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Review

The role of complement in trauma and fracture healing

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ABSTRACT

The complement system, as part of innate immunity, is activated immediately after trauma in response to various pathogen- and danger-associated molecular patterns (PAMPs and DAMPs), and helps to eliminate microorganisms and damaged cells. However, recent data indicate an extended role of complement far beyond pure "killing", which includes regulation of the cytokine/chemokine network, influencing physiological barriers, interaction with the coagulation cascade, and even involvement with bone metabolism and repair. Complement-induced hyper-activation and dysfunction reveal the dark side of this system, leading to complications such as sepsis, multiple-organ dysfunction, delayed fracture healing, and unfavorable outcome. Thus, the present review focuses on less known regulatory roles of the complement system after trauma and during fracture healing, rather than on its bacterial and cellular "killing functions". In particular, various complement crosstalks after trauma, including the coagulation cascade and apoptosis system, appear to be crucially involved early after trauma. Long-term effects of complement on tissue regeneration after fracture and bone turnover are also considered, providing new insights into innate immunity in local and systemic complement-driven effects after trauma.

1. Introduction

Traumata are accountable for an increasing portion of the global burden of disease [1,2], and reflect a major humane, socio-economic, clinical, and scientific challenge. Traffic accidents with multiple injuries and fractures are the number one killer of young people and the leading cause of death during the first half of life (up to age 45) [3]. Survival of patients after severe tissue trauma, for example, after multiple fractures, requires an adequate surgical management and even more importantly, as a *conditio sine qua non*, an effective molecular and cellular danger response to repair damaged tissue. Various fluid-phase and cellular defense systems have evolved to clear any pathogen- (PAMPs) or danger-associated molecular patterns (DAMPs) generated by microorganisms or the host tissue, respectively [4,5]. Early after trauma, the complement system, coagulation cascade, and neutrophils together with the cytokine/chemokine network act in conjunction as the "first line of defense" of innate immunity, initiating a systemic danger response to overcome the insult [6]. The complement system is considered to be a main trigger and driver of whole body inflammation, clinically manifested as systemic inflammatory response syndrome (SIRS). During trauma-induced SIRS, there is an almost synchronic generation and release of pro- and anti-inflammatory cytokines/chemokines [7,8]. In the additional presence of PAMPs or bacteria, defined as sepsis, complement activation results in C3b-dependent opsonization of microorganisms, C3a- and C5a-induced recruitment of leukocytes, and formation of the membrane attack complex (C5b-9) to clear bacteria and PAMPs. Even if the complement system with its potent activation products was designed to prevent host organism destruction by killing invading microorganisms and damaged cells, it might nevertheless also kill the host itself through an uncontrolled and extensive inflammatory and coagulatory response with resulting multiple organ dysfunction [9,10] (Fig. 1).

However, there is evidence, that the complement system in particular acts far beyond its apparent and obscure killing mechanisms by influencing tissue repair, especially after severe trauma and concomitant bone fractures [11].

2. Trauma-induced complement activation – too much killing?

How does the traumatized, dying cell warn the innate immune system of danger [12]? A most effective molecular "early alarm system" for both DAMPs and PAMPs consists of the coagulation [13] and complement cascades [14,15]. In particular, trauma-induced exposure of negatively charged surfaces, released tissue factors, the generation of antigen–antibody complexes, released bacterial

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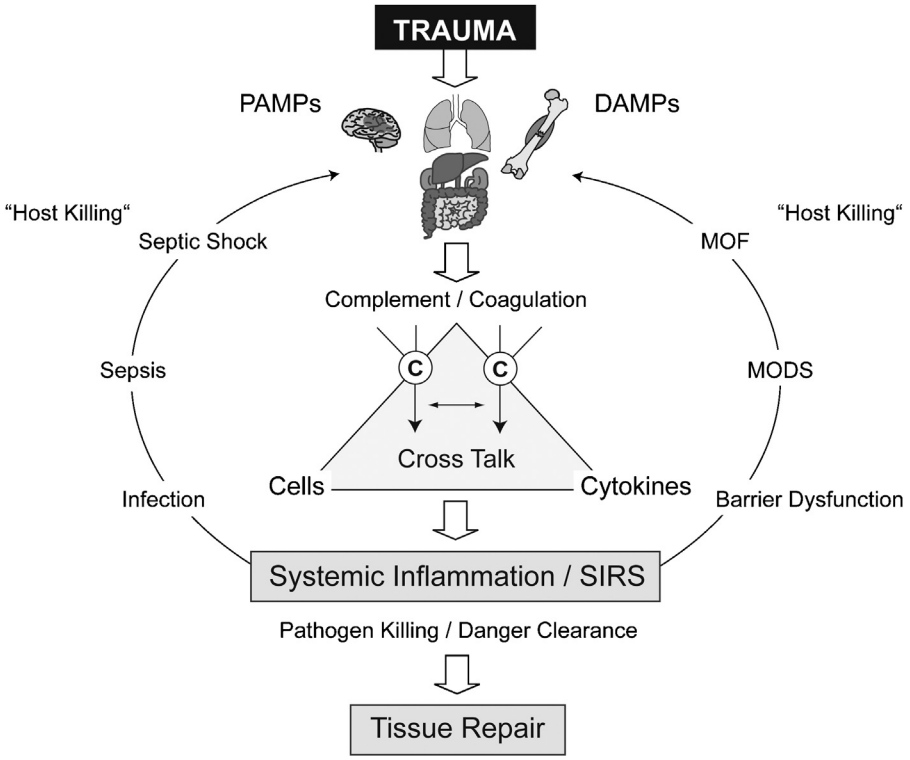

Fig. 1. Trauma-induced early activation of the complement and coagulation system and crosstalking systems, resulting in "killing" of pathogens and clearance of danger molecules. PAMPS, pathogen-associated molecular patterns; DAMPS, danger associated molecular patterns; SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; MOF, multiple organ failure.

lipopolysaccharides, and pathogenic carbohydrate structures among others can be sensed by the complement and coagulation cascades, which are excessively activated early after trauma. Recently, we have shown in a prospective multicenter study in 40 polytrauma patients (mean injury severity score [ISS] = 30.3 ± 2.9) an early, massive activation of complement (as evidenced by almost abolished CH50 values), discriminative between lethal and non-lethal outcome. Serum levels of the complement activation products C3a and C5a were significantly elevated throughout the 10-day observation period and correlated with the severity of traumatic brain injury and survival. The soluble terminal complement complex sC5b-9 and mannose-binding lectin (MBL) displayed a biphasic response after trauma, with an early increase and subsequent collapse over at least 24 h [16]. Other groups have also found in trauma patients who developed acute respiratory failure (ARDS) or multiple-organ dysfunction (MODS), a posttraumatic systemic depletion of C3 and C5 with enhanced C3a/C3 ratios [17–19]. In a mono-centered study including 208 major trauma patients, early generation of sC5b-9 was found to correlate with the ISS and development of MODS [20]. Furthermore, the alternative pathway was suggested as the predominant complement activation pathway after trauma [20]. The resulting SIRS appears to be associated with enhanced concentrations of complement activation products, as seen in various conditions [21]. In an experimental setting of rodent blunt thorax trauma, dramatic changes in blood neutrophil function were found, including an impairment of the phagocytic uptake and chemotactic activity, and an increase in the oxidative burst response, which may increase the bacterial impact and enhance cellular stress on the intact cells. Interestingly, these cellular defects were all prevented by blockade of C5a [22,23]. Systemic C5a is also known to effectively induce remote organ injury or delayed healing (e.g. delayed fracture healing). Because C5a is a potent inductor of all classical signs of inflammation (pain, heat, redness, swelling, and loss of function), this anaphylatoxin represents an ideal target for immune modulation to prevent development of direct and remote organ injury, such as ARDS and MODS [10,22], and even the development of non-unions [24,25]. Overall, in systemic inflammatory conditions, C5a may not only be considered as "too much of a good thing" [26], but as a consequence can result in "too much killing".

3. Complement crossroads after trauma

Trauma-induced rapid activation of the serial and interactive proteases of the complement and coagulation systems [27] leads not only to an early clotting response (to control blood loss), but also to a simultaneous early inflammatory response to control cell trauma (DAMPs) and to eliminate potentially invasive microorganisms (PAMPs). The coagulation factor Xa and thrombin as well as C3 and C5 have been particularly shown to be molecular triggers for both systems [14]. The activated coagulation- and complement-products (fibrin, C3a, and C5a) can chemotactically recruit polymorphonuclear neutrophils (PMNs) and macrophages to the cell-trauma site. There, the phagocytes recognize the danger signals via the DAMP receptors and convert them into a cellular response. This was postulated for example for the C5a receptor (C5aR), which can alone, or possibly in crosstalk with Toll-like receptors (TLRs) [28] or after dimerization with other receptors (e.g. CCR5) [29], translate the danger signals into a cellular response to induce an inflammatory reaction. In the clinical setting, trauma patients with low C3a levels demonstrated a correlation between sC5b-9 levels and plasma concentrations of prothrombin fragments 1 and 2 (produced upon thrombin generation), supporting the idea of a trauma-relevant, intensive crosstalk between the complement and coagulation cascades [14,27,30]. Furthermore, factor VII-activating protease (FSAP), which is activated by histones and nucleosomes from damaged cells, has recently been shown to be activated in multiple trauma patients and to generate C5a. Immediately after injury, a large increase in nucleosomes and circulating

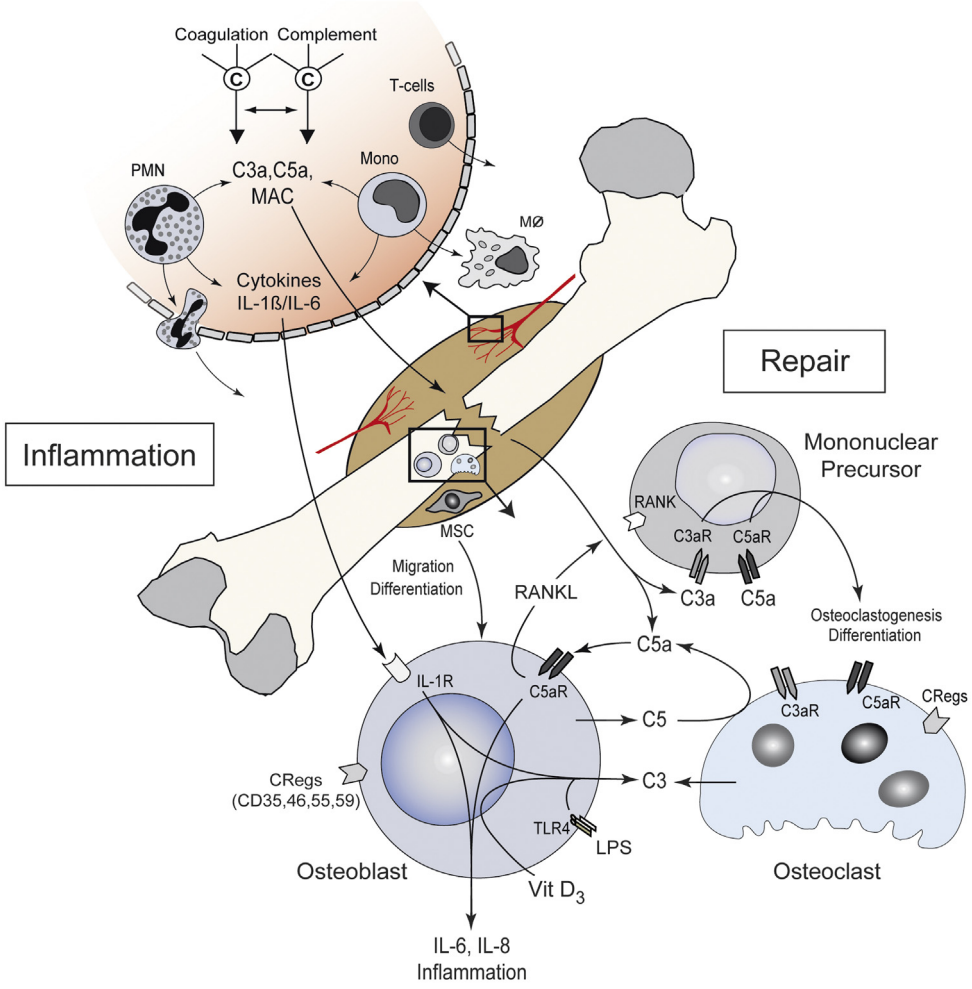

Fig. 2. Complement functions beyond "killing": systemic and local complement activation effecting fracture healing. MAC: IL, interleukin; MAC, membrane attack complex; MØ, macrophage; PMN, polymorphonuclear neutrophil; Mono, monocyte; MSC, mesenchymal stem cell; CRegs, complement regulatory protein; TLR4, Toll-like receptor 4; LPS, lipopolysaccharide; RANKL, receptor activator of NF-kB ligand.

FSAP activity was detected and a correlation between FSAP activity and C5a concentrations was found [31]. On a cellular level, there is also a procoagulant shift after exposure to C5a, as in response neutrophils generate tissue factor (TF) and mast cells enhance the production of plasminogen activator inhibitor (PAI-1) [30,32], which may not only play an important role in ARDS [33], but also influence hematoma formation after bone fractures.

The complement system also influences physiological barriers. For example, zonulin, which is related to the serine proteases MASP-1 and C1qrs, regulates tight junctions in epithelial and endothelial cells [34]. Recently, zonulin was found to generate C5a independently of the canonical pathways and thereby may contribute to development of organ dysfunction, as seen for acute lung injury [34].

Another important crosstalk in the pathophysiology after trauma and fracture involves the apoptosis cascades (for "programmed suicidal killing") and the complement system. The pro-apoptotic serine protease granzyme B was found in enhanced concentrations in neutrophils and lymphocytes early after multiple injury [35]. Furthermore, a new interaction interface for granzyme B and C3a-/C5a-generation has been presented [35] Similarly, the plasma level of pro-apoptotic aspartic protease cathepsin D was significantly increased in multiple injured patients and, moreover, was capable of cleaving C5, generating biologically active C5a [36]. C5a in turn can delay neutrophil apoptosis, as found after trauma and during sepsis [37], which may enhance host damage [6]. In terms of a fracture, this may theoretically mean prolonged hematoma clearance, sustained inflammation, and delayed recruitment of osteoblast and osteoclast progenitors required for regeneration.

Interestingly, viable neutrophils primed by granulocyte/macrophage colony-stimulating factor and stimulated with either C5a or TLR4 agonist also exhibited enhanced survival and released neutrophil extracellular traps (NETs), which bind and kill microorganisms [38]. The complement system and TLRs as two central columns of innate immunity are activated by most PAMPs and contribute to an intensive crosstalk between the innate and adaptive immune response [39–41], leading to the elimination of infection and induction of tissue repair.

4. Failure of danger management after trauma: complementopathy and complications

Molecular danger management is physiologically tightly controlled by many regulatory systems. After severe trauma and fractures, hyper-activation, imbalance and, finally, failure of different regulatory systems have been reported [6]. In the case of the serine protease system, this gives rise to an early trauma-induced coagulopathy [6,13] and a nearly simultaneous complementopathy [42]. Early coagulopathy is of critical prognostic importance for polytrauma patients, because regardless of the severity of the injury, the post-trauma mortality rate is elevated fourfold [43].

With regard to the complement system, in addition to early activation [16,19], an early dysregulation of the complement regulatory proteins (CRegs) in polytrauma patients was also described by our research group [42]. Thereby, a reduction in the complement inhibitors CD46 and CD59 found on neutrophil granulocytes strongly correlated with increasing trauma severity in the presence of hemorrhagic shock [42]. Furthermore, key fluid-phase inhibitors of complement, including factor I and C4b-binding protein (C4BP), were significantly reduced early after trauma [20]. Because both the coagulation and complement systems are important in the early acquisition and processing of PAMPs and DAMPs, the early post-traumatic occurrence of coagulopathy and complementopathy are clearly critical for the development of post-traumatic complications, organ and immune dysfunction, and delayed tissue regeneration. Development of MODS is regularly associated with blood barrier dysfunction and failure. Remarkably, the anaphylatoxin C5a and sC5-9 have also been proposed to play a decisive role in the development of blood barrier dysfunction, particularly after traumatic brain injury [23,44]. For a bench-bedside transfer, first complement inhibitor strategies are under investigation, including C1q inhibition early after trauma, targeting changes in the IL-6 serum levels (as primary endpoint) and occurrence of inflammatory complications (as secondary endpoint), such as ARDS or MODS [45].

5. Role of complement in fracture healing: terra incognita

Although the data regarding the role of complement in SIRS development and concomitant complications are incomplete, even less is known about its influence on bone. Recent findings indicated a constant crosstalk between the bone and immune system, which resulted in the emerging field of osteoimmunology [11]. As bone acts as a reservoir for bone marrow, and, therefore, numerous immune progenitors and mature cells, the “immune function” of bone cells themselves has not been extensively studied. Our group has shown that both osteoblasts and osteoclasts are important players in the regulation of the immune response after trauma and are able to act as inflammatory cells post trauma (Fig. 2). The bone cells are able to respond to inflammatory signals, including PAMPs and complement anaphylatoxins, amplify them, and recruit other cells necessary for tissue repair. However, to what extent bone cells regulate their local and systemic inflammatory environment is not clear. Moreover, as complement factors and complement receptors are locally expressed during the whole healing phase, and not only during the initial acute inflammatory phase, it is clear that complement is in this context a “regulator” rather than a “killer”.

6. New insights into the crosstalk of complement and bone

Clinical data indicate that several disorders, which are associated with complementopathies, may also affect bone. Examples are systemic lupus erythematosus (SLE) and rheumatoid arthritis. SLE, caused by a deficiency in complement component C1q, is known to be associated with bone loss and an increased risk of fractures [46,47]. Rheumatoid arthritis, which is characterized by a severe degeneration of both bone and cartilage tissues of the joints and by systemic osteopenia, is accompanied by C3c and C9 deposition in affected joints and decreased CD59 expression [48]. Recently, these observations were confirmed by Neumann et al., who described the expression of factor B, C3, C5b-C9, and complement receptors C3aR and C5aR in rheumatoid synovia [49]. Wang and colleagues observed a strongly increased expression of complement factors B, C5, C7, and C9 in synovial membranes of osteoarthritic patients, which correlated with increased membrane attack complex (MAC) formation [50]. Moreover, they observed that CD59-deficiency accentuated the osteoarthritic phenotype in a mouse model, subjected to destabilization of the medial meniscus. Additionally, mice deficient in different components of MAC, including C5 or C6, were protected against osteoarthritis. The authors suggested that the dysregulation of complement might play a key role in the pathogenesis of osteoarthritis. There is also evidence that C5aR may play a crucial role in periodontitis, because mice lacking the receptor were protected from periodontal bone loss [51,52].

Confirming the clinical data of delayed fracture healing after a major trauma impact, our group demonstrated in a rat model of severe trauma that fracture healing was considerably impaired after an additional blunt chest trauma, a strong inducer of posttraumatic systemic inflammation. We found that the acute systemic inflammation altered the cellular composition and the cytokine expression in the fracture hematoma and considerably decreased bone formation as well as the mechanical competence of the fracture callus in the late phase of healing [53,54]. Both C5aR [55] and C3aR (unpublished data) were strongly expressed not only by immune cells during the early inflammatory phase of fracture healing, but also by osteoblasts, hypertrophic chondroblasts, and osteoclasts during the entire healing period in a spatial and temporal pattern both in zones of intramembranous and endochondral ossification. Moreover, the disturbed bone healing in the combined trauma model was attenuated after the application of the C5a-receptor antagonist PMX-53, indicating a mechanistic role for complement activation [25].

Taken together, the clinical and experimental data clearly suggest a crucial function of complement in disorders affecting bone and in bone regeneration; however, the underlying molecular mechanisms remain unclear. Usually, complement proteins are considered to be expressed mainly by immune cells, such as monocytes and macrophages, as well as by hepatocytes and epithelial cells. The first evidence of the regulatory role of complement in bone was described more than two decades ago by Sakiyama et al., who reported C1s expression in the primary ossification center during bone development. C1s was expressed mainly by hypertrophic chondrocytes, suggesting that C1s, which can cleave collagen due to its serine protease activity, might play a role in cartilage degradation during ossification [56,57]. Andrades and colleagues observed the expression of factor B and C3 by chondroblasts in the growth plate, the site of endochondral bone formation during longitudinal growth of bones, whereas C5 and C9 were localized mainly in the hypertrophic zone, where chondrocytes are replaced by osteoblasts. Thus, they suggested that the alternative pathway of complement activation may play a role in the turnover of cartilage to bone during bone development [58]. However, the exact molecular mechanism of this regulation needs to be studied more precisely.

7. Bone cells: victims or offenders after fracture?

Our recent in vitro studies revealed the expression of the complement zymogens C3 and C5, the receptors C3aR and C5aR, and multiple regulators, such as CD46 (MCP, membrane co-factor of proteolysis), CD55 (DAF, decoy accelerating factor), and CD59 (MAC inhibitor) on undifferentiated and differentiated human mesenchymal stem cells (MSC) and osteoblasts [59] (Fig. 2). The strong up-regulation of both anaphylatoxin receptors during osteogenic differentiation indicates that osteoblasts might be effector cells for activated complement [11]. Indeed, we observed ligand-induced internalization of C3aR and C5aR in human osteoblasts [59], whereas Schraufstatter and colleagues demonstrated the same mechanism in undifferentiated MSC [60]. Receptor internalization is known to activate intracellular signaling pathways via Ras, MEK, and ERK1/2 [21,60,61]. As in immune cells, anaphylatoxins induce the release of pro-inflammatory cytokines from osteoblasts

(Fig. 2). Pobanz et al. found that C5a considerably increased IL-1β-induced release of IL-6 from osteoblast-like MG-63 cells [62]. Our group confirmed these data, demonstrating a synergistic effect of C5a and IL-1β on the expression and release of IL-6 and IL-8 by human osteoblasts [59]. The interaction of IL-1β and C5a observed in these studies may result from crosstalk down-stream in the signaling pathways. These observations indicate that activated complement may induce an inflammatory response of osteoblasts, particularly in a pro-inflammatory environment, such as the early fracture hematoma. Furthermore, osteoblasts can produce the complement zymogens C3 and C5 following stimulation with 1α,25-dihydroxyvitamin D3 [59,63,64], further indicating the crucial role of complement in bone metabolism.

Another important function of complement anaphylatoxins is the recruitment of immune cells to the inflammation site. Studies by Schraufstatter et al. and our group revealed that C3a and C5a are also powerful chemokines for MSC [60] and osteoblasts [55]. The migration of MSC and osteoblasts toward a C3a or C5a gradient could be blocked by the application of the corresponding receptor antagonist, confirming the specificity of the effect. We observed a significantly stronger migration of osteoblasts in comparison to undifferentiated MSC, which could be explained by an increase in C5aR expression during osteogenic differentiation [55]. Osteoclasts, the bone resorbing cells, can efficiently cleave C5 to its active form, C5a, thereby possibly attracting osteoblasts during fracture healing and bone remodeling [59]. These data indicate that complement anaphylatoxins may modulate the recruitment of osteoblasts during bone remodeling and regeneration, thus supporting bone formation.

However, the literature indicates that complement anaphylatoxins may also modulate bone erosion. Osteoclast formation has been shown to be directly and indirectly influenced by complement. Osteoblast and osteoclast activity are strictly coupled via the RANKL/RANK/OPG system [65]. Osteoclast formation and activity is induced by RANKL (receptor activator of NF-kB ligand), which is released by osteoblasts and binds to its receptor RANK on the surface of osteoclast precursor cells. OPG (osteoprotegerin) is also released by osteoblasts and acts as a decoy receptor, inhibiting RANKL activity [65]. C3a and C5a have been shown to stimulate RANKL expression in osteoblasts, thus indirectly increasing osteoclast formation [59,64,66]. By blocking C3aR and C5aR in mixed human bone marrow cultures, Tu et al. showed that the anaphylatoxin receptors are necessary for osteoclastogenesis [66]. There is evidence that complement anaphylatoxins may also provoke a direct influence on osteoclast formation. We demonstrated that osteoclast-like cells can be generated in vitro by stimulation of monocytes with C3a and C5a in the absence of RANKL [59].

In conclusion, clinical and experimental data suggest a role for complement in bone metabolism, inflammatory bone disorders, and bone healing. Central complement components, including C3 and C5, are produced by bone cells. Osteoclasts can efficiently cleave C5 to its active form, C5a. Complement anaphylatoxins induce the migration and inflammatory response of osteoblasts and directly and indirectly regulate osteoclast formation. Further studies are needed to elucidate the role of complement in inflammatory and infectious bone disorders and to clarify whether complement could be a promising therapeutic target.

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