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Review

Does complement play a role in bone development and regeneration?

Philipp Schoengraf[a], John D. Lambris[b], Stefan Recknagel[a], Ludwika Kreja[a], Astrid Liedert[a], Rolf E. Brenner[d], Markus Huber-Lang[c], Anita Ignatius[a,*]

[a] Institute of Orthopaedic Research and Biomechanics, Centre of Muskuloskelettal Research, University of Ulm, Helmholtzstrasse 14, 89081 Ulm, Germany
[b] Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104-6100, USA
[c] Division of Joint and Connective Tissue Diseases, Department of Orthopaedics, Centre of Musculoskeletal Research Ulm, University of Ulm, Oberer Eselsberg 45, 89081 Ulm, Germany
[d] Department of Traumatology, Hand-, Plastic-, and Reconstructive Surgery, Centre of Surgery, Centre of Musculoskeletal Research Ulm, University of Ulm, Steinhövelstrasse 9, 89075 Ulm, Germany

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ABSTRACT

The skeletal and the immune system are not two independent systems, rather, there are multifaceted and complex interactions between the different cell types of both systems and there are several shared cytokines. As a part of the innate immunity, the complement system was found to be an important link between bone and immunity. Complement proteins appear to be involved in bone development and homeostasis, and specifically influence osteoblast and osteoclast activity. This review describes the complex mutual regulation of the two systems, and indicates some of the negative side effects as a result of inappropriate or excessive complement activation.

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Introduction

What began in the seventies with the discovery of a bone resorbing activity in cell culture supernatants of PBMNC (Horton et al. 1972) is now a steadily growing scientific field of intense interest, termed osteoimmunology. The subject of osteoimmunology is the multifaceted mutual regulation of the skeletal and the immune system that reaches far beyond the function of bone to provide the location of haematopoiesis and the formation of immune cells in the bone marrow. Bone cells, such as the bone-forming osteoblasts, their precursors the mesenchymal stem cells (MSC), and the bone resorbing osteoclasts are all influenced by cytokines released from

Abbreviations: BMP, bone morphogenetic protein; C3aR, C3a receptor; C5aR, C5a receptor; DAF, decay accelerating factor, CD55; DAMP, danger associated molecular pattern; ERK, extracellular-signal related kinase; GPCR, G protein-coupled receptor; IGF, insulin-like growth factor; IL, interleukin; MAC, membrane attack complex; MACIF, MAC inhibitory factor, CD59; MCP, membrane co-factor of proteolysis, CD46; M-CSF, macrophage-colony stimulating factor; MMP, matrix metallo proteinase; MSC, mesenchymal stem cell; OPG, osteoprotegerin; PAMP, pathogen associated molecular pattern; PBMNC, peripheral blood mononuclear cells; PDGF, platelet derived growth factor; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; TGF, transforming growth factor; TNF, tumour necrosis factor.

* Corresponding author.
E-mail address: anita.ignatius@uni-ulm.de (A. Ignatius).

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immune cells while conversely immune cells, such as T cells, are a target for RANKL, a typical bone cytokine.

In this review we will first provide a short overview of the interactions of bone with immune cells or inflammatory cytokines and will then concentrate on an important pro-inflammatory protein cascade, the complement system. Finally, we will discuss some diseases, which are associated with various impaired complement-bone interactions.

Bone and bone homeostasis

Bone is an organ with crucial functions in providing stability, serving as the main calcium depot, and containing the bone marrow with haematopoietic as well as mesenchymal precursor cells. It is continuously rebuilt in a process termed bone remodelling, a dynamic balance of bone formation by osteoblasts and resorption by osteoclasts. Remodelling is essential for constant renewal and repair of bone, and for adaptation to changing mechanical requirements. Diseases associated with pathologically low or high bone density such as osteoporosis or osteopetrosis, respectively, are linked to an imbalance of osteoblast and osteoclast activity.

Osteoblasts are derived from mesenchymal precursor cells and secrete the bone matrix, which mainly consists of collagen and non-collagenous proteins, *e.g.* alkaline phosphatase, osteopontin, and osteocalcin that are important for example for the mineralization of the matrix (Kassem et al. 2008). Mature osteoblasts that are completely embedded in bone substance become osteocytes. Although metabolically barely active, they have signalling functions in bone remodelling, and in phosphate and calcium metabolism as well as in mechanosensing (Bonewald 2010). Osteoblast differentiation and function is regulated by numerous factors, among them hormones, nerve signals, and vascular agents. Moreover, paracrine factors are involved in osteoblast regulation, *e.g.* transforming growth factor-ß (TGF-ß), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and platelet-derived growth factors (PDGF), as well as inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumour necrosis factor (TNF-α) (Heng et al. 2004; Kreja et al. 2010).

Osteoclasts are highly specialized multinucleated cells derived from haematopoietic cells of the monocyte/macrophage lineage. Osteoclasts are able to degrade the bone matrix by providing an acid environment and secreting proteolytic enzymes (Boyle et al. 2003). Interactions of osteoclast precursors with osteoblasts and stromal cells are essential for osteoclast differentiation. The key molecules regulating osteoclastogenesis are receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumour necrosis factor (TNF) family, and osteoprotegerin (OPG). Both factors are expressed by osteoblasts. Osteoclast formation is induced by the binding of RANKL to its receptor RANK on osteoclast precursors. The presence of macrophage-colony stimulating factor (M-CSF) is also important. M-CSF binds to the c-fms receptor of early osteoclast precursors, regulating their proliferation, differentiation, and survival. Osteoclast formation and activity is inhibited by OPG, the soluble decoy receptor for RANKL, which is also released by osteoblasts. The RANK/RANKL/OPG and M-CSF/c-fms receptor regulatory axes tightly couple osteoblast and osteoclast activity, thus controlling skeletal mass homoeostasis (Asagiri and Takayanagi 2007; Boyce and Xing 2008; Boyle et al. 2003). This finely tuned regulatory mechanism can be disturbed for example by inflammatory cytokines during the systemic or local inflammatory processes of fracture healing, particularly in the case of severe multiple trauma and in diseases such as osteoarthritis.

Interactions between the immune system and bone

Although the interactions of the skeletal and the immune system are manifold and complex, a key component of the mutual regulation is the RANK/RANKL/OPG system. RANKL is mainly produced not only by osteoblasts but also by immune cells such as T cells and neutrophils (Chakravarti et al. 2009; Maruotti et al. 2010). Binding of RANKL to its receptor RANK on monocytes is essential for the formation of osteoclasts by fusion of monocytes. However, the RANK/RANKL system also influences immune cell interactions, such as dendritic cell-T cell interactions and is necessary for the maturation of dendritic cells (Page and Miossec 2005; Schiano de Colella et al. 2008). The RANK/RANKL signalling is regulated by the expression of OPG, a decoy receptor for RANKL, which like RANKL is expressed by osteoblasts. The expression of these cytokines and the proliferation and differentiation of osteoblasts during the transition from bone resorption to bone formation is regulated by coupling factors expressed by osteoclasts. These coupling factors are secreted or membrane bound proteins expressed by osteoclasts or are liberated from the bone matrix during osteoclastic bone resorption (Matsuo and Irie 2008; Zhao et al. 2006). However, the RANK/RANKL/OPG system is also regulated by immune cells. B cells for example can up-regulate OPG expression and down-regulate RANKL expression (Djaafar et al. 2010).

In addition to the RANK/RANKL/OPG system, there are also interactions between bone and the immune system that are mediated by cytokines secreted by immune cells. Some of the most important pro-inflammatory cytokines produced by immune cells, such as IL-1β, IL-6, and TNF-α, induce osteoclastogenesis and bone resorption (Fig. 1). Other cytokines, such as the B cell mitogen IL-14, produced by Th1 and Th2 cells, have an osteoprotective effect (Maruotti et al. 2010).

In the following sections we will concentrate on the interaction between bone and the complement system, as complement activation is a central part of inflammatory processes and may play a central role in both bone and the immune system.

The complement system

The complement system is an ancient system for sensing and fighting danger, and is an essential part of the innate immunity. It defends the organism against foreign materials and pathogens by direct lysis or by recruitment of leukocytes, which perform phagocytosis. Normally, complement activation appears locally at sites of danger such as injuries, cell damage, and invasion of pathogens, helping to fight infections or to degrade dead or damaged cells. In contrast, severe incidents, such as multiple trauma or sepsis as well as chronic inflammatory processes, such as in rheumatoid arthritis and autoimmune diseases, can cause systemic, excessive, or persisting complement activation (Ricklin et al. 2010).

Most of the complement serine proteases are present in plasma as zymogens, inactive precursors that require proteolytic cleavage for activation. Several danger- or pathogen-associated patterns (DAMPs, PAMPs) including DNA, ATP, and pathogen surfaces can trigger complement activation via three different established pathways: the classical, the alternative, and the lectin pathway. All three pathways lead to the formation of one of two different C3 convertases and to the cleavage of C3 into C3a and C3b (Fig. 2). The larger fragment, C3b, can become a part of a newly formed C3 convertase or bind to a C3 convertase and form a C5 convertase that cleaves C5 to C5a and C5b. C5b promotes the acquisition of the terminal complement components C6 to C9 to the pathogen surface and the formation of the membrane attack complex (MAC) (Fig. 2). C3b and C5b bound to the cell surface of pathogens also act as opsonins and promote phagocytosis by macrophages and neutrophils.

In addition to these three activation pathways, other, complement-independent mechanisms for the cleavage of the

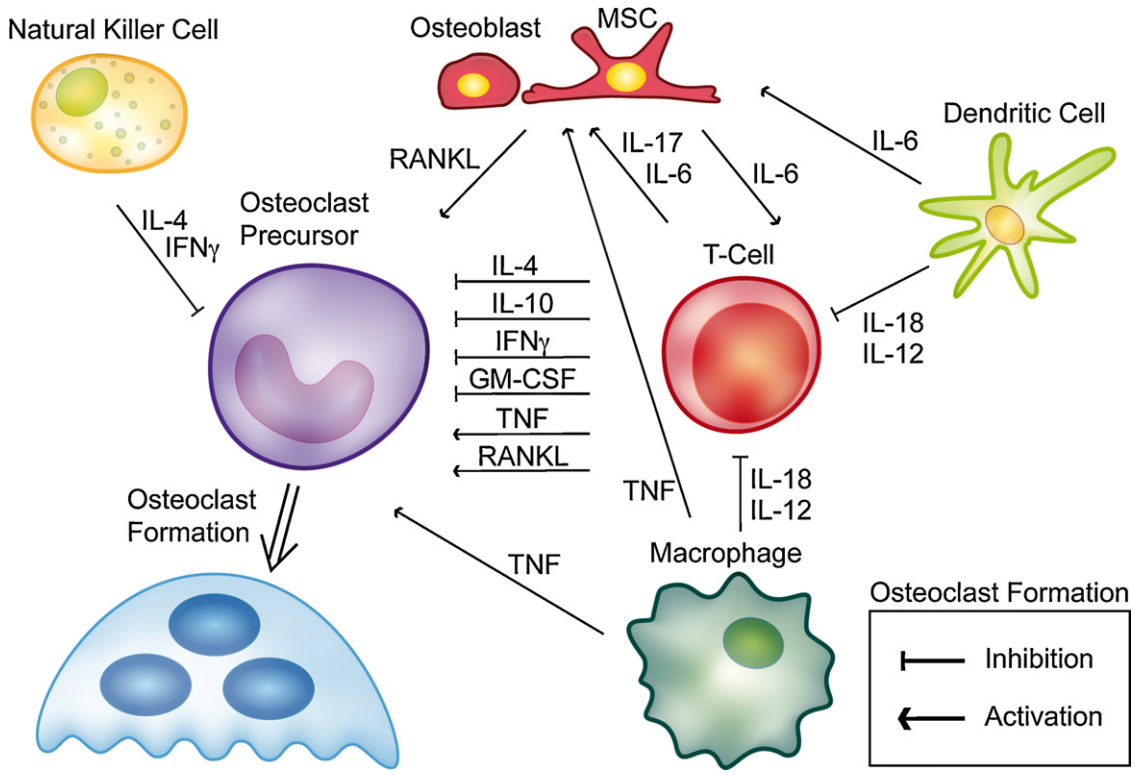

Fig. 1. Shared cytokines of bone and immune cells. Several cytokines that directly modulate OC formation are produced by immune cells while others indirectly influence osteoclast formation by affecting immune cells. A network of pro- and anti-inflammatory cytokines influencing osteoclast formation is shown. GM-CSF: granulocyte macrophage colony-stimulating factor; IFNγ: interferon-γ; IL: interleukin; RANKL: receptor activator of nuclear factor κB ligand; TNF: tumour necrosis factor.
Figure is based on data presented in Takayanagi (2007) and own data (Ignatius et al. 2011b).

complement zymogens C3a and C5a have been proposed. The first is a cross activation of C3 and C5 by cleavage via thrombin or factor Xa, central proteases of the other major serine protease system, the coagulation system (Amara et al. 2010; Huber-Lang et al. 2006). The other complement-independent mechanism is a cellular activation of complement. It was shown that activated macrophages are capable of cleaving C5 via a surface-bound serine protease, thereby producing chemotactically active C5a (Huber-Lang et al. 2002) (Fig. 2).

The small fragments resulting from cleavage of C3 and C5 are powerful pro-inflammatory mediators called anaphylatoxins. The anaphylatoxins induce release of histamine from basophils (Kretzschmar et al. 1993) and mast cells (el-Lati et al. 1994), and trigger the oxidative burst in macrophages (Murakami et al. 1993) and neutrophils (Elsner et al. 1994). C5a was also shown to act as a strong chemokine for macrophages (Aksamit et al. 1981), neutrophils (Ehrengruber et al. 1994), and mast cells (Hartmann et al. 1997) as well as B- and T-lymphocytes (Nataf et al. 1999; Ottonello et al. 1999). Recent studies have shown that anaphylatoxins can in addition to their effects in inflammation also influence the adaptive immune response, for example the functional modulation of macrophages and dendritic cells (Li et al. 2011; Zhou 2011). Their effects are mediated by a group of highly specific G-protein coupled receptors (GPCR), the C3a receptor (C3aR), and the C5a receptor (C5aR). There is a second C5a receptor called C5a receptor-like 2 (C5L2), whose role has not yet been completely elucidated. C5L2 is structurally homologous to C5aR but is deficient in G-protein coupling (Okinaga et al. 2003). It may act as a decoy receptor, negatively regulating the pro-inflammatory response (Gao et al. 2005; Gerard et al. 2005). Recently it was shown that C5L2 acts as a negative modulator of the C5aR-mediated response to C5a via the β-arrestin pathway in neutrophils (Bamberg et al. 2010). It was shown that C3a and C3a des-Arg are capable of binding to C5L2 at a second distinct binding site (Cain and Monk 2002). Recently, binding of C3a des-Arg was shown to increase triglyceride synthesis in adipocytes and preadipocytes (Cui et al. 2009). The same study suggests, that the functional consequences of C5L2 activation depend on both the ligand and the cell type.

While complement is a very powerful defence system, activated complement components can also have highly destructive side effects on healthy host tissue. In particular, the alternative pathway, which also functions as an amplification mechanism, has a high potential for damaging host-cells (Harboe and Mollnes 2008). To avoid self-damage, complement activation is tightly regulated by soluble and membrane-bound regulatory proteins. One mechanism of the control of anaphylatoxin activity is the cleavage of the arginine residue by carboxypeptidase N, resulting in the des-Arg forms of the anaphylatoxins (Bokisch and Muller-Eberhard 1970). Whereas C3a des-Arg has no remaining pro inflammatory activity, C5a des-Arg maintains 1–10 percent of the pro-inflammatory activity of C5a (Sayah et al. 2003). Several other soluble or membrane-bound proteins contribute to the control of the complement system. Soluble factors, such as C1-inhibitor (C1-INH), Factor H, and Factor I are mainly independent from the target cells as they act in the fluid phase or bind to host-specific cell surface structures. Most of the membrane-bound regulators, such as membrane cofactor of proteolysis (MCP, CD46), decay-accelerating factor (DAF, CD55), and MAC inhibitory factor (MACIF, Protectin, CD59) are expressed on most cell types with few exceptions (Kim and Song 2006). Erythrocytes express DAF and CD59 but not MCP. However, there are quantitative differences in the expression of complement regulatory proteins by different subsets of leukocytes (Christmas et al. 2006). Another exception is complement receptor 1 (CR1, CD35), which is mainly found on leukocytes and erythrocytes (Kim and Song 2006).

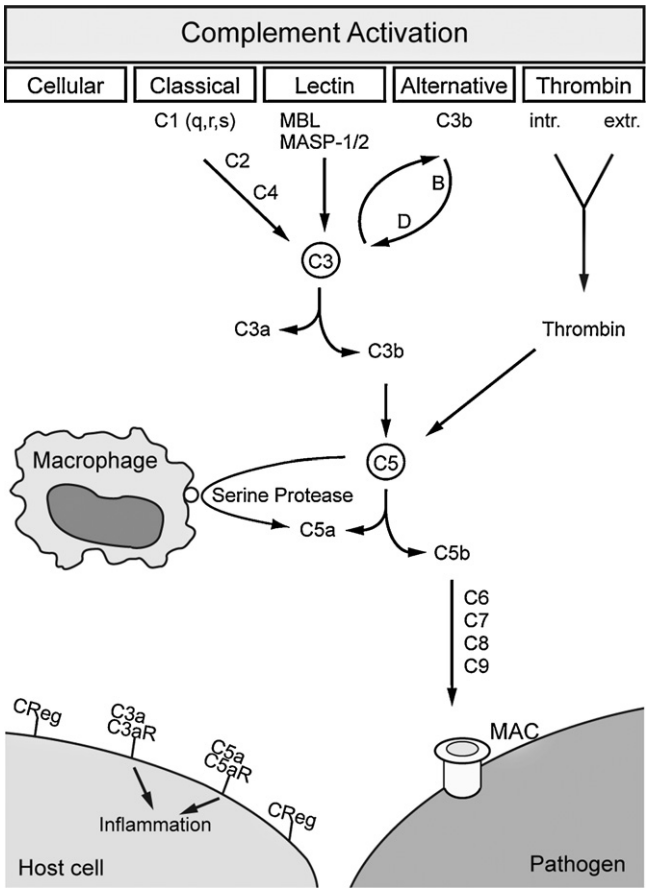

Fig. 2. Complement activation pathways. The complement system can be activated via several different activation pathways. The three well-established pathways, the classical, the alternative, and the lectin pathways are completed by the recently described cellular and thrombin pathways, which directly activate C5a and are independent of the formation of C5 convertases. Intr., intrinsic activation; Extr., extrinsic activation.
Adapted from Ehrnthaller et al. (2011).

Interactions of complement with bone

Bone development

Over the last several years evidence has accumulated indicating that various complement proteins are involved in endochondral bone formation during bone development. In endochondral bone formation a cartilage template is formed, which is then replaced by bone. In the growth plate there are three major zones of resting, proliferating, and pre-/hypertrophic chondrocytes. Longitudinal growth of bones proceeds by proliferation of chondroblasts spatially orientated in columns, which then up-regulate synthesis of matrix proteins and become resting cells. This is associated with an increase in cellular size (hypertrophy) and finally apoptosis of the chondrocytes and their replacement by osteoblasts accompanied by remodelling and mineralization of the collagen matrix. These sequential processes are tightly regulated by a complex network of stimulating and inhibiting growth/transcription factors as reviewed elsewhere (Wuelling and Vortkamp 2010). It was shown by Andrades et al. that complement proteins were also present in the zones of endochondral bone development in a distinct spatial pattern (Andrades et al. 1996). In the resting zone of foetal rat tibiae and femurs C3, factor B, and properdin were expressed. Factor B and properdin were also found in the proliferating zone. In the hypertrophic zone C5 and C9 were present (Andrades et al. 1996). These findings indicate that complement might contribute to the turnover of cartilage to bone in endochondral ossification.

This conclusion is supported by several other studies showing that C1s, the first component involved in the classical pathway of complement activation, plays a role in cartilage degradation during ossification. C1s was found in epiphyseal cartilage and in the fracture callus of hamsters (Toyoguchi et al. 1996), in the primary ossification centre of the human femur (Sakiyama et al. 1994), and in the secondary ossification centre of hamster tibia (Sakiyama et al. 1997) as well as in chondrocyte cultures *in vitro*. In all these studies, C1s was found to be located mainly in chondrocytes with an increase during differentiation and reached a maximum in hypertrophic chondrocytes (Nakagawa et al. 1997; Sakiyama et al. 1994; Sakiyama et al. 1997; Toyoguchi et al. 1996). An immunohistochemical analysis of the secondary ossification centre of hamster tibiae using an active form-specific antibody showed that active C1s was present mainly in degrading matrix around invading vessels, whereas the inactive form was found mainly inside hypertrophic chondrocytes (Sakiyama et al. 1997), indicating a role for C1s in matrix degradation. Indeed, C1s was found to be able to cleave type I and II collagen and gelatin through its serine protease activity (Yamaguchi et al. 1990). Additionally, C1s was shown to co-localize with matrix metalloproteinase (MMP)-9 in the primary ossification centre of the human femur and to be able to activate the MMP-9 zymogen (Sakiyama et al. 1994). In a recent review it was concluded from these findings that complement proteins could potentially stimulate turnover of cartilage to bone (Rutkowski et al. 2010).

Complement and osteoblasts

The fundamental role of complement in bone homeostasis is indicated by the presence of several complement regulators and receptors in different types of bone cells. C5aR was found to be expressed in osteoblasts and osteocytes in healthy human bone as well as on osteoblasts, osteoclasts, and chondroblast-like cells in the fracture callus of rats during the entire healing time (Ignatius et al. 2011a). In our most recent *in vitro* study we demonstrated the expression of the complement regulators MCP (CD46), DAF (CD55), MACIF (CD59), the anaphylatoxin receptors C3aR and C5aR, and the complement components C3 and C5 in differentiated and undifferentiated MSC and in osteoblasts (Ignatius et al. 2011b). We also showed a significant increase in the expression of MCP (CD46), MACIF (CD59), and C5aR during osteogenic differentiation of MSC (Ignatius et al. 2011b).

The expression of complement regulatory proteins in MSC and osteoblasts is not surprising, as most of these proteins are ubiquitously expressed, whereas the up-regulation in the expression of MCP (CD46) and MACIF (CD59) during osteogenic differentiation indicates a stronger protection against complement for osteoblasts compared to MSC.

In a recent study we showed that the presence of C3a and C5a, even in pathophysiological doses that may occur under systemic inflammatory conditions, did not affect osteogenic differentiation of osteoblasts (Ignatius et al. 2011b).

However, the presence of anaphylatoxin receptors and their up-regulation during osteogenic differentiation, demonstrate that osteoblasts are target cells for activated complement. Additionally, two independent studies showed that anaphylatoxin receptors are internalized following binding of their respective agonist. Schraufstatter et al. (2009) found an internalization of C3aR and C5aR by MSC, while our group showed that C5aR is internalized into osteoblasts (Ignatius et al. 2011b). Ligand-induced internalization of C3aR and C5aR was previously described for other cell types, such as granulocytes (Settmacher et al. 1999; Van Epps et al. 1990). There appears to be different effects of anaphylatoxin receptor internalization. Internalization of GPCR in general and anaphylatoxin

receptors in particular is commonly considered as a regulatory mechanism involved in homologous desensitization, which might be important in systemic or excessive inflammation (Bock et al. 1997; Meuer et al. 1981; Naik et al. 1997). In contrast, translocation to the nucleus or continuous location in the nucleus was previously described for different GPCR, leading to long-term effects including prolonged nuclear ERK1/2 activation (Gobeil et al. 2006; Goetzl 2007; Lu et al. 1998; Zhao et al. 2008). In MSC, internalization of both anaphylatoxin receptors was also accompanied by strong and prolonged phosphorylation of ERK1/2, which was shown previously for C5aR in various cell types. However, the strong and prolonged ERK1/2 phosphorylation following C3aR internalization was surprising, as the effect of C3aR internalization is transient and weak in most cell types (Schraufstatter et al. 2009).

As anaphylatoxins are known to be powerful chemokines, it is very likely that migration is one of the functions of anaphylatoxins on MSC and osteoblasts. It was shown that MSC display a strong chemotaxis towards a C3a or C5a gradient that could be blocked by the respective receptor antagonists, as well as by pertussis toxin, thus indicating a receptor-specific G_i-activation-dependent mechanism (Schraufstatter et al. 2009). The chemotactic effect of C5a via the C5aR was confirmed by a recent study from our group, not only for MSC but also for osteoblasts. In this study, significantly stronger cell migration was found in osteoblasts than in undifferentiated MSC, which might be explained by an increase in C5aR expression during osteogenic differentiation (Ignatius et al. 2011a), suggesting higher anaphylatoxin sensitivity of more mature cells of the osteogenic lineage. An earlier study also showed that C5a-induced migration in PBMNC but not in MSC (Schmal et al. 2007), which conforms to the study from our group. These contradictory findings regarding MSC migration capability might be explained by differences in the differentiation status of the MSC, as Schraufstatter et al. used MSC up to passage 7 whereas MSC used in our study were only in passages 2–4. This might be relevant as MSC of higher passages tend to spontaneous osteogenic differentiation (Banfi et al. 2002). In contrast to other cell types, chemotaxis of MSC was accompanied by a strong and extended ERK1/2 phosphorylation not only after C5aR activation but also following C3aR activation (Schraufstatter et al. 2009).

Another function of anaphylatoxins in bone cells could be the induction of pro-inflammatory cytokine release, as was shown for several other cell types, mainly immune cells. An initial study concentrating on complement-triggered IL-6 release from MG-63 osteoblast-like cells found a significant increase in the IL-1β-induced release of IL-6 when C5a was added. The authors concluded from these results that osteoblasts express a functional C5a receptor and that modifying critical C5a levels by C5a agonists and antagonists might be a promising therapeutic approach to inflammatory bone loss as found in periodontitis (Pobanz et al. 2000). Recently, our group published a similar result from an experiment using human primary osteoblasts. We found a significant increase in the production and release of the pro-inflammatory interleukins IL-6 and IL-8 after co-stimulation with IL-1β and C3a or C5a. This finding indicates an immune modulatory response of osteoblasts under pro-inflammatory conditions (Ignatius et al. 2011b). The synergism of IL-1β and complement anaphylatoxins observed in these studies might be due to cross-talk down-stream in the signalling pathways. IL-1β effects are mediated by activation of MEKK1 and proteins of the interleukin receptor associated kinase (IRAK) family, leading to activation of C-JUN or NF-κB (Flannery and Bowie 2010). C5aR signalling involves activation of ERK1/2, AKT, and MAPK p38 (Rousseau et al. 2006). C3aR activation was shown to induce ERK1/2 and NF-κB (Li et al. 2008). However, the exact mechanisms of the interactions of interleukins and anaphylatoxins remain unclear and should be subject to further investigations.

Previously, complex coactions of pro-inflammatory stimuli and complement anaphylatoxins have been reported for other cell types. IL-1β increased the expression of C5aR on mononuclear cells (Takabayashi et al. 2004), while C5a stimulated IL-1β release from PBMNC (Okusawa et al. 1987). C5a and LPS synergistically induced IL-6 release from Kupffer cells (Mack et al. 2001). These findings suggest that C3a and C5a can enhance the inflammatory response of osteoblasts in a pro-inflammatory environment, such as in inflammatory bone diseases or during bone healing.

Bone cells are not only targets for activated complement but may also express and activate complement zymogens. In a recent study from our group we demonstrated expression of the complement zymogens C3 and C5 by differentiated and undifferentiated MSC and by osteoblasts. Less C3 and no C5 expression were found in PBMNCs with a strong increase during osteoclastogenesis (Ignatius et al. 2011b). These findings are supported by older studies which found C3 expression by mouse marrow-derived stromal cells (ST2) and primary osteoblasts in a dose-dependent manner following stimulation with 1α,25-dihydroxyvitamin D_3 *in vitro* (Hong et al. 1991; Sato et al. 1991). This dependency on 1α,25-dihydroxyvitamin D_3 was tissue-specific in stromal cells and osteoblasts whereas C3 production from hepatocytes required no vitamin D_3 (Sato et al. 1991). It was found that C3 production is vitamin-D dependent in bone but not in liver or serum. In vitamin D-deficient mice, C3 production could be restored by supplemental administration of 1α,25-dihydroxyvitamin D_3 (Jin et al. 1992).

Complement and osteoclasts

Osteoclasts, like MSC and osteoblasts, express complement components. Osteoclasts express the complement regulator MACIF (CD59), the anaphylatoxin receptors C3aR and C5aR, and the complement zymogen C3. It was also shown that the anaphylatoxin receptors were not internalized by osteoclasts after 45 min of incubation with C3a or C5a, which is in contrast to the internalization of C3aR and C5aR by osteoblasts (Ignatius et al. 2011b).

There is evidence from several studies that complement positively influences osteoclast formation by direct and indirect effects. Osteoclasts can be derived from murine bone marrow cultures stimulated with 1α,25-dihydroxyvitamin D_3. Osteoclast formation was inhibited by an anti-C3 antibody, suggesting a crucial role for C3 in osteoclastogenesis. As this inhibition appeared strongest when the anti-C3 antibody was added at early time points of culture, the authors reasoned that C3 is required for the proliferation of precursor cells and in early differentiation (Sato et al. 1993). A similar, more recent study using bone marrow cells from wild type and C3-deficient ($C3^{-/-}$) mice showed that $C3^{-/-}$ bone marrow cultures generated fewer osteoclasts than wild type marrow cells, since several features of osteoclast formation are impaired in bone marrow cultures from $C3^{-/-}$ mice (Tu et al. 2010). By blocking C3aR and C5aR in human bone marrow cultures, Tu et al. showed that the anaphylatoxin receptors are necessary for osteoclastogenesis (Tu et al. 2010). As these studies investigated osteoclastogenesis from mixed bone marrow cultures, they cannot predicate whether osteoblasts or osteoclasts, or both are influenced by complement C3. Actually, there are indications of both direct and indirect effects on osteoclast formation. Bone marrow cells from $C3^{-/-}$ mice produced less M-CSF and a reduced RANKL/OPG expression ratio following stimulation with vitamin D_3 (Tu et al. 2010). Both M-CSF and an increased RANKL/OPG ratio are essential for efficient osteoclast formation, indicating an indirect effect of complement on osteoclastogenesis. Furthermore, it was shown that bone marrow cultures produce all the complement components necessary for activation of C3a and C5a via the alternative pathway, and that complement activation via this pathway as well as the anaphylatoxin receptors C3aR and C5aR are required for osteoclastogenesis

(Tu et al. 2010). This indirect effect was confirmed by a significantly increased expression of the osteoclast-affecting cytokines RANKL and OPG in osteoblasts after 24 h of co-stimulation with IL-1β and C3a or C5a (Ignatius et al. 2011b). We also showed a direct positive effect of C3a and C5a on osteoclastogenesis. Osteoclastogenesis experiments were performed with C3a and C5a in the absence of M-CSF and RANKL, and revealed a significant increase in osteoclast formation up to the level of control cultures with M-CSF and RANKL (Ignatius et al. 2011b).

Recently we showed that osteoclasts are capable of cleaving C5 directly, thereby generating chemotactically active C5a, indicating a role for osteoclasts in complement activation (Ignatius et al. 2011b).

Complement and bone in disease

Due to the important role of complement in the development and homeostasis of healthy bone, it is not surprising that complement proteins are involved in several diseases affecting bone, and that typical disorders of the complement system also have an influence on bone. Deficiencies of different complement components lead to a number of typical diseases. General deficiencies of early complement components of the classical and lectin activation pathways can cause immune-complex diseases. An example is the deficiency of C1q, which is one of the most important risk factors for the development of systemic lupus erythematosus (SLE) (Sontheimer et al. 2005). Another important complement disorder is a deficiency in the complement regulatory protein factor H, which is accompanied by uncontrolled activation of the alternative pathway, leading for instance to membranoproliferative glomerulonephritis type II. The same effect can be caused by an auto-antibody stabilizing the C3bBb complex.

At present there is no data available on bone disorders associated membranoproliferative glomerulonephritis type II, but it is generally accepted that complement-induced SLE, which is accompanied by a systemic inflammatory environment is a strong risk factor for bone loss (Segal and Lane 1996).

Another important chronic inflammatory disease affecting bone is rheumatoid arthritis (RA), a disease of the joint, affecting cartilage as well as bone, leading to severe damage of both tissues (Lories and Luyten 2011). Complement is involved in the development of RA as well as in acute inflammatory arthritis caused by trauma and cartilage degeneration. Deposits of C3c and C9 were found in the synovial vasculature and the intercellular matrix in both RA and acute arthritis, accompanied by a decreased expression of CD59, an inhibitor of MAC formation, by synovial lining cells, stromal cells, and endothelial cells. In contrast, no C3c and C9 deposits were found in degenerative diseases of the joint. Additionally, the expression of CD59 was prominent in synovial lining cells, stromal cells, and epithelial cells from degenerative diseases (Konttinen et al. 1996). Furthermore, there is longstanding evidence that rheumatoid arthritis also induces general bone loss dependent on disease activity, leading to osteoporosis (Gough et al. 1994). It is highly likely that the chronically and systemically high levels of pro-inflammatory cytokines, especially IL-1β and TNF-α that are pivotal in RA, lead to enhanced expression of RANKL by osteoblasts and, thereby, to increased osteoclast formation (O' Gradaigh et al. 2004).

Very recently, a central role of the complement system – more precisely sublytic concentrations of the membrane attack complex (MAC) – has been identified in osteoarthritis (Wang et al. 2011). In this study, mice deficient in C5 or C6 were protected against the development of osteoarthritis after meniscectomy, whereas deficiency of the complement regulatory protein CD59 increased disease severity in wild-type animals. Osteoarthritis is associated with subchondral bone sclerosis and the development of osteo- (chondro-)phytes at the margins of affected joints, which arise through endochondral ossification processes (Huch et al. 2003). Currently, it is not known whether sublytic MAC concentrations might also contribute to these disease manifestations of osteoarthritis affecting bone tissue.

Another typical chronic inflammatory disease is periodontitis, which eventually leads to periodontal bone loss. A recent study showed the need for C5aR in the development of periodontal bone loss in both mice infected with *Porphyromonas gingivalis* and aged mice with naturally occurring periodontitis (Liang et al. 2010). The same study indicated that *P. gingivalis* uses cross-talk of toll-like receptors (TLRs) to escape immune clearance and cause inflammatory bone loss (Hajishengallis and Lambris 2010; Liang et al. 2010). In a study using germ-free mice and mice lacking C3a and C5a receptors, it was also shown that both the commensal microbiota and complement where essential for the development of periodontal bone loss during *P. gingivalis* infection (Hajishengallis et al. 2011).

Not only chronic but also transient systemic inflammation interferes with bone homeostasis and fracture repair. It was shown that pro-inflammatory cytokines were significantly up-regulated during systemic inflammatory response syndrome (SIRS) after severe trauma in a rat model of blunt chest trauma. The pro-inflammatory effect of the experimental blunt chest trauma was abolished by treatment with an anti-C5a antibody, showing that the complement system is a key component in posttraumatic inflammatory response (Flierl et al. 2008). Recently, our group showed in a rat model of femur fracture that fracture healing was significantly impaired by a concomitant blunt chest trauma, resulting in reduced bending stiffness of the fracture callus after 35 days compared to rats without blunt chest trauma (Recknagel et al. 2012a). Additionally, it was shown that treatment with a C5aR antagonist completely restored the impaired fracture healing caused by blunt chest trauma (Recknagel et al. 2012b). In this study we concluded that complement influences fracture healing indirectly by inducing a strong inflammatory response but may also directly influence the balance of osteoblasts and osteoclasts in favour of osteoclasts (Recknagel et al. 2012b).

A possible complication in the use of biomaterials for fracture repair and joint replacement is aseptic loosening of implants due to complement-induced excessive local inflammation. When a biomaterial is exposed to blood, complement is activated via the alternative pathway (Andersson et al. 2002; Chenoweth 1987; Hed et al. 1984). One possibility to protect biomaterials from complement activation are complement inhibitors bound to functionalized coatings, as was shown with factor H specifically bound to functionalized polyethylene oxide (PEO) coated surfaces (Andersson et al. 2006; Neff et al. 1999). The role of complement in biomaterial-induced inflammation and the current progress in shielding biomaterials from complement activation were reviewed in detail some years ago (Nilsson et al. 2007, 2010).

Conclusions

Several studies show a tight interaction of the skeletal and the complement systems not only in the maintenance of bone homeostasis but also in bone development and fracture healing. The complement anaphylatoxins influence the migration of bone cells, the osteoblast–osteoclast interaction, and a modulation of the inflammatory response by osteoblasts (Fig. 3). Furthermore, by expressing anaphylatoxin precursors and cleaving them to their active forms, bone cells might even contribute to local complement activation at sites of bone injury or disease (Fig. 3). Taken together complement activation might increase bone resorption via enhancing osteoclastogenesis, while recruitment of osteoblast

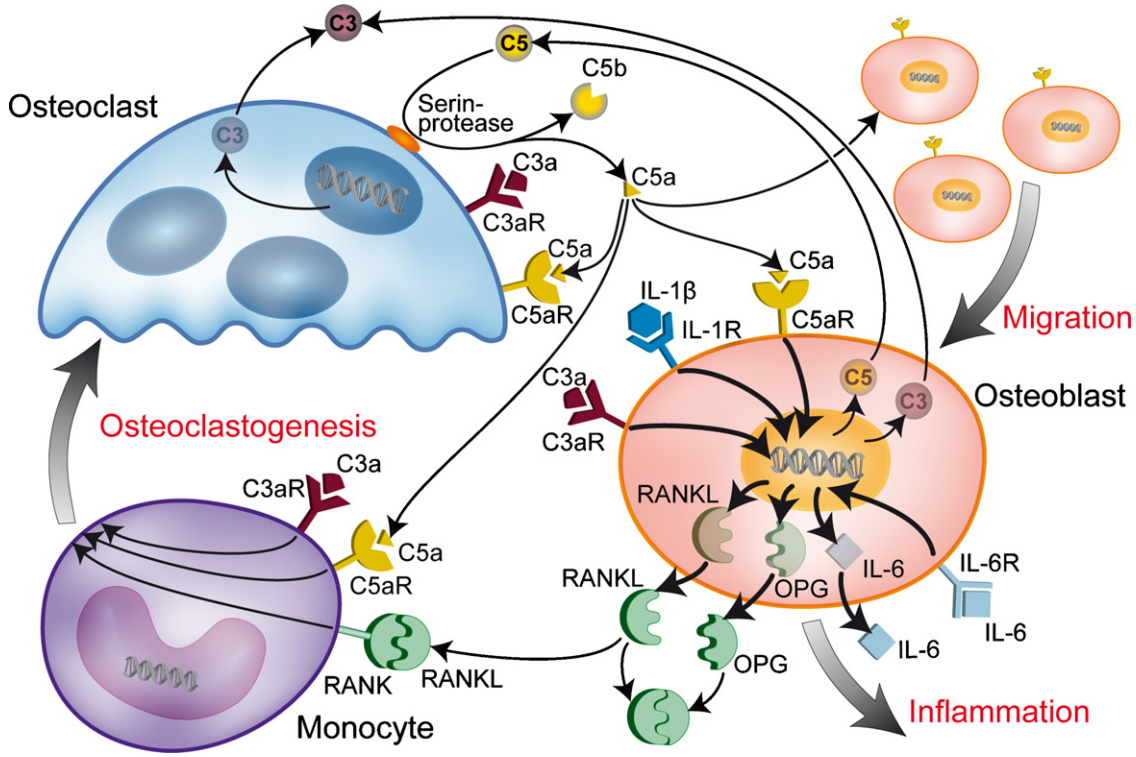

Fig. 3. Complement and bone cells. Osteoblasts produce the complement zymogens C3 and C5 while osteoclasts only produce C3. C5 can be activated not only by the well-established pathways but also directly by osteoclasts. MSC, osteoblasts, osteoclasts, and PBMNC express the receptors for C5a and C3a. C5a is a strong chemokine for MSC and osteoblasts (Schraufstatter et al. 2009; Ignatius et al. 2011a). Both anaphylatoxins in synergism with IL-1β induce a pro-inflammatory response in osteoblasts, marked by IL-6 production and release. Il-1β and anaphylatoxins also synergistically induce the production of the pro-osteoclastogenic RANKL and its competitor OPG. Additionally, C3 and C5 can directly induce the formation of osteoclasts from PBMNC in the absence of RANKL and M-CSF (Ignatius et al. 2011b).

precursors might favour bone formation. Further understanding of these interactions will be necessary to determine the pathomechanisms of bone-related immune diseases and immune-related bone diseases with the aim of therapeutic intervention.

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