Bone undergoes a continuous remodeling; a process which involves the activity of bone lining cells, osteocytes, osteoblasts and osteoclasts. Among these cell types, osteoclasts play an essential role in many bone diseases, which affect different skeletal locations in the body. As reported previously, osteoclasts at different locations are not identical, and such bone-site-specific osteoclasts suggest distinct osteoclast precursor pools. In this thesis we focused on the osteoclast precursors in mouse bone marrow. This marrow contains three different subsets of osteoclast precursors: early blasts, myeloid blasts and monocytes. In an attempt to understand how the distinct osteoclast precursors are affected in inflammatory bone pathologies, we analyzed the responses of these osteoclast precursor subsets to the inflammatory cytokines IL-1β (Chapter 2) and TNF-α (Chapter 3). In addition, we studied the response of these precursors in a mouse model for alleged non-inhibited IL-1 signaling (Chapter 4).

OSTEOCLAST PRECURSORS

Osteoclasts are generated by cells from the hematopoietic monocyte/macrophage lineages [1,2]. Osteoclast precursors are found at different sites in the body, including bone marrow [3], blood [4], spleen [5] and peritoneum [6]. The presence of different osteoclast precursors in bone marrow, spleen and blood can be analysed using flow cytometry. In long bone marrow, according to the expression of CD31 and Ly-6C, 6 subsets can be distinguished (Chapter 2, 3, 4). Among the 6 populations, only early blasts (CD31$^{hi}$ Ly-6C$^{-}$), myeloid blasts (CD31$^{+}$ Ly-6C$^{+}$) and monocytes (CD31$^{-}$ Ly-6C$^{hi}$) are M-CSF and RANKL responsive cells [7] and it was shown that they have the capacity to differentiate into osteoclasts [8]; a finding confirmed also in this thesis. In spleen, monocytes can be sorted as CD11b$^{+}$ Ly-6C$^{high}$ subsets which can differentiate into osteoclasts, and the CD11b$^{+}$ Ly-6C$^{++}$ subset is recognized as an inflammatory monocyte [9]. Regarding peripheral blood, monocytes can be categorized into two subsets: the classical monocytes (Ly-6C$^{hi}$), and non-classical monocytes (Ly-6C$^{lo}$) [10]. The precursors in bone marrow (early blasts, myeloid blasts and monocytes), are the origin of the other precursor cells found in the circulation and have a direct association with bone tissue. For these reasons, we limited ourselves to study these cell populations in bone marrows in this thesis.
Figure 1. The life span differs between osteoclasts generated from early blasts, myeloid blasts and monocytes. Micrographs were generated by live cell imaging during a period of 75 to 135 hours cell culturing. The cells were cultured with 30 ng/ml M-CSF, 20 ng/ml RANKL and 10 ng/ml IL-1β on plastic. Red arrows point to large osteoclasts.
Chapter 6

MONOCYTES: THE INFLAMMATORY OSTEOCLAST PRECURSOR

Monocyte recruitment

We studied the response of early blasts, myeloid blasts and monocytes to the inflammatory cytokines IL-1β and TNF-α \textit{in vitro}. Next to that, we assessed their percentage in an IL-1RA knockout arthritis mouse model \textit{in vivo}. Our data indicated that monocytes do not give rise to the highest number of osteoclasts, but monocyte-derived osteoclasts have the longest life span in the presence of IL-1β (Chapter 2). This cell type expresses much higher mRNA levels of TNF-α receptors than early blasts and myeloid blasts, which could make them more sensitive to TNF-α (Chapter 3). In Chapter 4, we showed that deletion of IL-1RA increased the proportion of monocytes, suggesting that monocytes are the most susceptible subset to IL-1 \textit{in vivo}. IL-1 is known to be the driving cytokine released under inflammatory conditions, during which monocytes are recruited to mediate host immune defense [11].

It still remains unclear whether the bone marrow-resident monocyte (CD31⁻Ly-6C$^{hi}$) characterized in the thesis, resembles the monocyte that is found in for instance blood and spleen. There are, however, some indications that this cell type shares features with monocytes outside the bone marrow [12]. Cell surface expression of the macrophage related marker Mac-1 also confirmed that this CD31⁻Ly-6C$^{hi}$ bone marrow cell type is like a monocyte [7]. The findings in the IL-1RA KO arthritis mouse model show that particularly monocytes were present at a higher percentage at most skeletal sites (Chapter 4). Since early blasts, myeloid blasts and monocytes follow successive stages in macrophage [7] and dendritic cell maturation [13], the increased proportion of monocytes in Chapter 4 suggested a maturation in the direction of macrophages or dendritic cells in the knockout animals. This possibly contributes to immune responses as well as osteoclastogenesis.

Sensitivity of monocytes to inflammatory cytokines

It is known that monocytes are recruited to inflamed tissues and are responsible for clearance of pathogens. They do so by killing and phagocytosis and also by secreting inflammatory cytokines, hereby attracting for instance T- and B-lymphocytes. Alternatively, they might be recruited at the bones of inflamed tissue, such as in periodontitis and rheumatoid arthritis, where they can contribute to the formation of osteoclasts. Therefore, it is likely that inflamed tissue contains the instructions for either cell fate. In inflamed tissue, high levels of inflammatory cytokines are present [14]. How some of
these cytokines induce or inhibit monocyte-derived osteoclastogenesis was studied in Chapters 2 and 3. Both IL-1β (Chapter 2) and IL-6 (Chapter 3) have stimulatory effects on monocytes’ osteoclast differentiation. Monocytes were shown to form the lowest number of osteoclasts compared to myeloid blasts and early blasts. This is probably due to their low proliferative capacity (Chapter 2), which is in agreement with de Bruijn et al., and Nikolic et al. who stated that these myeloid lineages have a decreasing proliferative capacity with an increasing maturity [7,13]. It is of considerable interest to note that monocyte-derived osteoclasts have the longest life span in the presence of IL-1β (Chapter 2) (also see Fig.1 unpublished data). In this respect the cells probably resemble the so-called “cell cycle-arrested quiescent osteoclast precursor (QuOPs)”. This cell type is characterized by its long life span without cell division [15]. In the latter study these QuOPs were harvested from murine bone marrow cells pre-cultured with M-CSF for 3 days [15]. This promoted macrophage maturation: most of the QuOPs were of later stages of myeloid lineage cells, resembling monocytes and macrophages. The long survival time of monocytes gives this cell type the potential to give rise to long life span-osteoclasts in the presence of IL-1β (Chapter 2). Therefore, we propose that this long life span-osteoclasts could contribute to a persisting bone degradation during inflammatory bone diseases.

Apart from the low proliferative capacity and long life span of monocytes, another intriguing finding of this subset is that TNF-α was shown to inhibit its osteoclastogenic potential when cultured on plastic (Chapter 3). This finding diverts from the generally proposed view that TNF-α stimulates osteoclast differentiation [16,17]. Interestingly, previous studies showed that TNF-α had different effects on osteoclast formation by precursors from different organs: it inhibited spleen cells’ osteoclastogenesis while this effect was not seen with bone marrow cells [18]. In this thesis, we also cultured splenocytes with combinations of different cytokines (Fig. 2, unpublished data), and found that the non-adherent spleen cells are the osteoclast precursors (Fig. 2A) while the adherent spleen cells failed to differentiate into osteoclasts (Fig. 2B). Regarding non-adherent spleen cells, TNF-α had no effect on osteoclast formation, whereas IL-1β or IL-6 were shown to stimulate this process (Fig. 2C). This indicates that splenocytes hardly respond to TNF-α, which is in line with Hayashi and co-workers’ finding [18]. Since spleen is known as the organ which stores half of the monocytes in mice [19], which are derived from bone marrow monocytes through circulation [11], the inhibitory effect of TNF-α found particularly with monocytes (Chapter 3) provides an explanation for the inhibition by TNF-α on splenocyte-derived osteoclastogenesis. Lam and
co-workers showed a similar inhibitory effect of TNF-α on purified myeloid precursors, which could be overcome by permissive RANKL exposure [20]. In Lam et al. ’s study, the cells were F4/80+/CD11b+/c-fms+/RANK+/CD106- murine bone marrow cells isolated by immunoselection [20]. This cell type likely resembles the monocytes in our study which express high F4/80 [8]. The responses of monocytes to TNF-α under different conditions (with/without RANKL, or with/without bone) makes this subset unique compared to the other two osteoclast precursor subsets.

Figure 2. Splenocytes’ osteoclastogenesis and the response to different combinations of cytokines. A. Non-adherent splenocytes have the capacity to differentiate into TRAcP+ osteoclasts. B. Adherent splenocytes do not differentiate into osteoclasts. C. Total number of TRAcP+ multinucleated cells formed under different cytokines’ conditions. IL-1β and IL-6
stimulated osteoclast formation, whereas TNF-α had no effect on this process. Splenocytes were isolated and seeded on plastic plates and cultured with M-CSF and RANKL overnight. After 20 hours, the splenocytes were categorized into non-adherent splenocytes (A) and adherent splenocytes (B). These cells were further cultured with 30 ng/ml M-CSF, 20 ng/ml RANKL, +/- 10 ng/ml IL-1β, +/- 10 ng/ml IL-6, +/- 10 ng/ml TNF-α on 96-well plastic plates. The cells were stained for TRAcP activity. TRAcP$^+$ multinucleated cells are purple and the nuclei are blue. TRAcP$^+$ multinucleated cells (>2 nuclei) were counted as osteoclasts. (n=6, $P<0.05$).

MYELOID BLASTS: THE DOMINANT OSTEOCLAST PRECURSOR

Myeloid blast-derived large osteoclasts in the presence of inflammatory cytokines

Among the three osteoclast precursor subsets myeloid blast is the dominant precursor in forming large osteoclasts as well as in achieving a high level of bone resorption, either in response to M-CSF [8], or to IL-1β (Chapter 2) and TNF-α (Chapter 3). Although it has been known for some time that the cytokine IL-1 stimulates the formation of large osteoclasts [21,22], the results of Chapter 2 show that one cell type in particular, the myeloid blast, contributes to that in vitro. Large osteoclasts were reported to resorb 2.5 times more bone per cell than small osteoclasts, and the resorbing activity correlated to the number of nuclei [23]. What is the mechanism causing such enhanced bone resorption activity by large osteoclasts induced by IL-1? It was reported that large osteoclasts have higher expression of IL-1RI, the receptor of IL-1 that induces the signaling, while small osteoclasts express higher levels of the decoy receptor IL-1RII [21]. In Chapter 2, we showed that myeloid blast-derived osteoclasts express a significantly higher level of IL-1RI in the presence of IL-1β, and myeloid blast-derived osteoclasts express a higher level of IL-1RI than early blasts and monocytes. These findings indicate that IL-1 predominantly affects the myeloid blasts which results in an increased size of the osteoclasts.

The stimulated multinucleation is likely due to enhanced cell-cell fusion. In line herewith, we found a significantly higher expression of DC-STAMP in myeloid blast cultures induced by IL-1β (Chapter 2). It is known that DC-STAMP plays a very important role in cell fusion [24]. Yet, how exactly DC-STAMP is involved in the fusion process is not known. Wang et al. stated that DC-STAMP was located at different sites of the osteoclasts as where fusion
took place. Moreover, osteoclast multinucleation is rather an actin-dependent process [25]. We have also analyzed the actin rings in osteoclasts generated by the three subsets (Fig. 3 unpublished data) and found that myeloid blast-derived osteoclasts cultured on bone and induced by IL-1β gave rise to a significantly higher number of actin rings per osteoclast compared to the other cultures (Fig. 3C). Actin rings are the prerequisite for forming sealing zones and further initialize bone resorption [26]. The high number of actin rings therefore is in line with the highest bone resorption activity in the myeloid blast cultures. In Chapter 5, we found that osteoclasts were smaller in an adseverin knockout mice (lack of proper actin severing and capping) compared to wild type mice. This proves that the size of osteoclasts is likely to be an actin dependent process. An in vitro study of this adseverin knockout mouse showed a similar effect on the size of osteoclasts indicating that adseverin regulates the actin cytoskeleton, which affects the size of osteoclasts [27]. Whether there is a link between myeloid blasts and the change of osteoclast size in adseverin knockout mice remains to be elucidated.

Myeloid blasts as osteoclast-ready precursors

Myeloid blasts form large osteoclasts the earliest both in response to IL-1β (Chapter 2; also see Fig. 1), and to M-CSF and RANKL [8]. Although myeloid blasts are not the most mature subset in the successive macrophage maturation process [7,13], they contain the highest number of M-CSF and RANKL responding cells as analyzed by a colony forming unit (CFU) assay, indicating that this cell lineage contains the most osteoclast-ready precursors [8]. As analyzed by live cell imaging (Fig.1), in the presence of IL-1β, myeloid blast generated large osteoclasts which can be visualized already after 85 hours of culture. This is 10 hours earlier than early blasts and 20 hours earlier than monocytes. Therefore, we hypothesize that in the hematopoietic system, CD31hi Ly-6C– bone marrow cells contain subsets with different differentiation capacities and can go into three different directions (Fig. 4): including the directions of the macrophage (Fig. 4A), dendritic cell (Fig. 4B), lymphoid stem cell (Fig. 4C) and osteoclast (Fig. 4D).

Early blasts, myeloid blasts and monocytes are successive stages during M-CSF-stimulated macrophage maturation [7]. These maturation stages in the macrophage lineages can all differentiate into osteoclasts in the presence of M-CSF and RANKL, but myeloid blast is the subset most mature/ready to differentiate into osteoclasts. De Vries and co-workers reported that the longer myeloid blasts were primed with M-CSF, the harder for them to differentiate
into TRAcP⁺ osteoclasts [28]. This is because that M-CSF priming led myeloid blasts to differentiate into monocytes/macrophages [7], which was proven by the positive expression of macrophage markers F4/80, Mac-1 and with microarray analysis [28]. These myeloid blast-derived monocytes/macrophages became less prone to differentiate into osteoclasts compared to myeloid blasts. Since myeloid blasts are destined with a short life span (Chapter 2 and Fig.1), priming without RANKL for up to 4 days may exceed their osteoclast differentiation time on plastic. Taken together, myeloid blasts appear to be the “osteoclast-ready cells”; they quickly respond to differentiate into osteoclasts.
Figure 3. Formation of actin rings in osteoclasts on bone differentiated from early blasts, myeloid blasts and monocytes. A. Confocal microscopic image of osteoclasts and actin ring structures. Cells were cultured with 30 ng/ml M-CSF, 20 ng/ml RANKL, with or without 10 ng/ml IL-1β for 5 days on bone slices. The membrane of osteoclasts was stained for CD44 (red), actin ring structure was stained with phalloidin (green, arrows point to actin rings) and nuclei stained with DAPI (blue). IL-1β increased the number of actin rings predominantly in myeloid blast cultures. B. Correlation analysis of the number of actin rings per osteoclast and the number of nuclei per osteoclast. C. The area of actin rings (μm²) per osteoclast showed that IL-1β stimulated the actin ring area in myeloid blast cultures. Cells with more than 2 nuclei were recognized as osteoclasts. 10 osteoclasts were analyzed per animal and the mean was calculated. Totally 6 animals were analyzed and these six conditions were compared with ANOVA test (n=6, ** P<0.01, ***P<0.001).

EARLY BLASTS: THE PROLIFERATING AND PLURIPOTENT PRECURSOR

Early blasts, known as the immature stage of macrophage development [7], were shown to have the highest proliferative capacity [13]. In line with this finding, we showed in Chapter 2 that IL-1β accelerated proliferation by early blasts; an effect not seen with myeloid blasts and monocytes. This was confirmed by the mRNA level of proliferation gene Cyclin D1 [29]. A possible explanation for such a response to the cytokine is its known proliferation-stimulatory effects on immature myeloid precursors [30]. Indeed, the earliest stage in the myeloid lineage, early blasts, responded to IL-1β by an enhanced proliferation. In Chapter 3, we have also tested a combination of different inflammatory cytokines, (IL-1β, TNF-α and IL-6). We found that there was an enhanced osteoclastogenesis by early blasts in response to any condition of combinations, except with IL-6 alone. Besides their high proliferative capacity, cells present within the early blast population also have the potential to differentiate into other cell types (Fig. 4C). One of the main characteristics of these CD31 hi Ly-6C- bone marrow cells is their thymus-repopulating capacity; they are able to differentiate into T cell subsets [31,32]. It was also reported that the CD31 hi/med Ly-6C population had a long-term repopulating ability. This population was defined as immature hematopoietic stem cells with high proliferation capacity [33]. 90% of these cells are lymphocytes (CD31 med Ly-6C-) and 10% are early blasts (CD31 hi Ly-6C-). Therefore early blasts contain progenitors of both the lymphoid lineage and the myeloid lineage, with a high proliferative capacity and contain the cells that can differentiate into different
types of hematopoietic cells.

**THE BLUNTED OSTEOCLASTOGENESIS**

Commonly used protocols for mouse bone marrow derived osteoclastogenesis make use of M-CSF and RANKL, which are regarded as the minimally required cytokines for osteoclast formation. Next to this cocktail, many inflammatory factors, such as IL-1 and TNF-α were shown to increase osteoclast formation and activity. However there are certain conditions during which osteoclast formation is no longer possible: IL-1β was generally recognized as a stimulatory factor on osteoclastogenesis, but Lee and co-authors showed early addition of IL-1β together with, or prior to RANKL significantly inhibited human osteoclastogenesis. This effect was no longer seen when IL-1β was added after a stimulation with RANKL [34]. Our own results indicate that certain myeloid stages, under certain cell culture conditions, may become osteoclastogenesis insensitive. Two examples are given, concerning two osteoclast progenitors and two cytokines. In this thesis we found that addition of TNF-α together with RANKL prevented murine monocyte-derived osteoclast differentiation on plastic, while such inhibitory effect was abolished by M-CSF and RANKL exposure as well as by seeding monocytes on bone (Chapter 3). Interestingly, the block of osteoclastogenesis by TNF-α and the previously shown myeloid blast-derived osteoclastogenesis by M-CSF priming [28] did not occur on bone. These observations led us to conclude that apparently osteoclastogenesis insensitivity can be specifically induced in certain, defined stages. This insensitivity is temporal, since it can be overcome when in contact with bone, that apparently nullifies osteoclastogenesis inhibition, making the precursors susceptible to RANKL.

A possibility of the blunted osteoclastogenesis is that in those conditions, the expression of RANK is significantly decreased, as seen in the study by Lee et al. [34] and in Chapter 3. This suggests that in order to stimulate osteoclast differentiation, activated RANK expression on the precursor cells is a prerequisite. From these examples of blunted osteoclastogenesis, bone can be used as a stimulatory factor to overcome the inhibitory effect [28] (also see Chapter 3). A possible explanation for such a stimulation may be the presence of certain compounds present in the matrix of bone. It was shown that bone tissue stimulates osteoclast formation by releasing high levels of IL-1 [35] and transforming growth factor-β [36]. Additionally, proteins such as osteopontin and bone sialoprotein present in bone could also stimulate the
osteoclast differentiation [37,38]. Recently, Yao and co-workers reported that RANKL stimulated osteoclast formation of TRAF6−/− precursors cells when cultured on bone, but not on plastic [39]. Therefore interaction with bone and bone matrix proteins is likely to have an important role on activation of the osteoclastogenic signaling pathway. The other way around: blunted osteoclastogenesis was only observed in cultures on plastic, which led us to propose that under certain conditions at a distance from bone, osteoclast formation should be prevented.

**HETEROGENEITY OF OSTEOCLAST PRECURSORS AT DIFFERENT SKELETAL SITES**

In many inflammation related bone pathologies, different types of bones might be affected differently. This thesis has studied the composition of bone marrow cells at different skeletal sites using an IL-1RA knockout mice model (Chapter 4). Four types of bones have been analyzed, being long bone, calvaria, vertebra and jaw. These different bones were chosen since many studies showed that heterogenic osteoclasts can be generated from different skeletal sites [40–43], and these functionally different osteoclasts could result from different osteoclast precursors [40]. In wild-type mice, long bone marrow contained a significantly higher amount of myeloid cells than the other sites. This finding is in line with data presented by de Souza Faloni et al., who showed that long bone contained a higher number of myeloid cells than jaw bone marrow [42]. If we assume that osteoclasts are derived locally, these findings may suggest that different skeletal sites differ in their dynamics to form osteoclasts locally.

In Chapter 4 we used IL-1RA knockout mice which is a model that develops rheumatoid arthritis without extra stimuli [44]. We studied the precursor composition and osteoclastogenesis at different locations. The bone marrow cells from the different skeletal sites were shown to be stimulated differently. A high number of large osteoclasts formed from the marrow cells obtained from long bone was probably related to the high percentage of myeloid blasts in this type of bone. These findings may suggest that due to the high number of myeloid blasts in IL-1RA KO mice, the long bones will be the most severely affected location influenced by joint inflammation. Is there a different susceptibility of different skeletal sites to the inflammatory cytokines? Studies comparing cytokine susceptibility of bone marrow from different bones are rare. It was shown, for example, that TNF-α stimulates osteoclastogenesis at
vertebra [45]; that calvaria was highly susceptible to IL-1 [46]; and that IL-6 together with sIL-6R were found to stimulate osteoclastic bone degradation in calvaria bones [47]. However no studies have compared the responses of bone marrow cells isolated from the different skeletal locations. In this thesis we analyzed the responses of the bone marrow cells isolated from the different skeletal sites under conditions of a deregulated IL-1. An increased percentage of myeloid lineage cells was found in both long bone and jaw, and the bone marrow cells from all of the four sites displayed an increased osteoclastogenic capacity in the IL-1RA knockout model. Further studies are needed to elucidate whether this may have an effect on the actual bone phenotype of these mice.

Figure 4. Different differentiation routes of early blasts, myeloid blasts and monocytes.
A. Early blasts, myeloid blasts and monocytes follow successive macrophage maturation stages when cultured with M-CSF. B. They follow dendritic cell developmental stages when cultured with GM-CSF. C. Early blasts contain the cells that have the potential to differentiate into various thymocyte subsets. D. All these three subsets follow their own route to differentiate into osteoclasts stimulated by M-CSF and RANKL: Myeloid blasts are the osteoclast-ready precursors. Different osteoclasts are formed by these three subsets if stimulated by the inflammatory cytokines IL-1β or TNF-α.

CONCLUSION

This thesis unraveled certain aspects of the heterogeneity of mouse bone marrow osteoclast precursors in the context of their responses to inflammatory cytokines. We showed that the different osteoclast precursor subsets respond differently to inflammatory cytokines:

1) **Early blasts** are the immature precursors characterized by their highly proliferative property, and IL-1β particularly stimulates early blast proliferation.

2) **Myeloid blasts** are the most ready osteoclast precursors: they respond the quickest and generate the highest number of large osteoclasts under the influence of both IL-1β and TNF-α. This cell type appears to be the dominant osteoclast precursor and gives a rapid response under both physiological conditions and in the presence of inflammatory cytokines.

3) **Monocytes** respond differently to TNF-α: this cytokine displays both inhibitory and stimulatory effects on monocytes’ osteoclastogenesis, depending on the timing of exposure to this cytokine. Monocytes are present at a higher percentage in the IL-1RA knockout mice, mainly in long bone and jaw sites. They do not give rise to very high numbers of osteoclasts compared to the other two subsets, but monocyte-derived osteoclasts survive the longest time in the presence of IL-1β, and they may give persistent bone degradation in the presence of IL-1β.

This thesis provides insight into the heterogenic properties of murine bone marrow osteoclast precursors and their possible contribution to bone-site-specific osteoclasts. Inflammation related bone pathologies such as rheumatoid arthritis and periodontitis arise clearly in complex microenvironments. This microenvironment is likely a “cytokine soup” where inflammatory cytokines
like IL-1, TNF-α and other mediators are present. In this thesis we conclude that these cytokines have diverse functions on different osteoclast precursors and these cytokine-induced osteoclastogenesis contribute differently to different bone sites.
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