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Osteoimmunology of Fracture Healing: A Brief Review on the Immune Systems' Cellular Milieu Role in Bone Injury

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ABSTRACT

Bone fracture healing is the most common medical treatment in the usual clinical practice. Bone has the unique property for self-healing, which is in addition to the ability of not forming a scar. Following fracture, a chain of events takes place, which include the activation of healing processes in both the cellular and tissue level. These events, lead to a full bridging of the gap between the two bone-ends of the fracture. In that process the immune system is active and the immune cells are known to play a significant role. The process of fracture healing can be divided in three main steps; these are sequential in nature and independent. The first phase includes the inflammation phase, which involves the acute activation of the immune system, the second phase includes the repair phase, which involves the recruitment of mesenchymal progenitor cells differentiating to chondrocytes and the final phase involves remodeling, where osteocytes are recruited in order to form the new bone. In conclusion, immunity serves as the initial responder at the skeletal damaged site, restores the vasculature, and initiates cascades of signals to recruit cells to carry out the repair processes. Therefore, it is believed that the immune system can be considered as a promising therapeutic target for bone fracture healing.

KEY WORDS: Fracture Healing, Immune System, Bone Fracture, Bone Healing, Immune Cells

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Introduction

Bone as a tissue has the unique ability of healing itself without forming a scar (1). However it is reported that 5%-10% of the fractures result to permanent failure of healing, yearly, a rate that increases with certain comorbidities and aging (2). There is a need for the regulation of skeletal homeostasis that is controlled not only by the musculoskeletal system but by the combination of multiple biological systems as well (2). There are various "regulatory molecules" that both, the skeletal and the immune systems, share such as cytokines and signaling molecules, and thus the immune system is in close relation with the bone system (3). This interaction between osteo-cells and parts of the immune system, which are known for their role in bone repair, retains a great research interest from scientists and clinical practitioners (4), as it is known that bone fracture healing is tightly in cooperation and under the strong regulation of the immune system (5).

As the bone marrow is the main site of hematopoiesis, it hosts Hematopoietic Stem Cells (HSCs), as well as mature immune cells, including B-cells, neutrophils, macrophages and T cells (6). Although previously neglected, the bone and the immune system share the same microenvironment, where they interact consisting of the "osteoimmune system". This system, involves all bone marrow cells. For historical purposes, the discipline of "osteoimmunology" was developed in order to investigate the underlying molecular mechanisms of bone healing linked to inflammatory processes. An older work of Horton *et al*. (1972) referred to the possible interactions between the skeletal system and immune cells, after studying cases of periodontitis (7), where immune cells stimulated by bacterial antigens procured the production of "osteoclast activating agents" (7). Much later on, the term "osteoimmunology" was coined by Arron & Choi (2000), which emphasized the T-cell-mediated regulation of osteoclastogenesis in the context of arthritis autoimmunity mechanisms (8, 9). This was one of the first studies concerning the correlation between osteocytes and the immune system, in terms of the autoimmunity machinery. Thus, immune/skeletal communication is a "round trip", where the key players are the osteoblasts/osteoclasts and the cells of the hematopoietic system. At the same time, bone has been shown to play a role both in the normal development of the immune system through the Hematopoietic Stem Cells (HSCs) in the bone marrow, as well as in acquired immunity. It is also known that memory T- and B-cells always return to the bone marrow. One of the key-molecules that are known to play a role is RANKL, whose part in osteoimmunology is still under investigation (10), while its role seems to extend beyond the bone. For example, its immunohistochemical expression has been studied in association with RANK and OPG in gingival tissues where they appear to play a major role in periodontal bone loss (11, 12).

There are three sequential phases that consist the process of fracture healing which however remain independent. These phases are: "*inflammation*, *repair* and *remodeling*". The triggered local inflammatory response ignites the fracture healing process that leads to the remodeling and repair phases (13). When a bone fracture occurs, a hematoma is formed because of the ruptured vasculature, embedded in the proximal bone and the bone marrow. In this hematoma, various immune cells infiltrate, including the complete niche of white blood cells (14). These cells, it is believed that they are activated by molecules derived by the injured tissues, and after activation the immune cells produce pro-inflammatory cytokines at the injury site, in order to induce acute inflammation (3). After the inflammatory phase, mesenchymal progenitor cells assimilate around the bone lesion, which in turn they differentiate to chondrocytes. The differentiated chondrocytes synthesize cartilage forming a matrix, which is known as "*callus*" and at that phase is soft. The soft "*callus*" bridges the injured bone fragments and afterwards this soft callus is replaced by a hard "*callus*", which also consists of bone tissue. This hard "*callus*" undergoes additional remodeling by activated osteoclasts and osteoblasts, which act further for the purpose of restoring the original morphology and function of the bone without any scar formation (3, 14-16).

Control of Fracture Healing

Repair and restructuring of bone injuries (fractures,

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lesions) are under the close surveillance of the immunological machinery. The immune system is considered to be crucial for this healing process because in the sequence of the events taking place, first inflammatory mechanisms are triggered, followed by bone healing. From observations in patients under immunosuppressive treatment, it became apparent that bone injury and fracture manifested delays in the healing process and higher frequency of nonunion. This conclusion was further supported by similar observations in HIV patients (3, 17-20).

Fracture healing consists of anabolic and catabolic phases during which both intrinsic and modulatory immune functions are essential (14). The first responses to bone injury include the cell-mediated immunological response, which causes the initial inflammation and thereafter followed by the removal of necrotic tissue, the induction of angiogenesis and the initiation of repair (21-23). While the inflammation response lasts for a short time, the immunological response extends at longer time intervals, ranging from the first response to later stages of bone healing (1). The immune cells participating in the process are thought to be activated by molecular mechanisms that is signaling molecules excreted at the injury site, which in turn release pro-inflammatory cytokines eliciting acute inflammation (3). The necessary first steps during the inflammation stages include clotting, tissue granulation, and cell recruitment, which depend on the coordination of various immune cells. Hematopoietic cells seem to direct mesenchymal cell differentiation and activity throughout the different phases of this procedure (1).

However, a controversial finding, suggests that a bone fracture leads to immunological suppression (probably due to the induction of the stress mechanisms induced). Immune suppression is believed to take place due to local activation and increase of induced T-regulatory (iT_{REG}) cells, which suppress active intrinsic immune responses within the fracture "*callus*" (24, 25). Several studies have highlighted the role of mesenchymal stem cells, which convey immune tolerance during the early stages of "endochondral bone formation" and provide a kind of "sheltering" for the developing tissues. This is known to take place by the suppression of T-cell alloproliferation during stem-cell recruitment and cartilage formation (14, 25). However, if inflammation insists or remains unresolved, like when there is a bacterial infection at the fracture site, healing is possible to fail (26).

Immune Cells function and origin

Platelets

Platelets have been shown to have a role in fracture healing although their primary function is related to blood clotting (27). "Platelets are non-nucleated cells of the myeloid lineage" (28-30). The injured or damaged vessel produces and releases thrombin, a serine protease, which stimulates platelets to initiate the clotting process (28-30). The activated platelets orchestrate the response by forming the "fibrin thrombus", which acts as a "scaffold" for cellular engraftment. At the same time, platelets respond to the stimulus by secreting inflammatory cytokines (IL-1, IL-6, TNF-α) and growth factors (PDGF, TGF-beta), which triggers a cascade of events, involving the activation of other immune cells (neutrophils and monocytes), as well as mesenchymal progenitor cells (28-30).

Macrophages

Macrophages are myeloid lineage cells that are differentiated from monocytes. Macrophages, consist of various other cell subsets with different functions and are responsible for arranging inflammation and tissue regeneration after tissue damage (31, 32). Macrophages are some of the first cellular responders, which appear at the local hematoma and retain their presence throughout the healing process (14, 33, 34). Macrophages have been found to play a major role in bone homeostasis and bone fracture repair, since they act as "helper" cells to the osteoblastic and osteoclastic cell lineages, by taking part in the "crosstalk" and communication to maintain the balance in bone remodeling (1, 35). There are two main macrophage categories, based on their polarization (36); the M1 macrophages, which are termed also as "classically activated macrophages" (37) and the M2 macrophages, which are known also as "alternatively activated macrophages" (38).

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In a previous study by Schlundt *et al*. (2018), it has been suggested that the M1 macrophages are the first to infiltrate the bone fracture location during the "acute phase", while the M2 macrophages have been found to multiply in numbers during the "subacute" phase (39). Yet, the combination of both the M1 and the M2 macrophages is the machinery that play the significant role during the healing process, as they regulate both the early and late phases. This is indicated by the fact that time-dependent reduction of macrophages in either the "acute" or "subacute" phase leads to significant delay of bone healing (40). Additionally, it has been shown that macrophage inhibition, leads to a decrease in mesenchymal progenitor cell population, and thus inhibiting the ability of these cells to differentiate to osteoblasts (35, 41).

The exact machinery and molecular mechanisms, behind the action of macrophages in bone fracture healing is still unclear. It is certain that macrophages play a significant part in bone healing, yet it is still unknown how macrophages interact with the injured bone in order to procure repair. This is still a topic of intensive investigation and research (1, 3).

Neutrophils

The role of neutrophils in fracture healing remains unclear and involves many aspects of tissue repair. Neutrophils, are the most abundant granulocytes, as well as they are part of the polymorphonuclear family of immune cells together with basophils and eosinophils (42). They are activated by the presence of IL-1, TNF-α, which are produced by platelets (43). Neutrophils contribute both to the early stages of the inflammatory phase, as well as the later. In the early stages of the inflammatory phase they deposit the "fibronectin matrix", while during the later phases of inflammation, neutrophils remove the cellular and tissue debris, as well as they are implicated at the *thrombus* removal (44). Neutrophils are the first to arrive, on the fractured bone site, as they manifest anti-septic effects and also remove the damaged cells and other debris (23, 45, 46). The most significant role of neutrophils appears yet to be the release of cytokines (IL-1, IL-6, IL- 10, TNF-α, MCP-1, CXCL-1α, MIP-1) attracting monocytes, which will subsequently differentiate to macrophages (1).

T-Lymphocytes

T-lymphocytes, also known as T cells, are hematopoietic cells of the lymphoid lineage characterized by the expression of T- cell receptors (TCRs) (1, 3). A recent study on animal models has shown that T- and B-lymphocytes, appear at the fracture site three days after injury and subsequently their population reduces, when the physiological process of cartilaginous formation begins (47). It has been noticed that depletion of T-cells leads to reduced skeletal health and subsequently to a decrease in fracture healing (47, 48). Studies with mice have shown that subjects with lack of T- and B-lymphocytes have more "stiff" bones, more susceptible to fracture (49).

As aforementioned, T-lymphocytes regulate bone fracture healing and it is also known that white blood cells are present during both the primary and final stages of the bone repair process (50). T-lymphocytes consist of different sub-classes, which include the $T_{\alpha\beta}$-cells or $T_{\gamma\delta}$-cells, and their differentiation is defined by the expression of T-Cell Receptors (TCRs). The population of the $T_{\alpha\beta}$-cells is tightly regulated by positive and negative feedback mechanisms in the thymus. The regulatory mechanisms have as a result the maturation of $T_{\alpha\beta}$-cells, which subsequently express αβ-TCRs and co-receptors (51). Similarly, these cells will produce CD4+ T-helper (Th) cells, CD8+ cytotoxic T-lymphocytes (CTLs), and CD4+ regulatory T-cells (Tregs) (52). On the other hand, the $T_{\gamma\delta}$-cells are derived from the common cell of origin, as in the case of $T_{\alpha\beta}$-cells, but they recognize not antigen–specific molecules (53). These cells function as "sentinels" for the detection of microbe antigens and "self-components" released due to tissue stress, contributing to the intrinsic immune response. This function is facilitated by the production of cytokines and/or cytotoxicity (54). Further on, $T_{\gamma\delta}$-cells are subdivided into cellular subsets based on the expression of TCR-Vγ chains. Each cellular subset possesses its own characteristic tissue distribution and cytokine production pattern (55).

While $T_{\alpha\beta}$-cells exhibit "pro-inflammatory and anti-inflammatory functions" that are crucial for

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antigen-specific immune responses, $T_{\gamma\delta}$-cells are reported to be able to spread selectively to the tissue epithelium, where they exert defense and tissue repair functions, in the periphery (3). Recent studies have indicated that $T_{\gamma\delta}$-cells' population increases vastly during bone fracture, thus contributing to its healing by the production of IL-17A, which enhances bone formation (56).

A contrasting mechanism to the healing properties of T-cells, is derived from the presence of effector memory CD8+ T-cells. Effector memory CD8+ T-cells are found in abundance in the peripheral blood of patients with delayed fracture healing, while another experimental model has shown that when injected in mice, bone repair manifests significant delay to bone healing. These findings supported that fact that effector memory CD8+ T-cells suppress bone repair (57). This is also supported by the fact that effector memory CD8+ T-cells can produce TNF-α and interferon-gamma (IFN-γ), which participate to the suppression of bone marrow mineralization mesenchymal stem cells (MSCs) (3).

B-Lymphocytes

B-lymphocytes, also known as B-cells, are "hematopoietic cells of the lymphoid lineage", just like T-lymphocytes, and play a vital role in humoral immunity (1, 3). Depletion of B-cells is thought to be responsible for lessened bone health and decreased fracture restoration, same as the lack of T cells (47, 48). As in the case of all immune cells, B-cells are known to produce cytokines, which participate, with their turn, in the inflammatory processes (58). The B-lymphocytes that encounter their respective antigens, respond by an increase in proliferation and subsequently differentiate to plasma cells, which are further capable to produce body-defending antibodies. Some B-lymphocytes differentiate into "memory B-cells" that are prepared to act in re-infection. B-cell biology is very complicated, since they consist of several cellular subsets with different functions (3).

In the case of bone fracture healing, B-lymphocytes and T-lymphocytes have been found to play a role at the end of the inflammatory phase, and are again activated during the mineralization phase (50). The two cell populations act in contradictory roles. This takes place at the later stages of the inflammatory phase. On one hand, T-cells produce RANKL, which recruits, differentiates and activates osteoclasts and on the other hand, B-cells suppress the pro-inflammatory signaling through the suppression of IFN-γ, TNF-α, and IL-2 (59). At the same time, B cells also produce OPG, regulating in that way osteoclastic differentiation and activity (60).

In a recent report by Raggatt *et al*. (2013), supported the theory that B-lymphocytes do not play an important role in bone fracture healing. This came from the finding that mice with B-cell deficiency and in particular, with a μ-chain deletion, manifested no hindrance in bone fracture healing (61). However, later studies have highlighted that during bone injury (fracture) B-lymphocytes increase at the injury site, as well as in the peripheral blood, where they release IL-10. Consequently, B-cell related IL-10 reduced production, has been found to be s associated with delayed fracture healing (4, 48, 50, 58, 62). Despite current knowledge advancements, the role of different B-cell subsets in bone regeneration, is still largely unknown and thus further research is required.

Natural Killer Cells

Natural killer cells (NK cells) are "hematopoietic cells of the lymphoid lineage" and their function in fracture healing is not fully known (1). NK-cells are responsible for the recognition of aberrant or "strange" cells, as well as virally infected cells and their subsequent extermination. NK-cells are able to induced target-cell apoptosis, as well as, cell lysis through cytotoxic granules (63). This function in fracture healing is possible to be expressed by removing damaged cells in the injury site, while it has been shown that conditions at the fracture site inhibit NK cell-based cell lysis (64). However, it appears that the vital role of NK-cells is the "clean-up" or "debridement" of the injured tissue. This is believed to take place through the ability of NK-cells to recruit other inflammatory cells, osteoclasts, as well as the release of IFN-γ and RANKL (65). Noteworthy, Furthermore, they are known to play a vital role in tissue regeneration, through the recruitment

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of mesenchymal progenitor cells during the later stages of fracture healing (66).

Pro-Inflammatory Cytokines

In case of bone injury, an early event taking place is the interruption of blood supply and platelet aggregation with the release of platelet-derived pro-inflammatory cytokines. Such cytokines include the presence of IL-6, IL-1 and TNF-a (5). In addition, the early stages of fracture, include the formation of a hematoma, which aggregates inflammatory cells that further produce pro-inflammatory inflammatory cytokines and growth factors. The formation of this hematoma is crucial and its removal causes a defective bone healing (5, 67, 68).

Interleukin IL-6

A major player of inflammation is IL-6. It is released by numerous cells, as well as receptors for IL-6 are found on an additional abundance of cells. Numerous reports have highlighted the role of IL-6, which is found to exert multiple effects, as well as it is believed to stimulate osteoblastogenesis (69-71). IL-6 functions in a temporal pattern, as it is released within the first 24 hours after the injury (the bone fracture) promoting osteoblastogenesis through the differentiation of mesenchymal cells (including cells in the bone repair site). Thus, it is believed that IL-6 depletion is linked to healing impairment, which yet is compensated by the release of complementary factors that counterbalance IL-6 deficiency (3, 72-74).

Tumor Necrosis Factor (TNF)-a

The multi-purpose cytokine TNF-α, is mainly released by activated macrophages and is able to bind to TNFR1 and TNFR2 in order to activate downstream signal transduction (3, 75). The role of TNF-α is crucial to bone physiology, since animal models have shown that aberrant expression of the cytokine is linked to chronic inflammatory poly-arthritis (76, 77). The exact mechanism of arthritis induction by TNF-α is still unknown, yet it is believed that induces a Wnt signaling inhibitor, in synovial fibroblasts, and thus suppresses bone formation in the arthritic joint. On the other hand, it is shown that TNF-α promotes bone restructuring in cases of fracture (78, 79). Interestingly, *in vitro* experiments, in which mesenchymal cells treated with TNF-α, manifested contradictory results, as in some cases TNF-α promoted and in other inhibited bone formation. These distinct functions bring up the role of bone fracture microenvironment, as it appears that it regulates TNF-α functions (3).

Interleukin IL-17A

Initially, IL-17A was known to be produced by Th17 cells and later it has been found that they are also produced by the CD8+ T-cells, $T_{\gamma\delta}$-cells, invariant NK T-cells, NK cells, Lymphoid Tissue inducer (LTi) cells, B-cells, and mesenchymal cells (80-84). The role of IL-17A, in bone physiology, has been found that is linked to inflammatory bone loss in erosive arthritis, as well as it has been found that it sustains the body physiology against bacteria and fungi by stimulating the release of antimicrobial peptides and neutrophils recruitment (3). Furthermore, several studies have reported that IL-17A promotes bone regeneration, as well as it has been used for therapeutic purposes, since anti-IL-17A antibody is effective against arthritis (85, 86).

In the case of bone fractures, IL-17A has been found to also play an important part. In particular, it has been reported that IL-17A is activated during the "early phase" of bone fracture healing and it is mostly released by $T_{\gamma\delta}$-cells (25, 56). In addition, it is reported that IL-17A promotes the mesenchymal cells proliferation on the injury site, and stimulates their differentiation to osteoblasts (56). As aforementioned, since IL-17A is released at the time-point immediately after the injury, it is believed that it is a cytokine that regulates the early, primary osteoblastogenesis (3).

Osteoclasts

Osteoclasts are "multinucleated cells of the myeloid lineage" and they can differentiate directly from monocytes, as well as from macrophages (87). Although, osteoclasts were initially believed to be strictly bone-related cells and thus not immune-related cells, recent findings have shown that they play a major role in the immune response. In par-

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ticular, they are thought to act as indirect mediators of immunity, since they are able to release factors that can activate the innate immunity, but also inflammatory signals lead to their differentiation and activation (1, 35, 88). Their main role in bone physiology is that of "bone phagocyte". This name is derived from their specialized property and ability to resorb bone matrix (89).

The primary mechanism of osteoclast function, comes from the differentiation of monocytes, which are assembled to the fracture-site, differentiating to osteoclasts. Osteoclasts are primarily activated by RANKL (1, 35), whose primary source are the osteoblasts. Besides osteoblasts as sources of RANKL, NK-cells and activated T-cells are also able to produce RANKL during fractures. Further on, another molecule that participates in osteoclast activation is osteoprotegerin (OPG). OPG is a "decoy" receptor, which binds to RANK and inhibits RANKL binding, thus preventing osteoclast activation. OPG is released by osteoblasts and B-cells during fracture healing (1, 35).

Conclusions

It has become evident that the immune system plays a critical role in the process of bone fracture healing and restructuring. The process of bone fracture healing includes three main phases. These are epigrammatically; Inflammation, repair and remodeling. During the stage of inflammation, which is considered crucial, the mechanisms of innate and adaptive immunity, along with the respective cells assist to the process of bone debris removal, anti-sepsis and preparation of the microenvironment for the upcoming event of mesenchymal differentiation. The stage of inflammation involves the interaction of macrophage/osteoclast lineages, both contributing to the phase of inflammation. During the next phase, the repair phase, the multi-potential stromal cells, are taking charge in order to start bone repair. Multi-potential stromal cells, are cells possessing a trait of adherence capacity, which express surface molecules such as CD90, CD73, CD105, but not hematopoietic lineage markers. Multi-potential stromal cells, are the ones that are able to differentiate into bone, fat and cartilage cells, where in the case of bone healing they differentiate to chondrocytes (5, 90).

Although, initially it was thought that immune cells participate during the phase of inflammation in the bone healing process, later studies have shown that they are also active during the repair phase. Immune cells, during the repair phase, function as mediators of mesenchymal differentiation, as well as they play an important role to convert "soft *callus*" into "hard *callus*". Another responsibility of immune cells is the assistance to the formation of vasculogenesis in the injured bone. Finally, it has been shown that the mesenchymal stromal cells, active during the repair phase, mediate the function and interactions of osteocytes and in particular, the interaction between osteoclasts and osteoblasts. Cells revealed to play such a role, have been reported to be the macrophages and probably the Th17 lymphocytes (5).

In conclusion, immunity is the first biological machinery implicated during injury and in particular, during bone injury and damage. Immuno-cells, play a significant role as they, restore vasculature, initiate signaling cascades and recruit cells to complete the repair processes. Thus, immune system can be considered to be a potential therapeutic target for bone fracture healing. However, the exact mechanisms on the interactions between bone physiology, homeostasis and the immune still are still largely unknown. Intense research is already at play, which is required in order to better appreciate the biological significance of immune cells in bone regeneration.

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