Chapter 1

General Introduction
INFLAMMATORY BONE DISEASES

Inflammatory bone diseases are characterized by local bone erosion and the patients suffering from these diseases have severely affected quality of life. Rheumatoid arthritis (RA) is one of the most common systemic inflammatory bone diseases and patients with RA are often associated with an increased fracture risk [1]. RA is an autoimmune disease which can result in local joint deformations due to joint erosion and bone loss [2]. Although the symptoms of RA patients are quite clear, the pathogenesis of this disease is still not completely understood. Up to date knowledge suggests that environmental factors, such as smoking [3], as well as a genetic basis both contribute to RA development [4,5]. It is well established that inflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) play an important role in RA development [6–10]. During the process of inflammation, immune modulators have both local and systemic effects and eventually lead to bone degradation [6].

Another prevalent inflammation-related bone disease is periodontitis, which affects billions of people worldwide [11]. Periodontitis is a common oral inflammatory disease that leads to the destruction of alveolar bone. This disease is initiated by bacteria; one of the most prevalent ones is Porphyromonas gingivalis [12]. Induction of the subsequent immune response results in an increased bone resorption by osteoclasts [13]. Such bone loss around the teeth, if left untreated, will eventually lead to the loss of teeth.

BONE CELLS AND BONE REMODELING

Bone is one of the most important tissues for the support and protection of the body. It is metabolically active and it remodels throughout the entire life. In this process four cell types are involved: osteoblasts, osteocytes, bone lining cells and osteoclasts.

Osteoblasts are the bone-forming cells and are derived from mesenchymal stem cells. They are characterized by a high expression of the key transcription factor Runt-related transcription factor 2 (Runx2), osterix and alkaline phosphatase (ALP) [14–17]. To build up bone tissue, osteoblasts start by secreting osteoid, an organic matrix which contains mostly collagen and several other proteins such as osteopontin. Subsequently, calcium and phosphate are secreted by these cells and hydroxyapatite is generated. Next to its role in the formation
of bone, osteoblasts can also provide osteoclastogenic factors that stimulate osteoclastogenesis [18].

Once the bone is produced by osteoblasts, some of these cells cover the surface of the newly formed bone, known as bone-lining cells. Other osteoblasts are trapped inside the bone tissue and become osteocytes. As the most abundant cell type of bone, osteocytes provide for the communication with the cells adjacent to the bone. Osteocytes play a crucial role in the modulation of bone metabolism. With their long cytoplasmic extensions they form a network in the bone. These slender extensions have the capacity to sense strain differences exerted upon the bone [19] and to respond by activating or deactivating bone resorption and formation, thus modulating the activity of osteoblasts and osteoclasts [20]. Recently, evidence showed that osteocytes express high amount of RANKL that support osteoclastogenesis [21].

To balance bone remodeling another important cell type, the osteoclast, is responsible for bone degradation. Different from the osteoblast lineage, these cells are derived from the monocytic lineage. Osteoclasts are large multinucleated cells and can degrade both mineral and matrix, e.g. collagen. The non-organic bone matrix (mineral) is first dissolved by acid released by osteoclasts through several pumps, which exposes the matrix of the bone. This is followed by secretion of a series of proteolytic enzymes, such as cathepsin K and matrix metalloproteinases (MMP) that degrade the organic bone matrix [22,23]. It has been reported that osteoclasts not only resorb bone, but they can also stimulate osteogenic differentiation by releasing matrix-derived growth factors [24], for instance transforming growth factor β1 (TGFβ1) [25], and regulate activity of other hematopoietic stem cells [26].

Bone remodeling involves the interaction between the mentioned cell types. Osteoblasts and osteocytes [27] express RANKL which is an essential molecule for osteoclastogenesis to occur. Mature osteoclasts release clastokines (e.g. tartrate-resistant acid phosphatase) which in turn stimulate osteogenesis [28]. The balance between bone formation and bone resorption ensures a healthy bone homeostasis. Bone diseases are characterized by an imbalance of these processes. For example during RA resorptive activity by osteoclasts exceeds the formation activity by osteoblasts [29].
Figure 1. Osteoclast precursor cells. Osteoclasts can be generated from bone marrow myeloid lineage (A), blood borne-monocytes (B) and splenocytes (C).
OSTEOCLASTS AND OSTEOCLASTOGENESIS

As mentioned above, osteoclasts are generated from mononuclear monocyte/macrophage lineage precursor cells. Two cytokines are necessary for osteoclast formation: macrophage colony-stimulating factor (M-CSF) [30] and receptor for activation of nuclear factor kappa B ligand (RANKL) [31], both are provided by cells from the osteoblast lineage. Osteoblasts release M-CSF, which binds to colony stimulating factor 1 receptor (c-fms) expressed on the membrane of monocytes, inducing proliferation of these osteoclast precursors [32,33]. Next to M-CSF, osteoblasts, bone lining cells and osteocytes express RANKL on their membrane [34]. RANKL can bind to its receptor RANK expressed on the membrane of osteoclast precursors, and induces osteoclast differentiation [31]. During osteoclastogenesis, however, the regulatory decoy receptor of RANKL, osteoprotegerin (OPG), is released also by osteoblasts. OPG inhibits osteoclast formation by competing with RANK by binding to RANKL [31].

When osteoclast precursors undergo osteoclastogenesis and fuse to form multinucleated osteoclasts, these differentiated osteoclasts attach to the bone surface. The interaction of the cell with the bone results in polarization of the cells. This allows osteoclasts to tightly adhere to the bone surface, where they form a structure called “sealing zone”. In the centre of this sealed off area, a resorption site is formed by generating an area of membrane protrusions, recognized as ruffled border. Osteoclast polarization is required to seclude a portion of bone where an acid microenvironment is established, with a pH of about 4.5 [35]. Protons are released into the resorption area through ion exchange by several proton pumps present in the ruffled border membrane. These pumps include the H+-ATPase proton pump, a chloride channel, and a bicarbonate/chloride exchanger [36,37]. These pumps generate an acid environment which initiates the demineralization, and subsequently ensures an optimal activity of cathepsin K to degrade the bone matrix. At later stages of resorption, evidence suggests that the pH rises, creating a more favorable environment for MMP-mediated bone matrix resorption [38].

OSTEOCLAST PRECURSORS

Numerous findings indicate that osteoclasts are not identical at different skeletal sites [39–44]. Osteoclasts from different sites are different in their bone-resorbing activities: MMPs and cysteine proteinases are involved in
calvarial bone resorption, while long bone resorption appears to depend on cysteine proteinases only [44]. Also osteoclasts in the scapula depend more on MMPs than cysteine proteinases for resorption while the opposite was found for long bone [43]. It has been shown that precursors from jaw and long bone marrow differ in their capacity to generate osteoclasts [40]. These data suggest that the occurrence of phenotypically different osteoclasts may be related to differences in the precursor composition of local bone marrow. The differences of osteoclast precursors and their possibly different response toward cytokines will be the subject of this thesis.

Figure 2. Mouse bone marrow osteoclast precursor subsets. Mouse bone marrow cells can be further categorized to several subsets by labeling them with CD31 and Ly-6C: Early blasts (CD31\(^{hi}\)Ly-6C\(^{i}\)), myeloid blasts (CD31\(^{i}\)Ly-6C\(^{i}\)), monocytes (CD31\(^{i}\)Ly-6C\(^{hi}\)), lymphocytes (P2), granulocytes (P4), and erythroid blasts (P7). Among these sorted cell subsets, early blasts, myeloid blasts and monocytes contain the osteoclast precursors.
The mononuclear precursors of osteoclasts originate in the bone marrow and move to many different sites such as blood and spleen (Fig. 1). Bone marrow contains stem cells which can develop into myeloid and lymphoid stem cells [45]. Myeloid stem cells give rise to either monocyte/macrophage progenitors, or to neutrophils, megakaryocytes and erythrocytes. Lymphoid stem cells differentiate into immune cells such as NK cells, T cells and B cells. Neither mature T, and B cells [46] or megakaryocytes and erythrocytes [47] have the potential to differentiate into osteoclasts. These cells, however, may play a role in regulating osteoclastogenesis by producing RANKL [48], and transforming growth factor-β (TGF-β) [49]. In bone marrow, only a small portion of cells, the myeloid lineage cells, have the potential to differentiate into osteoclasts (highlighted in blue in Fig. 1). Next to these cells blood-borne monocytes [50] as well as splenocytes [51] also have the capacity to differentiate into osteoclasts in mice.

**EARLY BLAST, MYELOID BLAST AND MONOCYTE**

Following discovery of cell surface markers, fluorescence-activated cell sorting (FACS) has been used to sort different types of osteoclast precursors based on specific surface markers. Three successive stages during myeloid lineage progression, have been recognized in mouse bone marrow by labeling the bone marrow cells with CD31 and Ly-6C: early blasts (CD31$^{hi}$ Ly-6C$^{-}$), myeloid blasts (CD31$^{+}$ Ly-6C$^{+}$) and monocytes (CD31$^{-}$ Ly-6C$^{hi}$) [52] (Fig. 2). These three subsets differ in their proliferation capacity [52], but each has the capacity to differentiate into osteoclasts [53]. It is known that these osteoclast precursor subsets contribute differently to osteoclastogenesis [53], but whether osteoclasts generated from these precursors are functionally different is not known. In the studies presented in this thesis we will focus on the role of early blasts, myeloid blasts and monocytes in osteoclastogenesis and the effect of inflammation related cytokines hereupon. More in particular, it is the aim of this thesis to investigate the responsiveness of different osteoclast precursor to inflammatory stimuli. The response of the three different osteoclast precursor subsets to IL-1β is studied in Chapter 2, and to TNF-α in Chapter 3. In Chapter 4 we studied the composition of the precursors at different skeletal sites in an IL-1RA knockout mouse model (Chapter 4).
Chapter 1

PROTEINS THAT MODULATE OSTEOCLAST FORMATION AND ACTIVITY

Inflammatory cytokines

Apart from the two essential cytokines, M-CSF and RANKL, some cytokines released during inflammation have been recognized to stimulate osteoclast generation. IL-1 is one of the driving cytokines that stimulates multinucleation and contributes to osteoclast bone resorption activity [54]. It was shown that IL-1 triggers the key osteoclastogenesis signaling pathway, including NF-κB, p38, c-fos and NFATc1, and it has the potential to induce osteoclast formation independent of RANKL [55]. Another important cytokine, TNF-α, released by activated macrophages and involved in systemic inflammation, also stimulates osteoclastogenesis. Like RANKL, it belongs to the TNF superfamily. TNF-α and RANKL synergistically stimulate the expression of NF-κB and JNK thus activate osteoclast signaling pathway [56]. TNF-α closely associates with RANKL and stimulates RANKL-pretreated osteoclastogenesis via its receptor TNF-R1 [57]. To improve our understanding on the role of key cytokines on osteoclastogenesis, and how individual osteoclast precursors respond to such cytokines, we analyzed the effects of IL-1β and TNF-α on these processes in Chapters 2, 3 and 4.

Adseverin

In the past decade, several studies tried to elucidate the role of an actin-capping and severing protein, adseverin (also called scinderin), in the formation and function of osteoclasts. Adseverin closely associates with actin filament organization and plays an important role in regulating cell-cell fusion and podosome organization in vitro [58]. It was also shown that knockdown of adseverin reduced bone resorption by suppressing the secretion of cathepsin K by osteoclasts [59]. However, studies performed on mice in which the protein was knocked out showed no effect on vertebrae as assessed by microCT. Yet deleting adseverin protected against alveolar bone loss [58]. These latter findings suggested that adseverin might play a different role in vivo at different skeletal sites. This will be investigated in Chapter 5.

THESIS OUTLINE

This thesis focused on different mouse osteoclast precursors, and specifically
studied their responses to different cytokines that are highly expressed under inflammatory conditions. The studies aimed to answer whether different osteoclast precursors respond differently to inflammation-related cytokines in their capacity to generate osteoclasts.

In **Chapter 2**, we cultured three mouse osteoclast precursor subsets without and with IL-1β, and studied osteoclastogenesis, bone resorption activity, proliferation and gene expression. Their response to another inflammatory cytokine, TNF-α, was studied in **Chapter 3**. Both Chapter 2 and 3 are *in vitro* studies in which single cytokines were used to test the response of osteoclast precursors from long bone marrow. However, the *in vivo* inflammatory situation is far more complex involving a wide variety of systemic responses. In an attempt to analyze osteoclastogenesis in an inflammatory condition, we used an IL-1RA knockout mouse model to study the osteoclast precursor composition at different skeletal sites and their osteoclastogenic capacity (**Chapter 4**). In this model that lacks a negative feed-back of IL-1, we explored in different bones how the proposed constant IL-1 signaling affected the distribution of osteoclast precursors. In **Chapter 5**, we used transmission electron microscopy to analyze osteoclast morphology *in vivo*, aimed to find out whether an actin-capping/severing protein, adseverin, plays a role in osteoclastogenesis at different skeletal sites in non-inflamed and inflamed tissue.
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