Title: Do immune cells lead the way in subchondral bone disturbance in osteoarthritis?

Running title: Osteoimmunology in OA

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Abstract

Osteoarthritis (OA) is a whole-joint disorder, and non-cartilage articular pathologies, e.g. subchondral bone disturbance, contribute substantially to the onset and progression of the disease. In the early stage of OA, abnormal mechanical loading leads to micro-cracks or micro-fractures that trigger a reparative process with angiogenesis and inflammatory response. With the progression of disease, cystic lesion, sclerosis and osteophytosis occur at tissue level, and osteoblast dysfunction at cellular level. Osteoblasts derived from OA sclerotic bone produce increased amount of type I collagen with aberrant Col1A1/A2 ratio and poor mineralization capability. The coupling mechanism of bone resorption with formation is also impaired with elevated osteoclastic activities. All these suggest a view that OA subchondral bone presents a defective fracture repair process in a chronic course. It has been found that T and B cells, the major effectors in the adaptive immunity, take part in the hard callus formation at fracture site in addition to the initial phase of haematoma and inflammation. Infiltration of lymphocytes could interplay with osteoclasts and osteoblasts via a direct physical cell-to-cell contact. Several lines of evidence have consistently shown the involvement of T and B cells in osteoclastogenesis and bone erosion in arthritic joints. Yet the biological link between immune cells and osteoblastic function remains ambiguous. This review will discuss the current knowledge regarding the role of immune cells in bone remodelling, and address its implications in emerging basic and clinical investigations into the pathogenesis and management of subchondral bone pathologies in OA.

Key words: osteoarthritis; subchondral bone; osteoblast; T cell; B cell; osteoimmunology

Introduction

Osteoarthritis (OA) is a prevalent debilitating musculoskeletal disorder mainly afflicting the load-bearing joints, e.g. knee and hip. Given the fact that current treatment options fail to delay or prevent OA, the disease is far more complex than we thought. The traditional view of OA was a "wear and tear" problem of articular cartilage. A growing body of evidence suggests that it is not the case. First, OA is a whole-joint disorder, and non-cartilage articular pathologies such as synovial inflammation and subchondral bone disturbance contribute substantially to the initiation and progression of disease(Wen et al., 2014). Second, OA is not simply a mechanical problem but an inflammatory disease(Berenbaum, 2013). OA resembles a chronic wound healing process with an activated innate immune system in a non-specific manner(Scanzello et al., 2008). It is believed that the toll-like receptors on articular chondrocytes bind ligands such as hyaluronic acid, fibronectin and alarmins in synovial fluid triggering inflammatory responses in OA cartilage lesion(Kim et al., 2006, Schelbergen et al., 2012). As a consequence, the elevated inflammatory mediators such as complement 5 lead to destruction of articular cartilage in OA(Kapoor et al., 2011). Besides, it has been noticed that inflammatory cells, consisting of macrophage and T lymphocytes, infiltrate in the synovial tissues among over 50% of OA patients(Sakkas and Platsoucas, 2007).

OA was firstly differentiated from the other types of joint disorders such as rheumatoid arthritis (RA) based on the hypertrophic changes of subchondral bone seen in OA. Radiological findings of subchondral bone changes have been adopted to assess the severity of OA clinically(Kornaat et al., 2006). It includes bone marrow lesions (BMLs), osteophytosis, subchondral cyst and sclerotic changes *etc*. The presence of BMLs and cystic lesion correlate with the severity of pain, a major complaint of OA patients(Kornaat et al., 2006), rather than articular cartilage damage(Torres et al., 2006). In addition, such alterations in subchondral bone appeared to predict the risk of cartilage loss and the needs for arthroplasty surgery(Ding et al., 2007, Tanamas et al., 2010b, Hunter et al., 2006, Tanamas et al., 2010a). It implies the subchondral bone as a therapeutic target to rescue OA(Wen et al., 2014). It was found that the micro-cracks or micro-fractures occur at osteochondral junction as a result of the abnormal mechanical loading in the early stage of OA(Zhen et al., 2013, Burr and Radin, 2003). The incurred angiogenesis and inflammation may not only alter the process of subchondral bone modelling and remodelling(Zhen et al., 2013), but also alter the metabolism of overlying cartilage(Ashraf et al., 2011, Mapp and Walsh, 2012, Suri and Walsh, 2012). Disturbances in subchondral bone were usually attributable to elevated osteoclastic and osteoblastic activities as suggested by a histomorphometric study performed a long time ago(Reimann et al., 1977). Accordingly, bone anti-resorptives and anabolics were proposed as treatment options for OA(Karsdal et al., 2014). Pippenger and colleagues' work which demonstrated high abundance of osteoblasts and macrophages in OA subchondral bone(Pippenger

et al., 2015) leads us to believe that the activation of the immune system in micro-fracture healing process in subchondral bone deserves far more attention. The aim of this review is to discuss the current knowledge regarding the crosstalk between immune and bone cells during bone remodelling and to identify the information gap between osteoimmunology and the pathogenesis of OA *(figure 1)*. It will potentially provide a new insight in the importance of osteoimmunology in OA.

Role of subchondral bone

Interplay between bone and cartilage in the pathogenesis of OA

Whilst the most obvious hallmark of OA is the loss of articular cartilage which cushions the joint and reduces gliding friction during movement, the subchondral bone is just as important - homeostasis and integrity of articular cartilage rely on the biochemical and biomechanical interplay with subchondral bone(Lories and Luyten, 2011). In the 1970s, it was proposed that hardening of subchondral bone would increase the risk of the "wear and tear" problem of overlying cartilage in OA(Radin et al., 1972). The mechanical properties of subchondral plate and subchondral trabeculae were examined later on(Li and Aspden, 1997b, Li and Aspden, 1997a). Reports on stiffness or elastic modulus of OA bone were mixed – Li and Aspden saw an increase(Li and Aspden, 1997a), Coats and colleagues observed a reduction(Coats et al., 2003), while Brown and colleagues could not see any significant change(Brown et al., 2002). In addition, the size of subchondral bone does matter for the progression of OA - expansion of subchondral bone was found to be associated with cartilage loss in OA patients(Ding et al., 2007). Together, the relationship between bone and cartilage in pathogenesis of OA resembles a "shoe" and "foot" relationship. In this sense, the disproportional changes of subchondral bone ("shoe") and overlying cartilage ("foot") will wear the "foot" more rapidly, by which it contributes to the disease progression.

The bone-cartilage interface is composed of hyaline and mineralized cartilage, subchondral bone plate and underneath trabecular bone(Madry et al., 2010). As a transitional zone from soft to hard tissues, it is susceptible to injury under mechanical loading, thus "micro-fractures" and "neuro-vascularization" are often observed at the bone-cartilage interface in human OA specimens(Burr and Radin, 2003, Suri and Walsh, 2012, Mapp and Walsh, 2012). The accumulation of micro-fractures at the interface may activate bone remodelling process(Bettica et al., 2002, Botter et al., 2011). As a result, the growth factors embedded in bone matrix such as transforming growth factor-β (TGFβ) are released in the osteoclast-mediated resorption process at the early stage of OA as shown in a rodent model with anterior cruciate ligament (ACL) transection(Zhen et al., 2013). Enhanced TGFβ signalling is accompanied by perfusion abnormality, enhanced angiogenesis and marrow oedema in subchondral bone. In the meantime, subchondral bone plate porosity is also increased at the early stage of OA(Botter et al., 2011), which facilitates growth of blood vessels and nerves into articular cartilage from subchondral bone.

It is believed that the degeneration of articular cartilage is related to a complex network of biochemical pathways involving the diffusion of catabolic factors and mediators within and between different joint tissues, particularly in bone and cartilage. An osteoblasts/chondrocyte co-culture system was employed to explore the role of bone/cartilage tissue communication in OA(Sanchez et al., 2005, Prasadam et al., 2010). The release of matrix-degrading enzymes such as

matrix metalloproteinases (MMPs), mainly MMP-3 and -13, are responsible for cartilaginous matrix degradation in OA. It was found that osteoblasts derived from OA subchondral bone could induce healthy chondrocytes to a pro-catabolic phenotype, which was characterized by increased expressions of MMP-3 and -13, and reduced production of aggrecans(Prasadam et al., 2012, Sanchez et al., 2005). On the other hand, the chondrocytes in osteoarthritic cartilage may also be influential on the fate of osteoblasts(Prasadam et al., 2012, Prasadam et al., 2010). OA chondrocytes are able to increase the expression of MMP-1 of normal osteoblasts and enhance osteoblast differentiation, whereas normal chondrocytes are not(Prasadam et al., 2012). These studies may not reflect the real bone-cartilage communication at the initiation and progression of OA as the cells were harvested from end-stage disease tissues. On the other hand, Priam and colleagues established a novel bone–cartilage communication model to screen for soluble mediators released by loaded osteoblasts/osteocytes which may induce a pro-catabolic phenotype of articular chondrocytes(Priam et al., 2013). It was postulated that novel soluble mediators secreted by OA bone cells upon mechanical stimuli may activate the chondrocytes to produce degradative enzymes. In order to screen for soluble proteins involved in this cross-talk, iTRAQ secretome was adopted to identify differentially secreted proteins when the osteoblasts are under mechanical stress. Although the preliminary results were encouraging and a soluble protein (14-3-3ε) has been identified, the role of this protein in the pathophysiology of OA remains to be elucidated.

Pathophysiology of subchondral bone disturbance

Subchondral bone undergoes constant adaptation in response to physiological and biomechanical changes. It may suffer from abnormal mechanical loading, e.g. joint instability after ligament injury, overweight, or weakening muscles with aging. In the situation of abnormal mechanical loading, subchondral bone may develop bone marrow oedema, also known as "bone bruising" or "bone marrow lesion" (BML) (Link et al., 2003). BML is an early MRI diagnostic feature of OA closely correlated with severity of pain(Carotti et al., 2017) and is useful in predicting the rate of cartilage loss in knee OA patients(Tanamas et al., 2010b, Felson et al., 2007, Hunter et al., 2006). Histomorphologically, the presence of BML is associated with perfusion abnormalities, e.g. increased vascular permeability and ischemia(Lee et al., 2009, Aaron et al., 2007), formation of fibro-vascular tissue and under-mineralised sclerotic bone (Hunter et al., 2009, Shabestari et al., 2016). Whilst the key feature of bone marrow oedema is excessive accumulation of fluid in the marrow, studies have also demonstrated an increase of inflammatory cytokines like IL-6, IL-17F and IL-23(Zhu et al., 2017) and infiltration of immune cells in BML as well as a pro-inflammatory transcription profile with a significant portion involving cytokine signalling pathway and B-cell & T-cell activation(Kuttapitiya et al., 2017).

Subchondral bone cyst (SBC), previously known as "pseudo-cysts" or "geodes", is a major radiological finding in OA closely associated with BML. It is often present in femur, tibia, patella, and shoulder of OA patients(Tanamas et al., 2010a, Tanamas et al., 2010b). SBC commonly occurs underneath the joint's surface, subjected to major mechanical loading where the articular cartilage is severely damaged(Guermazi et al., 2012, Crema et al., 2010). It was reported in a longitudinal MRI study that SBC originates in the very same region as BMLs in knee OA patients(Crema et al., 2010). The presence of SBC, in conjunction with BMLs, is associated with the severity of pain(Link et al., 2003), and is a significant predictor for tibial cartilage volume loss and risk of joint replacement surgery in patients with knee OA(Tanamas et al., 2009). Occurrence of SBC can increase intraosseous pressure(McErlain et al., 2011) and correlates with increased bone turnover and greater cartilage deterioration (Chen et al., 2015). Moreover, the size of SBCs correlates with the degree of mineralization of surrounding trabecular bone(Chiba et al., 2012) – this implies SBC plays a significant part in high turnover of subchondral bone disturbance in the pathophysiology of OA(Chiba et al., 2012).

To understand the progression of subchondral bone disturbance in OA, researchers have looked into various rodent posttraumatic OA models, and several have successfully reproduced the development of BML and SBC in these models(Jones et al., 2010b, McErlain et al., 2012, Zhen et al., 2013). Zhen and colleagues observed an initial increase in blood flow in subchondral region in response to ligament injury, and increased diffused hyperintensity in subchondral region of knee

joints at one-month post-injury in MRI, signifying water retention otherwise known as bone marrow oedema. Histologically, they have observed an increase of osteoclast-mediated bone resorption and angiogenesis(Zhen et al., 2013). The MRI hyperintensity lasted for 2 months after severe triad injury(Jones et al., 2010b). Impaired vascular supplies contribute to BML, osteonecrosis and subsequently to the development of SBC after ACL transection plus medial meniscectomy in a rodent model(McErlain et al., 2012). BML and SBC are likely results of uncoupled bone resorption and formation due to the incurred mechanical instability.

The composition of subchondral bone matrix is dramatically changed in OA, especially at late stage of disease(Coats et al., 2003, Li and Aspden, 1997a, Li and Aspden, 1997b, Mansell and Bailey, 1998). Naturally, the mineral content of bone decreases with aging process, but the organic content increases in proportion accordingly(Li and Aspden, 1997a, Li and Aspden, 1997b). However, the organic phase of OA bone does not increase when the mineral content decreases, suggesting a defect in extracellular matrix formation(Li and Aspden, 1997a, Li and Aspden, 1997b). Low mineral to collagen ratio suggests a greater proportion of osteoid in the diseased tissue. The hypo-mineralization can be attributed to the altered alpha-1 to alpha-2 chain ratio in the composition of collagen type I in the subchondral zone of osteoarthritic samples(Kerns et al., 2014, Couchourel et al., 2009). This mechanically weak matrix, which provides poorer physical support to the articular cartilage, is thought to be one of the factors that worsen OA progression.

Temporal changes of subchondral bone have been thoroughly investigated in experimental OA models(Jones et al., 2010b, McErlain et al., 2012, Zhen et al., 2013). Surgical transection of anterior cruciate ligament (ACL) or medial meniscectomy was performed in order to mimic incurred mechanical joint instability in clinical scenarios. In the situation of abnormal mechanical loading, subchondral bone presented diffused water signals in bone marrow in the initial phase(McErlain et al., 2012, Zhen et al., 2013). Such detained water signals might arise from leakage of the newly formed blood vessels with high permeability, which leads to haematoma or oedema formation(McErlain et al., 2012, Zhen et al., 2013, Lee et al., 2009, Aaron et al., 2007). Normally, bone tissue is capable of self-regeneration without scar tissue formation. However, OA subchondral bone appears to be incapable of full recovery. Instead, fibro-vascular tissue and under-mineralised sclerotic bone develop in the injured site(Hunter et al., 2009). In more severe cases, osteonecrosis and cystic lesions may arise from chronic bone marrow oedema (McErlain et al., 2012). All these findings support a view that the pathological process of OA subchondral bone resembles delayed or failed fracture healing.

Cellular basis of osteoblast dysfunction

Naturally, osteoclastic and osteoblastic activities are precisely orchestrated to maintain balance between bone resorption and formation(Henriksen et al., 2009). Imbalance between osteoclastic and osteoblastic activities will lead to metabolic disorders of the bone such as Osteoporosis (OP) and OA. Reimann and colleagues have done a thorough quantitative histomorphometric analysis about uncoupled bone remodelling in hip OA (Reimann et al., 1977). Osteoclastic and osteoblastic activity was increased in OA subchondral bone by 190% and 96% respectively, indicating enhanced bone turnover rate. It is worth noting that the increase in activity was lower for osteoblasts than osteoclasts, yet paradoxically net bone gain rather than bone loss was observed in OA. It implies that dysregulated osteoblast-mediated bone formation may play a distinct role in the pathophysiology of OA in addition to the elevated osteoclastic activity.

The bone remodelling process involves local communication between osteoclasts and osteoblasts as well as interplay of osteocytes and mesenchymal stem cells (MSCs)(Henriksen et al., 2009). It is known that bone marrow MSCs rather than mature osteoblasts (lining cells) migrate to resorption pits on bone surface for osteogenic differentiation and bone formation to replace the damaged bone tissue(Park et al., 2012). Rollin and colleagues performed a couple of studies to characterise OA MSCs. The authors reported altered proteome with high percentage of metabolic enzymes and significant increase in the migration response of OA MSCs to platelet-derived growth factor-BB(Rollin et al., 2008b). Furthermore, they demonstrated significantly increased total TGF-β, TGF-β1 isoform, TBR-II, and TBR-III mRNA expression in OA MSCs(Rollin et al., 2008a). These results indicate activation of OA BMMSCs in response to chemotactic signals sent by the altered subchondral bone in an attempt to heal damaged tissue. Although OA MSCs display aging-related loss of proliferation capacities, they did not show gross osteogenic abnormality(Jones et al., 2010a). So, it was postulated that OA osteoblast dysfunction is attributed to the altered niche for osteogenic differentiation of MSCs in bone marrow. MSCs derived from BML in OA subchondral bone show slower proliferation and lower mineralization capacity(Campbell et al., 2016). It is speculated that the inflammatory milieu of the BML alters properties of MSCs, but the exact underlining biomolecular mechanism remains unclear.

OA osteoblasts tend to produce more type I collagen compared to normal osteoblasts, albeit at an abnormal alpha-1 to alpha-2 chain ratio. Subsequently, this atypical matrix cannot be fully mineralised, resulting in formation of sclerotic bone. Aside from altered matrix production, OA osteoblasts also express high levels of inflammatory cytokines, e.g. transforming growth factor β1 (TGFβ1), prostaglandin E_2 (PGE_2), cyclooxygenase-2 (COX-2), TNFα, IL1β, interleukin-6 (IL6), etc.(Massicotte et al., 2002, Sanchez et al., 2008). The overexpression of inflammatory cytokines is believed to signifcantly contribute in subchondral bone disturbance, as neutralizing excessive

TGFβ1 in OA osteoblasts is able to correct the abnormal collagen production(Couchourel et al., 2009).

Osteocyte is an often overlooked cell type which plays a vital role in the regulation of bone remodelling(Henriksen et al., 2009). Apoptotic signals released by osteocytes near micro-cracks, formed by excessive mechanical stress, can lead to increased osteoclastogenesis and thereby resorption of the deteriorated bone matrix. Meanwhile, osteocytes regulate bone formation through the production of sclerostin (SOST). SOST, a ligand for LRP5, prevents the WNTs from activating bone formation. Emerging evidence showed that OA osteocytes undergo significant morphologic and biochemical alterations(Jaiprakash et al., 2012, Appel et al., 2009). The number of osteocyte lacunae in OA bone is much higher than in osteoporotic fractured bone and the individual lacuna size in OA is smaller. OA osteocytes also differ drastically in terms of appearance as well as gene expression profiles. They appear to be rougher with rounded cell bodies and fewer disorganized dendrites compared to normal osteocytes(Jaiprakash et al., 2012). The expression of SOST is significantly reduced in OA osteocytes, which in turn augments WNT signalling to promote osteogenic differentiation of MSCs and bone formation(Jaiprakash et al., 2012, Appel et al., 2009).

Immune cells activation in fracture healing

Bone fracture healing is a complex process involving four main steps, namely hematoma, inflammation, callus formation and bone remodelling(Ono and Takayanagi, 2017). Immune cells i.e. macrophages, T and B cells participate actively in the healing process by migrating into the fracture site to serve various functions(Ono and Takayanagi, 2017). T and B cells, the major effectors in immune response, are involved in osteoclastogenesis and bone catabolism. It is believed that the crosstalk between immune cells and bone cells lies in the OPG/RANKL (osteoprotegerin/receptor activator of nuclear factor kappa-B ligand) system. Typically, RANKL secreted by osteocytes can bind to RANK on the surface of osteoclast precursors to initiate osteoclastogenesis for bone resorption during remodelling process(Xiong et al., 2011). However, it was more recently found that immune cells could be another source of RANKL for osteoclastogenesis, e.g. B lymphocytes(Manilay and Zouali, 2014). Specific knockout of RANKL in B cells can partially prevent bone loss as demonstrated in an ovariectomised rodent model(Onal et al., 2012). On the other side, B cells can also produce OPG, a soluble decoy receptor of RANKL that suppresses bone resorption process by interacting with RANK(Kong et al., 1999), which might paradoxically limit bone loss under certain circumstances(Choi and Kim, 2003). The intimate relationship between B cells and skeletal system implies B cells could be a potential therapeutic target for inflammatory bone loss in arthritis(Manilay and Zouali, 2014). Putting this into perspective, Rituximab, an anti-CD20 antibody that depletes mature B cells, has been approved for the treatment of RA(Chan and Carter, 2010). It is worth bearing in mind, however, that B-cells play a more pivotal, prominent role in the pathogenesis of this specific type of arthritis.

On the other hand, the interplay between immune cells and osteoblasts remains ambiguous. It was once reported that osteoblast function is compromised in inflamed joints(Walsh et al., 2009). Inflammatory cytokines produced by the immune cells, such as TNF-α and interleukin 1β (IL1β), might interfere with osteoblastic function via up-regulation of Wnt signalling antagonists in arthritic joints, e.g. Dickkopf and secreted Frizzled-related protein families. It is worth noting that there are still debates on the actual effects of these inflammatory cytokines on osteoblasts. For example, the effects of TNF-α on bone cells, stimulatory or inhibitory, are dose-dependent *in vitro*(Osta et al., 2014).

The role of immune cells in bone regeneration has also been investigated in fracture healing models(Konnecke et al., 2014, Toben et al., 2011, Nam et al., 2012, Reinke et al., 2013). It was reported that during fracture healing, there is an initial surge of T and B cells infiltration into the injured site. Then during soft callus formation, T and B cells are withdrawn from the site. A second surge occurs during mineralization of the callus. In the time course of the crucial mineralization stage, immune cells developed direct cell-to-cell contact with osteoclasts and osteoblasts(Konnecke

et al., 2014). Thus, it is believed that T and B cells play a regulatory role in bone modelling (regeneration) and remodelling. In an animal study performed by Toben and colleagues, fracture healing process was accelerated with faster mineralized callus formation in $RAG1^{-/-}$ mice, which cannot form mature T and B cells(Toben et al., 2011). This finding connotes that infiltration of immune cells into the callus is detrimental for fracture healing. B cell is a dominant immune cell type in the hard callus both in terms of cell abundance and function(Konnecke et al., 2014). It was underdetermined whether B cells also play a dominant role in the late stage of fracture healing. The effects of T cells on osteoblasts are far more complex and the subsets of T cells may play different or even contradictory roles in bone regeneration(Nam et al., 2012, Reinke et al., 2013). The terminally differentiated effector memory CD8+ T cells (T_{EMRA}) are long-lasting in peripheral blood of patients with delayed fracture healing(Reinke et al., 2013). CD8+ T_{EMRA} cells overexpress interferon-γ (IFN γ) which inhibits osteogenic differentiation of MSCs. Understandably, depletion of CD8+ T cells promotes the formation of hard callus, and transplantation of CD8+ T cells does the opposite and leads to fracture non-union(Reinke et al., 2013). In contrast, another subset of T cells, IL-17F-producing T cells, is believed to trigger osteoblast maturation in the early stage of fracture healing(Nam et al., 2012).

Current knowledge of osteoimmune crosstalk in OA

Subchondral bone disturbance plays a pivotal role in OA(Hugle and Geurts, 2017). To further understand OA progression, biomolecular signalling which interferes with bone metabolism needs to be considered. Since bone-turnover and inflammation are closely connected, they share a lot of cytokines, transcription factors and molecular pathways(Ginaldi and De Martinis, 2016). To start with, T and B lymphocytes are able to express RANKL and can consequently directly influence bone resorption once they have invaded into the subchondral bone(Ginaldi and De Martinis, 2016). Immune cell infiltration in subchondral bone marrow has been documented in OA *(figure 1)*, although it is relatively mild compared to the other types of arthritis, in which inflammation takes the central stage, e.g. rheumatoid arthritis and ankylosing spondylitis(Appel et al., 2006, Bugatti et al., 2005). T cells play an important role in osteoimmunology in posttraumatic OA. In an ACL transection OA mouse model, CD4+ T cells were found to infiltrate into the entire joint tissue, not only in synovium but also in subchondral bone and articular cartilage one month after the surgery(Shen et al., 2011). Klein-Wieringa and colleagues' study on human end-stage knee OA confirmed this finding by demonstrating presence of activated CD 4+ cells in the synovium and infrapatellar fat pad(Klein-Wieringa et al., 2016). Activation of CD4+ T cells can induce the expression of macrophage inflammatory protein 1γ (MIP-1γ) and provoke osteoclastogenesis in OA joint(Shen et al., 2011), although the number of CD4+ T cells gradually decrease later on with the progression of OA.

Regulatory T (Treg) cells enrichment in the joint is not specific to inflammatory arthritis. Like RA, Treg cells are recruited to OA synovial fluid and synovium. There are a few differences in the frequency and function of CD4+/CD25+ Treg cells between OA and RA(Moradi et al., 2014). One major difference between OA and RA is that the function of Treg cells is believed to be intact in OA but impaired in RA joints(Moradi et al., 2014, Flores-Borja et al., 2008). Li and colleagues showed that during OA progression, decreased CD4+/CD25+/Tim-3+ causes reduction in IL-10 secretion(Li et al., 2016).

Very interestingly, in the late-stage of OA, the number of CD68+ macrophages and CD20+ B-lymphocytes were significantly higher in the sclerotic region of subchondral bone than in non-sclerotic areas(Geurts et al., 2016). It has been shown, that B cells and its secretion of IL-10 are associated with delayed bone fracture healing(Sun et al., 2017b). Therefore, B cells are also suspected to have a hand in defective repair of subchondral bone in OA and further investigation in their function and crosstalk with subchondral bone and osteoclasts would bring up valuable knowledge. The influence of macrophages on the bone homeostasis has been widely discussed(Kaur et al., 2017). Its impact on OA is largely unknown, but should be carefully considered, as macrophages are known to play various roles in fracture response, tissue

regeneration and bone healing(Forbes and Rosenthal, 2014, Alexander et al., 2011). In addition to the previously mentioned elevation in number of macrophages in sclerotic subchondral bone of knee OA, increased number of macrophages have been described in subchondral bone of OA facet joints, in which it is accompanied by enhanced de novo bone formation(Netzer et al., 2016). To test whether depleting macrophages can rescue OA, Wu and colleagues looked into an obese mouse model and found that depletion of macrophages not only failed to rescue post-trauma OA phenotype, but also caused higher level of systemic inflammation and invasion of CD3+ cells and neutrophils in the injured joint(Wu et al., 2017). Since depletion of differentiated macrophages can transform the bone marrow to an osteogenic environment with enhanced PTH anabolism(Cho et al., 2014), a specific macrophages subtype depletion might be able to prevent OA progression. All these data underline the importance of macrophages in subchondral bone homeostasis. Further understanding of their phenotypes and interactions is very valuable for a better understanding of the disease.

OA is not only a local, but plausibly a systemic inflammatory induced disease. In peripheral blood of OA patients, a significant aberrant ratio of CD4+/CD8+ cells were found(Apinun et al., 2016, Ponchel et al., 2015). The highly vascularized synovium could be a gateway for systemic inflammatory cytokines to affect the joint(Hugle and Geurts, 2017). In addition to be a gateway for systemic inflammation to affect the joint, synovial membrane is also a known source of inflammatory cytokines such as IL-1, IL-6 and TNF(Larsson et al., 2015). Mast cells, which are present in greater abundance in OA synovium(de Lange-Brokaar et al., 2016), can release inflammatory cytokines and recruit innate immune cells(Kroner et al., 2017), and thereby contribute to inflammation and aggravation of subchondral bone disturbance. Another often neglected immune cell type is the natural killer cell. In synovial fluid of end-stage OA, a high presence of CD56(+)brightCD16(-)low cytotoxic NK cells was found. This cell type produces high levels of granzyme A, which may contribute to the joint inflammation(Jaime et al., 2017). Benigni and colleagues also demonstrated that among the immune cells, NK and neutrophils are the first to infiltrate the synovium during the development of OA using a collagenase-induced OA model. Furthermore, they showed that Cxcr3-/- mice were protected from disease development after injury, suggesting that the NK and neutrophils functional interaction is promoted by the CXL10/CXCR3 axis(Benigni et al., 2017). Aside from synovial membrane, OA infrapatellar fat pad also contributes to the inflammatory milieu. Being colonized by macrophages and T-cells, it releases inflammatory cytokines without additional stimulation(Klein-Wieringa et al., 2016). All this evidence strongly suggests the involvement of inflammatory cells and mediators in the pathophysiology of OA, although the exact orchestra remains largely unknown.

Effects of bone derived growth factors on immune cells

Whilst bone tissue is composed mostly of calcified collagenous extracellular matrix – around 90% by weight, other proteins are also present in the matrix. It is already known that bone tissue matrix holds a plethora of bone derived growth factors, including TGF-β superfamily which consists of TGF-βs and various bone morphogenetic proteins (BMPs), osteopontin, insulin-like growth factors (IGFs) and platelet-derived growth factors (PDGF)(Capanna et al., 2010). Upon damage of the matrix these otherwise latent growth factors could be activated and made available to the quiescent cells as signalling cues and stimulatory factors for reparative purposes, may that be endothelial cells or mesenchymal stem cells. At the same time, these factors very often interact with the immune system, since the acute phase reaction of the immune system plays a vital role in wound healing by "cleaning up" the site of injury i.e. to remove potential pathogens and debris present prior to rebuilding of the tissue *(figure 1).*

TGF-β superfamily

Members of the TGF-β superfamily are known to be abundantly present in bone matrices(Wu et al., 2016). Aside from controlling cell proliferation and differentiation during wound healing process, the TGF-β superfamily is indispensable in modulating the immune system. TGFβ1 is a multifunctional protein with dual effects in mediating inflammatory response. TGFβ1 is required for the development of interleukin-17 producing T cells (Th17) at a relatively low concentration(Mangan et al., 2006). Th17 is conceived to initiate the inflammatory phase of arthritis and induces osteoclastogenesis(Takayanagi, 2012, Kotake et al., 1999). With enhanced osteoclastogenesis, the local level of active TGFβ signalling in subchondral bone reaches its peak two weeks after induction of OA and gradually resolves afterwards(Zhen et al., 2013). High level of TGFβ1 can induce Foxp3 gene expression in the CD4+CD25- T cells and mediate the phenotypic changes toward CD4+CD25+ Treg cells with immunosuppressive potential(Liu et al., 2008). It is postulated that TGFβ1 is a possible linking factor between bone modelling/remodelling and Th17 (inflammatory)/Treg (anti-inflammatory) cells development and survival. Whilst TGF-β1 is found to be a suppressor of the immune system, the effects of other factors within the TGF-β superfamily on the immune system vary and could be totally different since the BMP pathway differs siginificantly from the TGFβ pathway. BMPs act on separate sets of receptors, namely the type I BMP receptors (ALK2 &3) and type II BMP receptors (BMPRII, ACTRIIA and ACTRIIB)(Wang et al., 2014). In stark contrast against TGFβ's effect on macrophages, BMP-6 does not inhibit macrophage activity. Quite the opposite, it is known to activate macrophages, induce the production of iNOS, TNF-alpha(Hong et al., 2009) and IL-6. BMP signalling promotes Th17 differentiation(Yoshioka et al., 2012) but suppresses Treg cell generation and regulate IL-2

production. BMP-2 and -4 produced by vascular smooth muscle cells can induce monocyte recruitment and inflammation(Simoes Sato et al., 2014)

Osteopontin

Osteopontin (OPN), also known as bone sialoprotein I (BSP-1), is a multifunctional 44-75kDa non-collagenous extracellular matrix protein. It is expressed by a huge variety of cells, including osteoblasts, osteoclasts, chondrocytes, synoviocytes, macrophages, activated T cells, etc (Clemente et al., 2016). OPN is known to play important roles in modulating inflammation, bone remodelling & mineralisation, immune functions, chemotaxis, cell activation and apoptosis(Wang and Denhardt, 2008). It is considered to be a pro-inflammatory cytokine and is known to be associated with autoimmune disorders & inflammatory diseases like multiple sclerosis, systemic lupus erythematosus, irritable bowel syndrome, rheumatoid arthritis(Rittling and Singh, 2015) and bone diseases like osteoporosis and osteoarthritis. Being associated with various autoimmune disorders and inflammatory diseases of numerous organs, it comes to no surprise that OPN plays a central role in recruiting of inflammatory cells and driving the production of cytokines. It was reported that OPN regulates T cell development, enhances differentiation along Th1 pathway, suppress Th2, and supports Th17 differentiation(Cantor and Shinohara, 2009). In an animal study, OPN was found to induce signalling pathways that lead to survival of pathogenic T cells and induce production of IL-17 by CD4+ T cells(Murugaiyan et al., 2008). OPN is also found to directly increase the proliferation of human monocytes, activate motility and proliferation of macrophages(Tardelli et al., 2016). OPN is an intrinsic regulator that plays an important role in OA progression. Increased expression of OPN has been observed in OA joints and is correlated with severity of joint lesion and inflammatory status of OA. OPN level is significantly elevated in OA patient plasma and synovial fluid(Honsawek et al., 2009, Gao et al., 2010). Release of OPN leads to induction of MMP-13 and also activates NFkB pathway(Ding et al., 2017), initiating production of inflammatory cytokines like NO, PGE2, IL-1b and IL-6.

Effects of inflammation on bone remodelling in OA

Inflammation is an inevitable phase of bone fracture healing, thus intrinsically inflammatory cells regulate the process by secreting inflammatory cytokines and chemotactic mediators(Osta et al., 2014), as well as growth factors, to recruit and stimulate both immune cells and progenitor cells for remodelling of the injured site. Monocytes and macrophages are capable of releasing cytokines like BMP-2, BMP-4 and TGF-β1, which can stimulate osteoblast differentiation and proliferation(Loi et al., 2016). Without sufficient macrophages, new bone deposition and mineralisation would be suppressed, as demonstrated by previous macrophage-depletion models(Vi et al., 2015). With that being said, chronic inflammation is a totally different story and may delay bone fracture healing(Claes et al., 2012).

The inflammatory cytokine TNF- α has a paradoxical role in bone fracture healing. On one hand, transient TNF- α is in favour of bone regeneration(Chan et al., 2015). It can trigger a release of secondary signalling molecules and recruitment of MSC, and together with IL-1β, promote matrix mineralisation. On the other hand, high persistent level of TNF- α can delay bone regeneration, leads to chronic inflammation and even results in rheumatoid arthritis-like symptoms(Osta et al., 2014). Other inflammatory cytokines like IL-1 and IL-6 were reported to have similar roles in bone repairing – it is evident that inflammation is vital in preparing for bone regeneration, yet chronic unresolved inflammation does the complete opposite.

Whilst there is no direct evidence on how inflammation can cause osteosclerosis of the subchondral bone, it was previously demonstrated that abundance of B-lymphocytes and macrophages were significantly higher in sclerotic subchondral bone than in non-sclerotic subchondral bone(Geurts et al., 2016), hinting inflammatory cells do participate in causing osteosclerosis in OA. A study on periosteal macrophages in bone healing pointed out that osteomacs, a subtype of macrophages, are prominent in areas of high Col1a1 deposition in activated periosteum(Alexander et al., 2017), pointing out the possible role of macrophage in osteosclerosis.

Other authors also suggested TGF-β is the bridge between inflammation and fibrosis of the bone marrow(Desterke et al., 2015). Our postulation is that turbulence of TGFβ1 level after tissue injury such as subchondral bone micro-fractures might lead to activation of the adaptive immune system. Imbalance in inflammatory and anti-inflammatory cells might contribute to delayed union or non-union of micro-fractures, contributing to the hypertrophic changes of subchondral bone and the chronicity of disease. If this concept could be proven by identifying the unique pattern of adaptive immune system activation in OA, it would throw light on the development of new diagnostic and therapeutic strategies for OA.

Perspectives for bone drugs as treatment of OA

Recently a lot of studies investigated the feasibility of rescuing OA cartilage by means of intraarticular injection(Takayama et al., 2014, Cheng et al., 2016, Nagai et al., 2014). One study demonstrated intraarticular injection of Torin 1, a mTOR inhibitor, can reduce articular cartilage damage in collagen-induced OA rabbit model (Cheng et al., 2016). Another study investigated intraarticular application of Bevacizumab, an anti-vascular endothelial factor antibody, and showed the treatment can lessen cartilage deterioration, osteophyte formation and synovitis(Nagai et al., 2014). Others have conducted intraarticular therapy studies in humans. Intraarticular injection of platelet-rich plasma could relief pain in Knee OA patients(Shen et al., 2017). Despite being able to slow down cartilage damage and provide pain relief, the proposed therapies fail to stop the eventual onset of OA.

Since it is known that inflammation, especially synovitis, does occur in OA and that it is one of the major sources of pain, clinicians also administered the patients with immunosuppressive drugs and anti-inflammatory drugs like steroids(Freire and Bureau, 2016) and non-steroid anti-inflammatory drugs (NSAIDs)(Petite et al.). Corticosteroids are often applied intraarticular to give the patients a temporary relief of pain and gain of function of the affected joint(Richards et al., 2016). NSAIDs are often used orally over long time period to reduce pain(Guyot et al., 2017). In addition, NSAIDs are reported to be able to decrease inflammatory cytokines in human chondrocyte cell lines(Sun et al., 2017a). Whilst being effective in management of symptoms, these drugs also come with potentially serious side effects – chronic use of corticosteroids is known to be associated with higher risks of cataracts, diabetes, infection and osteoporosis, and use of NSAIDs is associated with higher risk of gastrointestinal disorders and cardiovascular diseases(da Costa et al., 2017). In addition, neither class of drug is capable of attenuating or modifying the progression of OA(Richards et al., 2016, Lapane et al., 2015). This hints more specific regimen that targets the downstream effectors of OA inflammatory response may potentially be more beneficial.

Amongst the pathways triggered by inflammation during OA, the Wnt and TGF-β signalling pathway are known to be essential for homeostasis of bone and cartilage metabolism(Yang et al., 2001). Inhibition of TGF-β signalling specifically in subchondral bone can restore subchondral bone disturbance and attenuate the severity of articular cartilage degeneration(Zhen et al., 2013), yet TGFβ/Smad3 signalling is essential to maintain the structural integrity of articular cartilage(Yang et al., 2001). Wnt and BMP signalling are indeed activated in attempt to repair the mechanical damages of articular cartilage(Dell'accio et al., 2008, Dell'Accio et al., 2006). Injection of TGFβ1 can regenerate damaged cartilage via stimulation of chondrocytes proliferation and proteoglycan synthesis. However, it also causes osteophyte formation(van Beuningen et al., 1994). It is a dilemma whether to supplement or inhibit Wnt/ TGF-β signalling to rescue OA. Therefore, it will

be useful to identify the downstream mediators of Wnt/TGF-β signalling accounting for the side effects(van den Bosch et al., 2016) that occur with their reparative effects.

Excessive osteoclastogenesis is another hallmark of subchondral bone inflammation. Bone antiresorptives and anabolics are candidates for structure-modifying drugs in OA(Karsdal et al., 2014), e.g. alendronate and teriparatide(Hayami et al., 2004, Sampson et al., 2011). It was reported that alendronate can effectively restore subchondral bone architecture, and reduce osteophyte formation and cartilage degeneration in spontaneous(Ding et al., 2008) and instability-induced OA models(Hayami et al., 2004, Jones et al., 2010b). A more recently conducted study showed it can prevent early cartilage degeneration but it is ineffective in the long run(Khorasani et al., 2015). Teriparatide, an FDA-approved bone anabolics for osteoporosis, was introduced for treating OA(Sampson et al., 2011, Lugo et al., 2012, Bellido et al., 2011, Chang et al., 2009). Teriparatide can restore the bone loss prior to the degeneration of cartilage, as demonstrated in an instability-induced OA model(Bellido et al., 2011). Teriparatide also exerts beneficial effects on the synoviopathy in OA(Lugo et al., 2012). Importantly, teriparatide promotes the proliferation of chondrocytes and inhibits the terminal differentiation towards hypertrophy, which favours the regeneration of articular cartilage in OA(Sampson et al., 2011). More investigation in bone remodelling and its modulation are needed to bring further understanding in its impact on load induced OA progression(Adebayo et al., 2017)

Recently, A genome-wide screening of OA subchondral bone identified periostin and leptin as novel signalling pathways involved in the uncoupled bone remodelling(Chou et al., 2013). These findings align with the current discussions on the relationship between metabolic syndrome and OA(Berenbaum, 2012, Berenbaum et al., 2013, Zhuo et al., 2012). It has been shown that periostin is upregulated in articular cartilage(Chijimatsu et al., 2015) and might be upregulated by IL-13 in synoviocytes in OA and may upregulate the production of MMP(Tajika et al., 2017). Furthermore, expression of periostin was also found in subchondral bone(Chijimatsu et al., 2015). We hypothesize that disorders of lipid metabolism may alter the niche of MSCs in bone marrow and contribute to the osteoblast lineage cell dysfunction in OA. A systemic biology approach is proposed to analyse the osteoblast dysfunction with the integration of genome, transcriptome, proteomics and metabolomics for metabolites.

Conclusion

In summary, osteoblasts dysfunction plays an important part in the uncoupled bone remodelling and bone-cartilage communication at the onset and progression of posttraumatic OA. However, there is still a big knowledge gap to be filled between the influence of the immune system on osteoblasts dysfunction and subchondral disturbance. Importance of osteoimmunology on the

onset and progression of OA needs further substantiation. Investigation of underlying causes may identify innovative biomarkers and novel therapeutic targets in the management of OA.

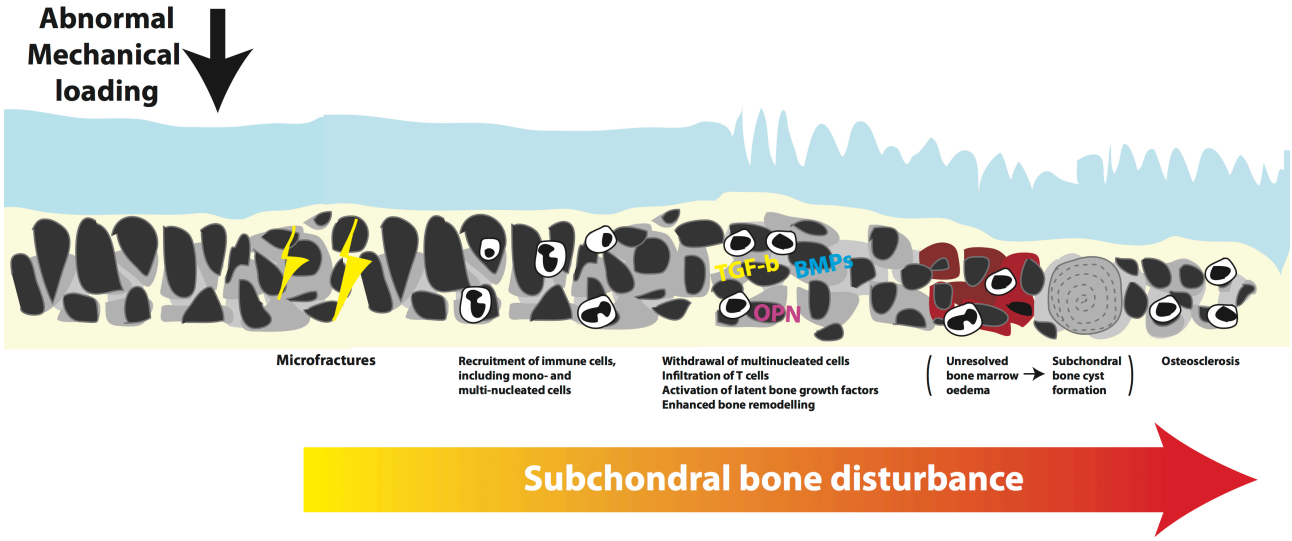

Figure 1: Proposed contribution of immune cells to subchondral bone disturbance in post-traumatic OA

Contributions

AW, PMC and CYW formulated the idea, drafted the manuscript and proofread the the manuscript before submission.

Conflicts of interests

None

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