK*, and *TRAF6* (data not shown), and hardly any difference in bone resorption per osteoclast was found (Fig. 4D), the increased bone resorption induced by IL-1β is possibly due to the generation of more and larger osteoclasts. Studies have shown that the number of nuclei per osteoclast is proportional to the volume of the bone resorbed by the osteoclasts [45]. Also, the number of actin rings formed by an osteoclast on bone or dentin was shown to increase with size [16]. Since it was shown previously that IL-1β can stimulate bone resorption by large mature osteoclasts [46], we can speculate that the increased number of large osteoclasts induced by IL-1β contributed to an increased bone resorption.

In summary, we conclude that IL-1β stimulates the osteoclast precursors, early blasts, myeloid blasts and monocytes in different ways with respect to proliferation and multinucleation: IL-1β accelerates early blast proliferation, but this parameter is not affected with myeloid blasts and monocytes. The cytokine stimulates bone resorption by inducing multinucleation in all three subsets. Myeloid blasts respond in the formation of the highest number of large osteoclasts as well as the highest level of bone resorption. Our results indicate that under the influence of the cytokine IL-1β, different murine osteoclast precursors differ in their capacity to generate osteoclasts. We propose that the differently generated osteoclasts may play different roles in bone degradation during inflammation.
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