Chapter 9

The Role of Immune Reactivity in Bone Regeneration

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Additional information is available at the end of the chapter

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Abstract

Bone is a complex organ with the capacity to regenerate. Even with this healing potential, healing results in fractured bone are unsatisfactory in a considerable patient cohort even with a good treatment regimen. These delayed healing cases encourage further research into possible new treatment approaches. The recently developed field of osteoimmunology addressing the tight interconnectivity of the skeletal system and the immune system could be a promising opportunity in this regard. In this review, the complexity of bone and the bone healing process are highlighted with an emphasis on the early healing phase. Specific immune cell subsets are considered for their potential to enhance bone healing and thus to develop new treatment strategies for patients in need.

Keywords: Regeneration, Fracture, Immune system, Inflammatory reaction, Healing

1. Introduction

1.1. Fracture Incidences

Bone injuries are frequent occurrences in daily life. Considering Germany as an example for a country with a health system guaranteeing treatment for fracture patients at a high standard, fractures of the extremities ranged between 560,000 and 640,000 cases per year over the past 10 years, with around 150,000 fractures of the femur and tibia, respectively (**Figure 1**). The statistical federal ministry recorded 802,662 fractures in Germany in the year 2014 (Statistisches Bundesamt, Wiesbaden, 2016-01-11). These numbers can be split up even further by age, where 38% of the patients with fractures of the extremities were older than 75 years, 33% between the age of 50 and 75 years, 16% between 25 and 50 years, and only 13% were younger than 25 years (**Figure 2A, B**).

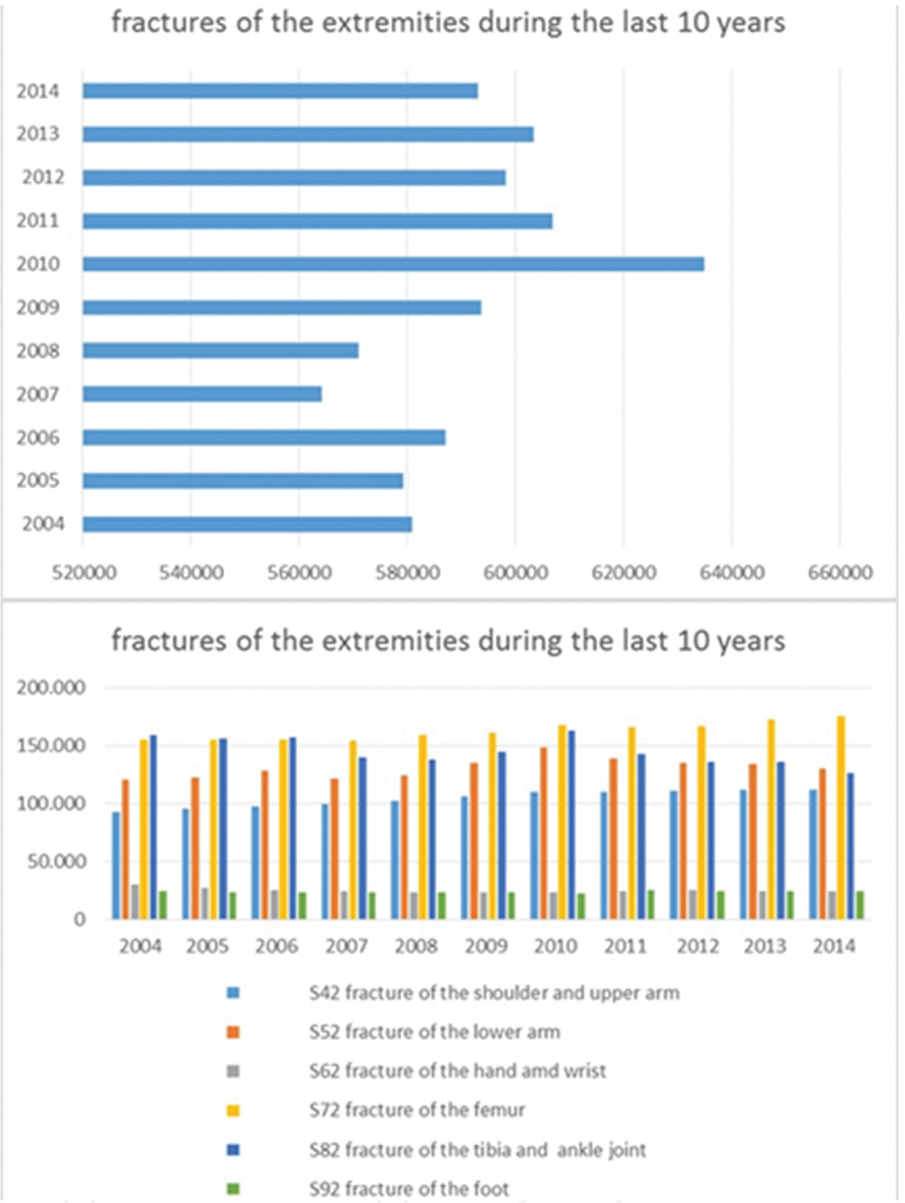

Figure 1. Fracture incidence in Germany (Gesundheitsberichterstattung des Bundes, 2016-01-11)—fractures of hand, arm, shoulder, leg and foot—incidence for 2004–2014.

Even in an environment with a good healthcare system and the normally very good healing potential of bone, 10–20% of all fracture patients still experience a delayed or nonunion after osseous injury [1–3] (**Figure 2C**). To overcome these delays in healing or reduce the nonhealing ratio, further research to gain understanding on the causes of healing delay or lack of healing is essential to enable new treatment strategies that support bone regeneration even under compromised conditions. With respect to the development of our population, the research into fracture treatment strategies becomes even more important as demography predicts an aging of the population. In Europe, it is Germany with the highest percentage of people over 65 years of age, and this percentage is rising (**Figure 2A**). In 1990, about 15% of the Germans were older than 65 years, and in 2011, this percentage had grown to 21% of people being over 65 years

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old (Statistisches Bundesamt, Eurostat 2011). This is important because the fracture incidence is higher in elderly people (**Figure 2B**). The demographic projection of the UN World Population Projections for the years up to 2025 foresees an increase of over 50-year-old people of 20%, which equals 219 million people in 2025. Further stratifying this by age groups, the highest growth of 32% is expected for people aged 80 years or older. Consequently, the fracture incidence in elderly will increase by 28% of the 4.5 million fractures estimated for 2025. With this high number of fracture patients with an advanced age, it is eminent to consider age-related alterations that might influence the capacity of osseous tissue to regenerate normally. With increasing age, it is the immune system that undergoes major transformation influencing bone regeneration considerably. To provide adequate treatment options, it is essential to unravel the interactions of the immune and skeletal system.

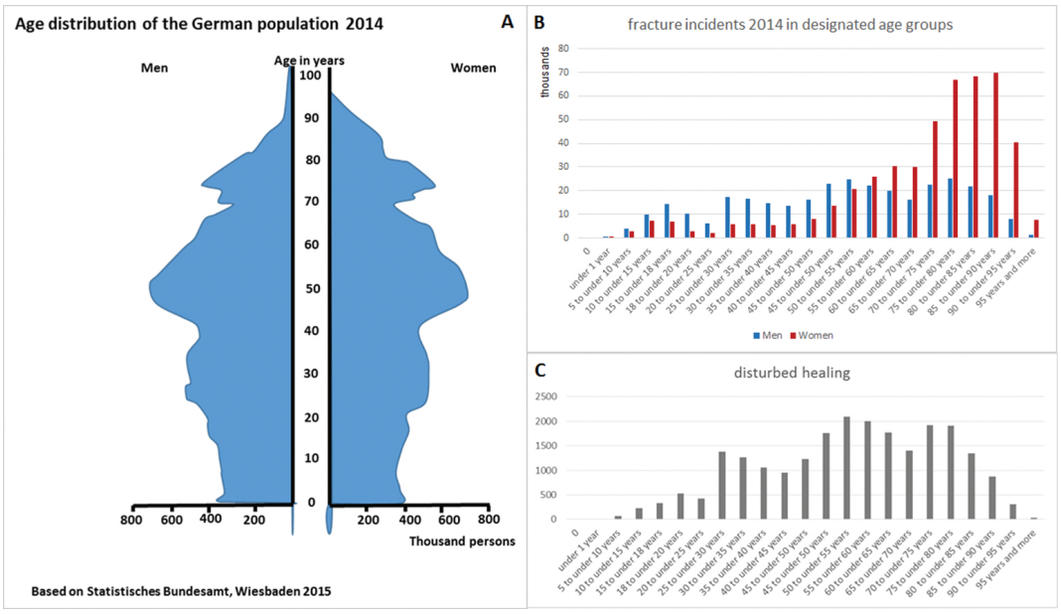

Figure 2. (A) Age distribution in Germany 2014 and (B) fracture incidence according to designated age groups. (C) Unsatisfactory healing results in fracture patients in corresponding age groups are shown, this includes malalignment, delayed healing and pseudarthrosis (nonunion) (M84 classification) (based on Statistisches Bundesamt, Wiesbaden 2016).

1.2. Primary and secondary healing

Bone is a remarkable organ because it is capable of regeneration and complete restoration of the osseous integrity both in form and function. Bone repair and fracture healing are unique because they recapitulate many of the ontological events that occur during the embryological development of the skeleton [4, 5]. To reach the "restitutio ad integrum," bone provides two mechanisms of scarless healing and regeneration: primary and secondary bone healing. Primary bone healing is only possible when the bone fragments are realigned anatomically, and the fracture zone is held under compression by an adequate fixation without a gap between

the bony ends (**Figure 3A**). Stable fixation and no relative movement are required when basic multicellular units consisting of cutting cones with osteoclasts and following bone-forming osteoblasts cross the fracture line to directly rebuild bone and thus re-establishing the osseous integrity at the fracture side [6, 7]. During this process, the new bone is directly organized as osteons and oriented along the dominant mechanical loading direction [8, 9]. Primary bone healing was for a long time considered as the best possible healing process and thus was the aim when fractured bone was clinically treated [10].

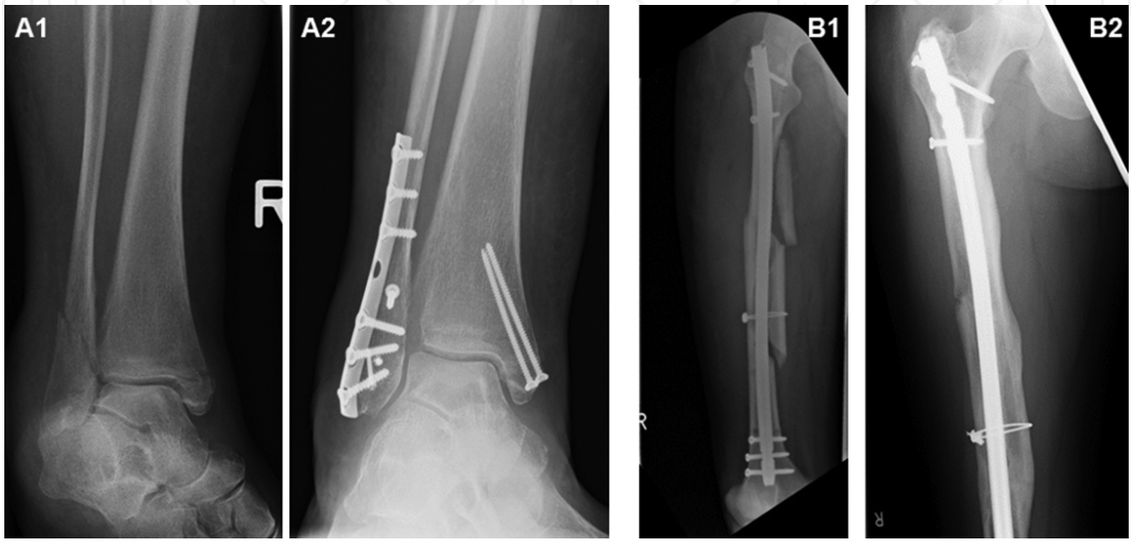

Figure 3. X-ray images from fracture patients: (A) fracture treated with an open reposition and internal fixation (ORIP) procedure with correct anatomical reconstruction of the fracture ends without fracture gap consistency—the bone will heal without callus formation through primary bone healing. (B) Comminuted fracture treated with an internal nail. Several gaps between the fractured bone ends remain and healing takes place by secondary bone healing as the callus visible in the image B2 taken 3 months after treatment clearly shows.

Secondary bone healing occurs whenever a gap persists between the fractured ends or when there is instability and thus interfragmentary movement (**Figure 3B**). This for example is the case if anatomical repositioning is not possible due to comminuted fractures or large bone defects. In secondary bone healing, a substitute tissue is formed to regain stability as fast as possible: an intermediate cartilage callus ensues. While intramembranous bone formation starts to consolidate the injured bone in the periosteal regions of the fracture gap, endochondral ossification processes start with the formation of cartilage islands in the gap between the fracture ends, forming an intermediate soft callus. Cartilage mineralization starts the woven bone formation process, which results in a hard callus. The final remodeling then restores the form of the continuous bone [11]. The intermediate cartilage step that provides a fast regaining of stability and reduces any interfragmentary movements often has a larger diameter than the original bone, especially if, as it would occur in nature, the bone remains untreated. It provides an increased polar moment of inertia against torsion and also withstands bending loads [12, 13]. While the large callus provides an evolutionary advantage to quickly regain mobility, it can be prevented in clinical settings by a stable fixation of the fractured bone [14].

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1.3. Fracture treatment

In the wild, a fractured long bone often leads to death of the injured animal. However, it seems that the younger the animal is when the fracture occurs, the higher are the chances of survival [15]. If an animal survives a long bone fracture, the bones most likely heals with a severe misalignment. The potent remodeling capacity of the bones will however strive to restore the mechanically defined form of the bone, which is dictated by the surface strains the bone sense during physiological activities.

In our society, most fractures are treated in such an efficient way that only in rare cases bone fractures lead to death. Fracture treatment in the form of stabilizing the fractured bone goes back at least to 2400 years before Christ as excavated mummies from an Egyptian tomb proved. Prof. G. Elliott Smith discovered the splintered bones during the Hearst Egyptian expedition at Naga-ed-Der in 1903 on two mummies [16]. Both died shortly after the fracture because no healing signs were observed on the bones even though the Egyptians seemed to have reached some proficiency in fracture treatment as other relicts with healed fractures, found later on, could prove. In most cases, healed femoral fractures showed limb shortening or deformation, whereas forearm fractures healed well, demonstrating the challenge of reestablishing weight bearing capacity with the fracture treatment. An Arab surgeon, El Zahrawi (936–1013 AD) described in his treatise "The Surgery" a splinting technique, which was used for a long time, consisting of several layers of bandages combined with splints to provide stability for the fractured limb [17]—a fracture treatment also described by Hippocrates and Celsus [18] and one that is to an extend still valid today.

In the early 1770, first records on internal fracture fixation using ligatures or wire fixation are reported from France [19]. This was followed by the introduction of screws around 1850, again in France [20], and the development of plate fixation reported in 1886 by Hansmann [21] of Hamburg.

Robert Danis (1880–1962) furthered the development of the concept of internal fixation to permit functional rehabilitation. He stated that an osteosynthesis is not entirely successful until it provides immediate mobilization, complete restoration of the form of the bone, and enables primary bone healing without the formation of a callus. This thesis was published in "Danis R.: *Théorie et Pratique de l'Ostéosynthèse*, Paris, Masson, 1949". Between the 15th and 17th of March 1958, a number of orthopedics met in the Kantonsspital of Chur and based on the work of Danis they formulated a number of papers on osteosynthesis and thus the AO—Arbeitsgemeinschaft für Osteosynthesefragen—was founded. The AO has continued to improve the principles of fracture treatment since then and is still a renowned entity in the orthopedic community.

Even with these tremendous progresses in fracture treatment, there are still several open questions concerning the treatment regimen: mal-fixation with too stable or too unstable fixation [22–25], critical gap size [26, 27], a deficit in angiogenesis together with the formation of atrophic pseudarthrosis [28–31], and deficits in the control of the inflammatory cascades [32–34] are challenging clinical situations that still lead to unsatisfactory healing results for patients and surgeons as well.

2. Immune cells and bone regeneration

2.1. Bone – a complex organ

Bone is not simply a hard nonorganic material that functions as an anchor for muscles and tendons providing stability and form for our bodies and enabling movement through the interplay of our musculoskeletal system; it is also protecting vital organs, such as the brain, lungs, and heart, and it is a living organ regulating homeostasis. Additionally, it is an organ that is essential for our immune system, as these cells arise and/or mature from stem cells in the bone marrow, it is also an organ that interacts with our hormonal balance through a multitude of factors, including the hormone osteocalcin [35], and acts as a storage not only for calcium, phosphate, and magnesium but also for growth factors, as for example transforming growth factor-β (TGF-β).

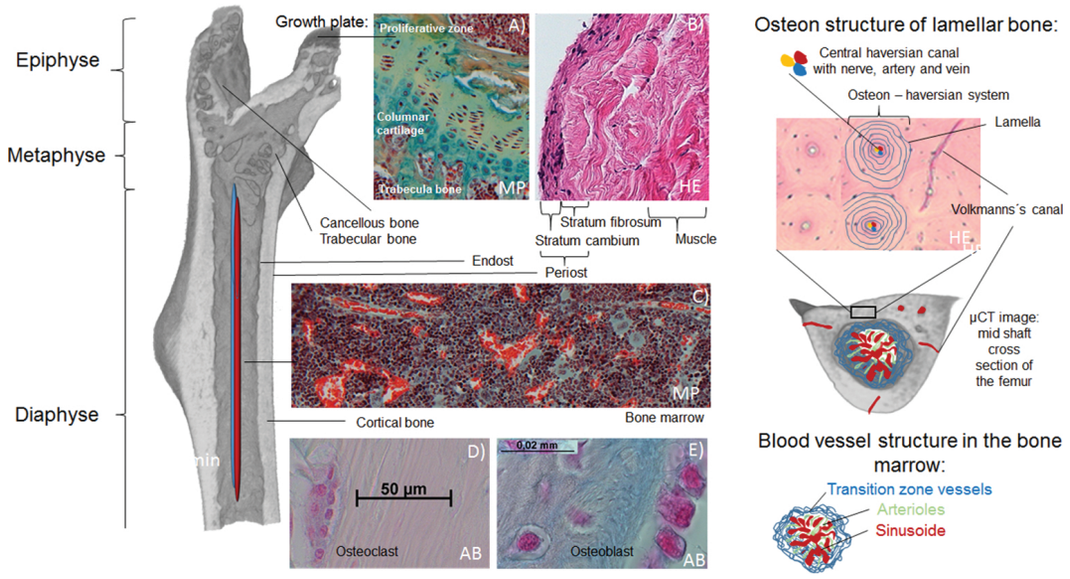

Figure 4. Bone is a complex organ. A long bone can be divided into epi-, meta-, and diaphyseal regions. The epiphyseal region contains the growth plate—the region of length growth of the bone. The epiphyseal zone is broad in young individuals and diminishes with age. Details are shown in a histological image where the transition from cartilage to trabecular bone is shown (A). Bone building cells are the osteoblasts. On the bone surface, they are arranged in palisade formation while synthesizing new bone matrix, the osteoid. They mature while they encase themselves in osteoid and finally mineralized bone matrix and become osteocytes (E). Osteoclasts on the other hand degrade bone; they are large multinucleated cells with a ruffled border directed at the bone surface (D). To emphasize the size difference, scale bars are enclosed in the image of the osteoclast and osteoblast. The bone marrow cavity is filled with bone marrow cells and a network of vessels (C). The vessel structure is explained more in detail with a cross section of long bone on the right-hand side. The cortical bone is covered by the endosteum on the inside and the periosteum on the outside. The periost is a rich source of cells, which are located in the stratum cambium (indicated in the histological out take), here visible by their dark nuclei. The stratum fibrosum covers the stratum cambium and is followed by a fascia and muscle closely adjacent to the bone (B). **Blood vessel structure in the bone marrow**: The bone is highly vascularized, next to a central vein and an artery system of sinusoids, arterials and transient zone vessels pervade the bone marrow cavity as indicated in the cross section of the bone on the right. **Osteon structure of lamellar bone**: The histological out take of the cross section shows the osteon structure of lamellar bone with its Haversian system. The bones are depicted as µCT 3D reconstruction images of mouse femura. Histological stainings are HE, hematoxylin eosin; MP, Movat pentachrome; and Ab, Alcian blue on paraffin- or plastic-embedded sections of long bone samples of mouse and sheep.

Bone healing is a complex process that involves a variety of different cells and signaling molecules, which originate not only from the bone, and here specifically from the periosteum, the cortical or cancellous bone, the endosteum and the bone marrow, but also from surrounding muscle tissue (**Figure 4**). An important supplier for cells and signals is the vasculature and thus the blood as a carrier. Bone is a very well-vascularized organ. Osteons are tube-shaped structures within the bone with an open space for blood vessels, veins, and nerves in the center. Small capillaries are found in the bone marrow near the endosteum, which continue into arterioles and sinusoids (with fenestrated basal membranes) towards the center where a large artery and central sinusoid transverse longitudinally through the bone marrow space [36]. Through the vessel connectivity, any osseous injury is prone to be influenced by systemic effects and vice versa to influence the systemic homeostasis. For example, the callus formation of injured bone is heightened in patients with traumatic brain injury. In this case, systemic changes caused by the brain injury influence the bone healing, most likely due to a competition for nutrients between the two injury sites and an altered hormone homeostasis [37, 38]. Another systemic effect that is most likely communicated to the bone is a change in the inflammatory state of an injured person—a higher systemic inflammatory reactivity will disturb the bone healing process and prolong the healing time necessary to achieve bridging [39]. Upon fracture, the vascular system of the bone is disrupted at the injury site, and it is imperative that revascularization swiftly occurs in order for a successful healing process. Tissue formation relies on the supply through the vasculature with oxygen, nutrients, signaling molecules and cells [29, 31, 40–42]. Restoration of the vasculature also enables cell recruitment of circulating regenerative cells towards the fracture site [41–44].

The cells partaking in the bone healing process do not only originate from the bone itself, but they also migrate out of different cell sources, which contribute finally to the healing process. A rich cell source for cells contributing to bone healing after injury is the periosteum as well as the bone marrow from where cells are attracted to migrate towards the injury site [45–47]. The muscle surrounding the fractured bone is also a valuable source for growth factors and stem cells, promoting revascularization and thus the bone healing process [48].

On analyzing bone healing, it is important to keep in mind that there are several different compartments involved, including the bone itself, the medullary cavity, the surrounding muscle and connective tissue, the blood supply, the metabolism, and the immune system.

2.2. Fracture healing

The fracture healing process itself is a strictly controlled complex process composed of consecutive and partly overlapping phases, which progress towards rebuilding bone integrity in form and function. Different cell types (immune cells, progenitor cells, and mesenchymal cells) [11] and their signaling molecules (cytokines, growth factors, and chemokines) [49] are partaking during a successful regenerative process.

Several growth factors involved in the healing cascade are currently under investigation to develop new therapeutic approaches to enhance bone healing: fibroblast growth factor [50], insulin-like growth factor [51], platelet-derived growth factor [52], transforming growth factor-β [53], vascular endothelial growth factor [50], and growth and differentiation factor 5 [54,

55]. However, the only growth factors so far clinically applied to further bone healing are bone morphogenetic protein 2 and 7 [56, 57].

The bone healing process can be roughly divided according to the healing steps into an inflammatory phase, a soft callus phase, and a hard callus phase (**Figure 5**). Upon closer observation, however, it becomes apparent that the healing process is more complicated than that. A more in-depth sequence of the healing cascade would be hematoma phase, proinflammatory phase, hypoxic phase, anti-inflammatory phase, revascularization phase, organized connective tissue phase, cartilage phase, hypertrophic cartilage phase, revascularization phase, cartilage mineralization phase, woven bone formation phase and remodeling phase [58].

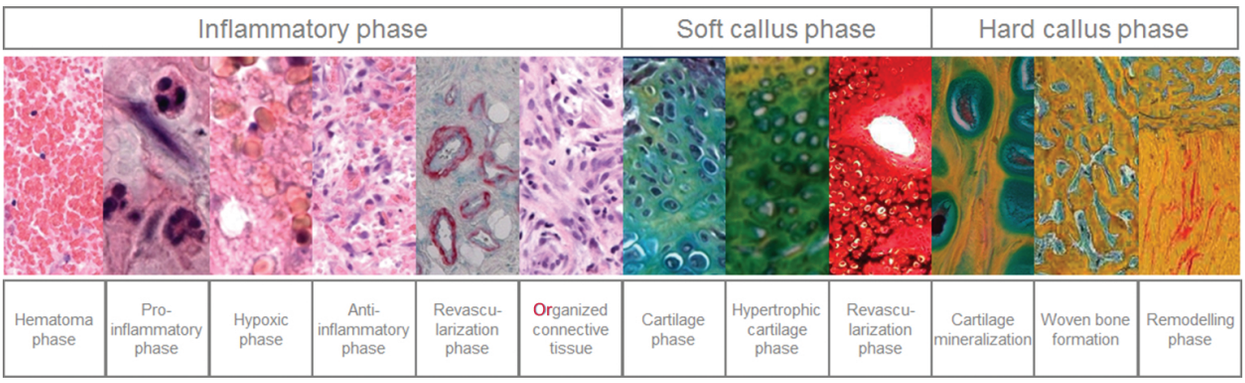

Figure 5. Fracture healing cascade: On closer examination, the inflammatory phase can be divided into at least six consecutive and partly overlapping phases showing the transition from the hematoma (red blood cells with some lymphocytes with dark stained nuclei) towards fibrocytes in the organized connective tissue (hematoxylin–eosin staining, different magnifications and an immunohistological staining for alpha smooth muscle for the revascularization phase). Soft callus phase can be divided into three phases (Movat pentachrome staining and Safranin van Kossa staining for the revascularization). The hard callus phase is divided into cartilage mineralization, woven bone formation and remodeling (Movat pentachrome staining).

Due to the complexity of the bone healing cascade with the multitude of different cell types involved and the plethora of tightly interacting and simultaneously highly controlled signaling molecules aiming to rebuild an organ consisting of periosteum, cortical bone, endosteum, and bone marrow in a way that optimally withstands the ruling mechanical strains, the process of bone regeneration is so far not understood. Therefore, research is compelled to use heuristic approaches to gain a more in-depth understanding and in conclusion develop new treatment approaches for patients in need.

2.3. Osteoimmunology

For a long time, bone homeostasis was explained with the balanced interaction of bone-forming osteoblasts and bone resorbing osteoclasts (**Figure 4**), however, this simple concept has changed. The interconnectivity of the skeletal system and the immune system has come into the focus of current research, consecutively leading to the founding of the new research field of "osteoimmunology." This new research field aims to elucidate the complex interactions

between these two systems in health and disease and already more and more knowledge has accumulated [59–63], enabling us to consider new treatment possibilities for regeneration in general and also specifically for bone [64]. The opportunity to control the inflammatory cascade to stimulate successful bone healing has now been confirmed [32–34, 65].

Both cell systems, the skeletal system and the immune system, originate in the bone marrow. They share progenitor cells (e.g. osteoclasts/macrophages) and signaling pathways, and due to their colocalization, which often cross react with each other. This is apparent for example when considering the RANK/RANKL/OPG system, the system controlling osteoclast differentiation/activity and thus bone resorption. Activated T cells and osteoblasts are able to express the membrane-bound and the soluble form of RANKL (receptor activator of nuclear factor kappa-B ligand) promoting osteoclastogenesis. B cells and osteoblasts produce and secrete OPG (osteoprotegerin), a decoy receptor blocking the RANK-RANKL ligation, thus inhibiting osteoclastogenesis [59, 62, 66]. This example illustrates that immune cells are involved in bone homeostatic processes directing either bone resorption or bone apposition.

Due to the interdependency of the two systems, any considered treatment option of immune modulation must take into account that by affecting the immune system the skeletal systems could also be targeted unintentionally.

2.4. The initial inflammatory phase

Vessels are disrupted and bleeding occurs upon injury and the fracturing of bone. The infiltrating blood coagulates and forms the initial hematoma in the fracture gap. The formation of a fracture hematoma in the early healing phase is an indispensable step for successful healing because it develops an angiogenic and osteogenic potential [29, 67]. The removal of the early fracture hematoma can delay bone healing as it has been demonstrated in animal studies, where the transplantation of a fracture hematoma can lead to ectopic bone formation [68, 69], demonstrating its osteogenic potential. The coagulation process and a simultaneous proinflammatory reaction are phylogenetically connected [70]. During evolution, the closure of a breached outer shell and the defense against possible pathogenic intruders were performed by one cell, the amebocytes, capable of clotting and a defensive immune response. This connection has survived evolutionary diversification of the clotting system and the immune system—both reactions still occur simultaneously upon bleeding. The amebocytes can still be found today in living fossils, such as the horse shoe crab [70]. Their immune response is so potent that it is used to monitor endotoxin levels within solutions by pharmaceutical companies. The limulus amebocyte lysate (LAL) test is capable of detecting contaminations as low as one part per trillion [71]. In evolutionary younger organisms, this highly effective immune cell is being replaced by a whole array of immune cells, which can be divided into an innate immunity and an adapted immunity, the latter is only found in vertebrates (**Figure 6**). Each of these is composed of various different cells: macrophages, neutrophils/granulocytes, mast cells, natural killer cells, dendritic cells and the complement system belong to the innate immune system, whereas T and B cells and the humoral immunity belong to the adaptive immune system. The cells of the adaptive immune system provide their host with a long lasting and protective immunity by maturing from naïve T and B cells to effector cells, when they

come in contact with their cognate antigen, and in some cases to memory cells, which allow a rapid immune response upon recurrent infection with an antigen previously encountered by the host. It has to be pointed out that the immune system is not only a barrier for extracellular microbes but also a regulatory system for body homeostasis. The immune system senses alteration in the environment, for instance damaged or aged cells [72, 73], expressing Toll-like receptors and other pattern-recognition receptors (PRRs).

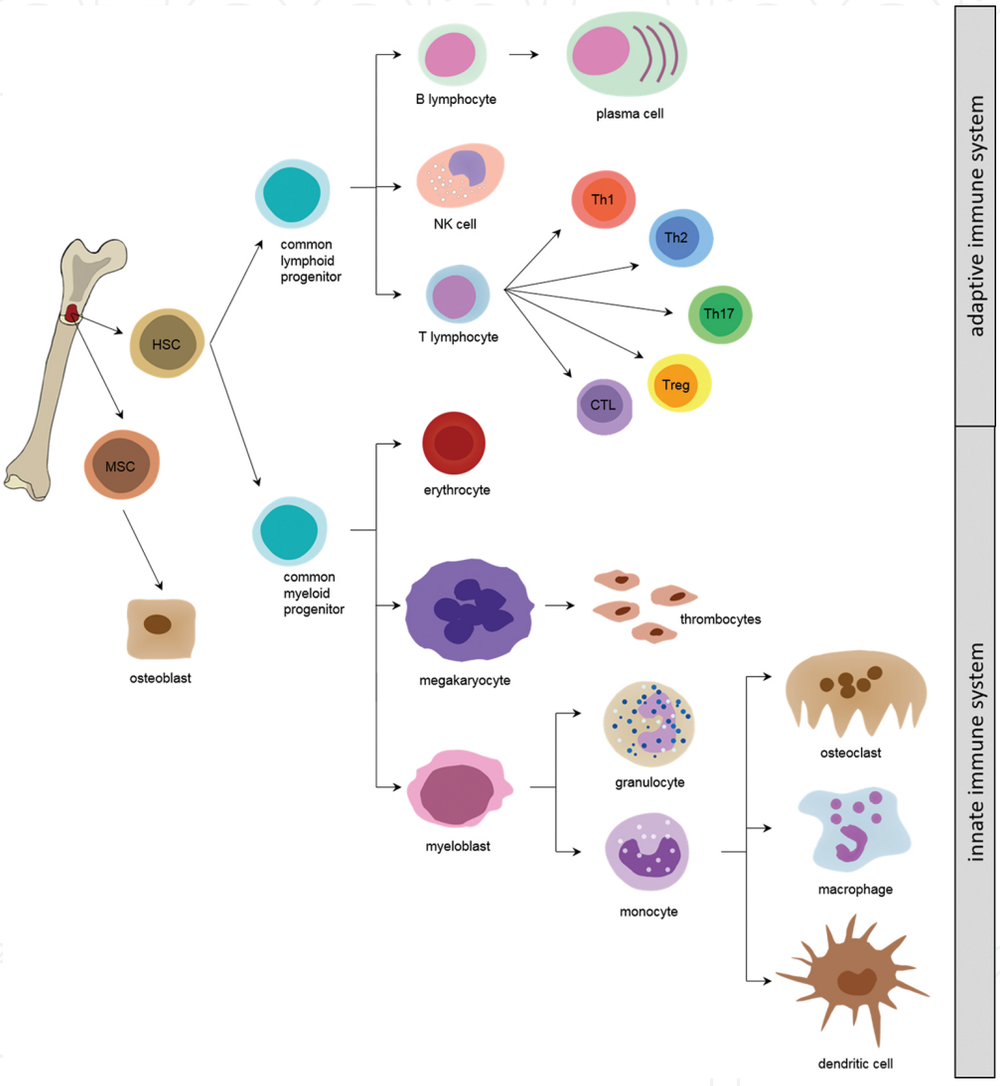

Figure 6. Diversity of cells of the immune system. Cells from the bone marrow give rise to the immune cells of the innate and adaptive immune system and also to the osteoblasts and osteoclasts of the skeletal system.

During fracture healing, both the cells of the innate and the adaptive immunity are involved, and immune cells play essential roles during all the fracture healing phases [74–77]. The initial inflammatory reaction ensuing upon hematoma formation initiates the healing cascade and thus can significantly affect the healing outcome [33, 34]. This initial inflammatory reaction is characteristic for bone, tightly controlled and different from other tissue healing with scar formation [32]. In fracture repair, the anti-inflammatory signaling is up-regulated between 24

and 36 hours after injury to terminate the proinflammatory reaction needed to attract necessary cells to the injury side [32, 33]. In parallel, the angiogenic signaling is up-regulated to initiate the essential revascularization process. The timely down-regulation of the initial proinflammatory reaction has been shown to be important as a prolonged proinflammatory reaction delays the bone healing process [29, 33].

The complexity of the initial immune reaction becomes even more apparent when considering cytokines expressed by immune cells during the different stages of the bone healing cascade. Tumor necrosis factor-α (TNF-α) has been reported to peak 24 hours after injury and return to baseline levels afterwards. During the remodeling phase, TNF-α shows a second expression peak during normal bone healing [64]. It is suggested that the first wave is due to activated tissue-resident cells, like macrophages, triggered through PRRs, and the second wave directly and indirectly by activated T cells. Looking closer into the role of this factor during bone healing is has been shown that too little, but also too much TNF-α leads to a delay in bone healing [78–80]. This demonstrates that the cytokine pattern has to be tightly controlled during the regenerative healing cascade to lead to a satisfactory healing outcome. Interleukin (IL)-17 is another cytokine that has been acknowledged to influence bone formation. On one hand, this cytokine has been reported to enable osteoblast formation [81], thus supporting bone formation; on the other hand, in the context of osteoporosis treatment, evidence occurred that IL-17 furthered osteoclastogenesis [82], thus supporting bone degradation. Contradictory reports can also be found for IL-6, which enhances fracture healing [83, 84] but reduces the mechanical strength of noninjured bone [85]. The microenvironment seems to be highly important for determination of the effect the cytokines have on the bone healing process, a fact that indicates the difficulties in using inflammatory cytokines to improve bone healing. The balanced immune response is highly important for a successful bone regenerative cascade [32, 33, 67].

Upon injury and disruption of the blood vessels, the nutrient and oxygen supply as well as the transport of metabolic waste is interrupted. The early tissue in the fracture gap consisting of the hematoma becomes hypoxic because oxygen is no longer provided by the vasculature. Therefore, cells trapped in the hematoma have to switch towards an anaerobic energy supply. The use of the remaining glucose in glycolysis to produce adenosine triphosphate (ATP), the energy molecule of the cellular metabolism, without the consecutive citrate cycle, results in lactate, an acid that consecutively lowers the pH value during the initial healing phase. Simultaneously, the sodium and potassium concentrations rise. These conditions present a milieu that is difficult for some cells, such as progenitor cells [86]. However, innate immune cells are well equipped to deal with these conditions and thus can be seen as the first responders to an injury. They express a range of cytokines that attract scavenger cells to clear the detritus that ensued upon tissue disruption and also direct the cells needed for the regenerative process towards the injury side. They readily switch from an aerobic energy supply towards an anaerobic and are often activated upon injury. Not only macrophages but also some T cell subsets are the most important actors during this first response [87, 88]. Hypoxia is a strong inducer of hypoxia inducible factor 1α (HIF1α), a transcription factor that is important for revascularization, cell migration, energy metabolism and growth factor expression, and therefore involved in the regenerative bone healing cascade [89]. HIF1α is expressed by most

innate and adaptive immune cells, including macrophages and lymphocytes; they stabilize HIF1α and are being influenced by HIF1α in their immune cell function [90].

The swift up-regulation of a proinflammatory reaction upon injury activates immune cells, which are capable to withstand the unfavorable environment and initiate the healing cascade through a very specific and highly controlled release of cytokines. Hypoxia is an important trigger for the transcription factor HIF1α that in turn initiates gene expression to instigate revascularization. For this process to succeed, effective anti-inflammatory signaling has to begin to terminate the initial proinflammatory reaction. During this initial phase, the track for a successful healing is thus determined, and it becomes apparent that a skewed first reaction leads to a delayed healing by consecutively retarding the following healing steps.

2.5. Challenging immune constraints

The interdependency of the immune and skeletal system indicates that there is a change in the interaction as the immune system changes with the advancement of age. Due to the memory function of the adaptive immunity in vertebrates, the naïve T and B cell population diminishes upon aging, whereas the compartment of memory T and B cells grows. More and more lymphocytes encounter their antigens and the library of known pathogens enlarges. Recent studies could show that CD8 positive terminally differentiated memory and effector cells (CD8+ T_{EMRA} cells) have a negative impact on bone healing and osteogenic differentiation of stem cells [91, 92]. Elderly people with a longer exposure time to antigens thus are prone to experience delayed healing.

Mice, a common laboratory animal to investigate bone healing, are mostly kept under sterile conditions. If these animals are housed under less sterile conditions, their immune cell composition changes so that after 4 weeks of semi-sterile housing the percentage of memory and effector (CD8+) T cells was markedly enhanced. If bone healing is compared between sterile raised mice and those exposed mice, our group could show that the regenerative capacity was reduced [91, 93]. This is an important aspect that should be kept in mind during future research questions, which are analyzed in mice.

Nonsteroidal anti-inflammatory drugs (NSAIDs) offer pain relief and are commonly used also on fracture patients. As the name already indicates, these selective cyclooxygenase-2 (COX-2) inhibitors have anti-inflammatory functions. After reviewing the importance of the initial inflammatory reaction, the question arises whether this pain medication could delay fracture healing or not. Indeed there are numerous reports that state that NSAIDs delay healing [94–98]. The effect, however, depends on the dose and time frame of application and seems to be more pronounced in older nonselective anti–COX-2 agents [99]. Clinically, NSAIDs are a valuable alternative to opioids (painkillers directly addressing the nervous system) and still remain in use also in fracture patients for short-term pain relief.

Several diseases have also been reported to delay bone healing through a changed immune response. Diabetic-related delay of fracture healing has been linked to higher TNF-α levels [100]. A weakened immune response in diabetic patients results in a dampened chemotactic function and defective macrophage activity—two factors that are needed in a successful bone

healing cascade [101]. A systemic disease with a high impact on the immune system is human immunodeficiency virus (HIV), and these patients have a bone phenotype with a high prevalence of osteoporosis and fragility fractures [102]. The impact on fracture healing, however, is unclear and difficult to determine due to the highly active antiretroviral therapy that these patients receive [102, 103]. Transplant patients receiving severe immune suppressive medication also show a higher risk for fractures and delayed healing outcomes. In contrast to these examples – where the immune system is weakened – conditions where a patient has a heightened immune answer or is already in a chronic proinflammatory systemic state, such as rheumatoid and arthritis patients, the prolonged proinflammatory reaction can result in delays in fracture healing [104–106].

Currently, the patient's immune status is not being evaluated when a fracture treatment is considered. However, this could help in the future to stratify patients who would benefit from an immune modulatory intervention to prevent a delay in fracture healing. This would especially be true in elderly patients because being bed-ridden for longer periods of time enhances frailty considerably.

2.6. Specific immune cell subsets that have been identified as important players in the bone regenerative process

In fracture healing, immune cells from the innate immune system and from the adaptive immune system are involved with specific and essential roles. Main cell types of the adaptive immunity are B and T cells with highly specific antigen receptors. Another important aspect of the adaptive immune system is its memory that enables its fast reaction towards recurring pathogen invasion. Adaptive immune cells can be activated not only through their antigen receptors, but also probably more important for the bone healing process through signals released by the innate immune system. From the innate immune system, especially macrophages have been in the current focus of osteoimmunology.

2.6.1. Macrophages

Macrophages are an important part of the innate immune system; they are among the first responders in case of an injury. Not only do they prevent pathogen invasion, but they also help in clearing ensuing cell debris [107]. However, their role in bone healing is even more complex and even today we have not yet unraveled their participation completely. Tissue-resident macrophages have been determined as key players in the orchestration of the recovery process towards a re-establishment of tissue integrity [108]. It was only in 1992 that it was recovered that macrophages are capable of a phenotype change from a proinflammatory type towards a prohealing phenotype [109]. The proinflammatory phenotype is named M1 or classically activated macrophage, and the second phenotype is termed M2 or alternatively activated macrophage. Since then, these "M2" macrophages have been associated with the resolution of wound healing *in vivo* in chronic leg ulcers [110], atherosclerotic lesions [111], traumatic spinal cord injury [112] and inflammatory renal disease [113]. It turned out that the M2 population is more divers and therefore subclassifications have been introduced: M2a (anti-inflammatory), M2b (immune-regulatory) and M2c (remodeling) [114]. In bone healing,

the prominent macrophage phenotype during the initial phase is M1. Upon attenuating of the proinflammatory phase, the macrophage phenotype changes towards the M2 phenotype [77]. In a proof of concept study in mice, we were able to show that an induction of the M2 phenotype early in the fracture healing cascade can enhance bone healing [77].

2.6.2. Regulatory T cells

The T cell population is highly divers and probably pleiotropic as well as interchangeable. Among the T cells, there seem to be subpopulations supporting the fracture healing process and also other subpopulations, which have negative effects on the healing process. CD4+ and CD8+ T cell subsets have been addressed in this context. CD4+ T cells have been shown to increase osteogenic differentiation in human mesenchymal stem cell cultures in *in vitro* assays using their conditioned medium, whereas this effect was missing when observing CD8+ T cells [115]. The osteogenic effect of CD4+ T cells was further supported through their positive effects during wound healing [116], however without a more specific determination of the responsible CD4+ T cell subset. In later studies, regulatory T cells came more and more into the focus as a CD4+ T cell subset with positive effects on bone healing. Mice with an increased percentage of regulatory T cells showed higher bone mass and decreased bone resorption when compared to wild type mice [117, 118]. Regulatory T cells support osteoblast differentiation and have a negative impact on osteoclast differentiation and function [119]. In a skull defect model in mice, it was possible to enhance bone healing through the addition of regulatory T cells in combination with applied autologous bone graft [120]. Currently under investigation is the possibility of a direct interaction of regulatory T cells and bone-forming cells or their progenitor cells, the mesenchymal stromal/stem cells. This interaction is supported by the fact that mesenchymal stromal/stem cells, as osteoblast precursors, and regulatory T cells use similar suppression mechanisms for an immune response [121]. The direct interaction between regulatory T cells and bone-forming cells as well as mesenchymal stromal/stem cells could proceed through coordination of the CD39-CD73-(adenosine)-ADOR pathway. This purinergic signaling would potentiate the differentiation of mesenchymal stromal/stem cells and thus facilitate bone regeneration [122]. Another direct interaction between osteoblasts and regulatory T cells could be the induction of IDO (indoleamine 2,3-dioxygenase) and HO-1 (heme oxygenase-1) by regulatory T cells [123] or the fact that regulatory T cells can inhibit CD40L and thus regulating the RANKL-OPG balance in favor of osteoblast differentiation [124].

2.6.3. T helper 17 cells

The lead cytokine expressed by Th17 (T helper 17) is IL-17. The dual effect of IL-17 on osteoclasts and osteoblasts has been mentioned before. However, these cells are of interest as novel therapeutics targeting IL-12, IL-23, IL-17, and IL-17 receptor and which are now used to successfully treat psoriasis by either repressing Th17 differentiation (IL-12/IL-23) or by directly targeting IL-17. Psoriasis has two manifestations, one in skin (psoriasis vulgaris) and one in bone (psoriasis arthritis), and the immune modulatory treatment shows positive results in both [125]. Th-17 cell differentiation is induced by IL-1β, IL-6 and TGF-β [126, 127], with TGF-β

being responsible for an increase in responsiveness of Th17 cells to IL-23. IL-23 is necessary for stabilization, survival and proliferation of Th17 cells [128]. This IL-23/Th17 axis is the target of the immune modulatory therapies currently introduced. For example, a cytokine neutralizing antibody against the p40 subunit of IL-23 inhibiting Th17 differentiation and survival, which in consequence lowers IL-17 concentrations, underwent clinical trials [129, 130].

2.6.4. CD8 + T_{EMRA} cells

A direct crosstalk between activated T cells and bone-forming cells can be assumed during the healing process. Among these T cells, CD8+ T_{EMRA} cells were confirmed to have a negative effect on the bone regenerative process. High expression levels of TNF-α and interferon-γ (IFN-γ) of CD8+ T cells decreased the osteogenic differentiation capacity *in vitro* [91]. CD8+ T_{EMRA} cells can be triggered to express these cytokines without antigen-presenting cells and do not necessarily need costimulatory molecules like CD80/86-CD28 but are activated by bystander responsiveness [131–133]. These cells accumulate in the fracture hematoma due to their tissue homing qualities and they occur in higher numbers in patients experiencing a delayed healing [91]. In the clinical setting, the recognition of a delayed or missing bone healing is so far only possible when these healing disturbances become visible in X-ray or computed tomography evaluations of the fractured bone. An early identification of patients at risk of a delayed or disturbed fracture healing is still missing. CD8+ T_{EMRA} cells could proof to be a marker for delayed healing risk in patients, since these cells also show elevated values in peripheral blood. Predicting patients with an extended need for special fracture treatment could thus just be done by analyzing the CD8+ T_{EMRA} percentage in peripheral blood early on in the healing process.

2.6.5. Outlook

Not only the interaction of the skeletal and immune system in fracture healing is not well understood so far, the immune reaction in itself is also still not unraveled. Aside from the complexity of the cytokine pattern guiding the regenerative process, the plasticity of the immune cells is still a vast challenge: M1 macrophage phenotype changing towards M2, Th1 changing towards Th2 response, regulatory T cells changing into Th17 cells and vice versa, to mention only a few aspects that still have to be understood. First approaches have been successful in influencing the fracture treatment through immune modulation (NSAIDs or IL-23 neutralization antibodies) but the possibilities are far from being exploited. A stratification of patients can help to decide, which treatment is optimal for which patient, especially with respect to the current immune status of these patients. With the numbers of delayed healing fracture patients still vastly unknown and possibly massively underestimated, and the demographic prognostic of a substantial increase in the elderly population during the next years, the need for further treatment options is rising together with the necessity of enhanced basic research in the field of osteoimmunology.

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