Upregulation of early inflammation to enhance fracture repair

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Abstract

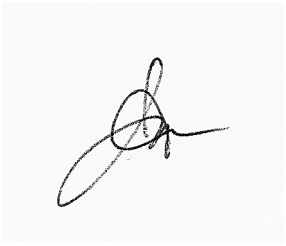
Fractures of bone are very common, affecting 2% of the population per annum. Whilst the majority heal uneventfully, 10-15% exhibit delayed or non-union. These complications tend to occur in patients who have sustained high-energy open fractures, which are limb-threatening injuries, or low-energy osteoporotic fractures, which are associated with high morbidity and mortality rates. Enhancement or acceleration of fracture repair would confer significant benefit to these patients as well as reduce the public health burden.

Inflammation represents the earliest response following trauma and initiates a cascade of downstream events crucial for wound healing. However, the mechanism by which this occurs remains poorly defined. A detailed understanding of how these upstream events initiate fracture healing is a necessary step in the development of therapeutics to enhance this process. Our group previously reported that addition of low dose recombinant human TNF (rhTNF) at the fracture site accelerated fracture repair in a murine tibial fracture model. Here I show that local rhTNF treatment is only effective when administered within 24 hours of injury, when neutrophils represent the major inflammatory cell infiltrate. Endogenous TNF was expressed at the fracture site initially by neutrophils and after 3 days by monocytes/macrophages. Systemic administration of anti-TNF resulted in impaired fracture healing. The addition of rhTNF to the fracture environment in an air pouch model enhanced neutrophil recruitment, and promoted the recruitment of monocytes through CCL2 production. Conversely, inhibition of either neutrophils or the chemokine receptor

CCR2 resulted in significantly impaired fracture healing. Fragility fractures represent a major unmet medical need and they are associated with permanent disability and premature death. Using a murine model of fragility fractures, rhTNF treatment improved fracture healing during the early phase of repair. Translated clinically, accelerated healing would permit earlier load bearing and reduce the morbidity and mortality associated with delayed patient mobilisation.

Statement of originality

I hereby declare that this submission is my own work and to the best of my knowledge contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at the University of Oxford or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked, is explicitly acknowledged in this thesis.



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Chapter 1: Introduction

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1.1 Fractures – the clinical perspective

1.1.1 The Clinical Problem

Bone fractures are a very common clinical problem, affecting 3.6% of the UK population every year(Donaldson, Reckless et al. 2008). While bone exhibits a remarkable ability to heal following injury and the majority of fractures heal uneventfully, certain subgroups of patients would benefit from strategies to enhance and accelerate healing. The first of these comprise high-energy open fractures. These are limb-threatening injuries prone to delayed or non-union and account for most of the 10-15% of all fractures that exhibit impaired bone healing (Einhorn 1995; Klamut, Chen et al. 2004; Evans 2010; Komatsu and Warden 2010; Pelled, Ben-Arav et al. 2010). The second group are fractures of osteoporotic bone, or 'fragility fractures', which represent the greatest unmet clinical need.

1.1.2 Treatment of fractures

The treatment of fractures to provide the optimal mechanical and biological environment for healing depends on a number of factors, including the patient demographic, pre-injury status, fracture pattern and presence of other injuries. Fractures may be managed conservatively or operatively. The key principle involves reduction and fixation of the fracture to achieve the pre-injury skeletal alignment and immobilisation at the fracture site for optimal healing. Poorly reduced fractures result in permanent deformity such as angulation, leading to malunion, and excessive movement at the fracture site compromises union,

leading either to delayed or non-union. The current mainstay treatment option for these complications is repeat surgery, often aided by bone graft or substitutes to promote fracture healing. Bone transport may be required to restore limb length. Usually, surgical intervention achieves the best reduction and stabilization. Yet despite best efforts, high-energy open fractures of the tibia remain prone to delayed and non-union while surgical implants remain prone to failure in fragility fractures. Enhancement and acceleration of bone healing would benefit these patients significantly by allowing earlier loading and confer considerable health and socioeconomic benefits.

1.1.3 High energy, open fractures

Open tibial fractures are severe limb threatening injuries associated with considerable morbidity. They largely affect young men of working age and often occur as a result of motor vehicle accidents. Typically, a large amount of energy is transferred, leading to stripping of the periosteal covering and fragmentation at the fracture site. Recovery from these injuries is prolonged and the average time to fracture union is between 41 and 43 weeks (Keating, Blachut et al. 2000; Gopal, Giannoudis et al. 2004; Nanchahal, Nayagam et al. 2009). The rate of fracture non-union or delayed union following high-energy trauma is estimated to be 10%-31% (Audige, Griffin et al. 2005; Harris, Althausen et al. 2009). Moreover, as the soft tissue envelope is breached, all open fractures are by definition contaminated by microbes and susceptible to infection. Indeed, 8-13% of Gustilo IIIB fractures are complicated by deep tissue infection, which may result in delayed amputation (Glass, Barrett et al. 2011). A multicenter

prospective observational study conducted by the Lower Extremity Assessment Project study group found that in the salvage group, 8.6% suffered from osteomyelitis and 31% developed non-unions (Harris, Althausen et al. 2009). Furthermore, between 20% and 50% of patients with open lower limb fractures are permanently disabled (Seekamp, Regel et al. 1996) and almost 40% of patients have not been able to return to work by 3.4 years (Keating, Blachut et al. 2000). One retrospective study found that despite a successful limb salvage rate of 93% in open tibial fractures, only 28% of patients returned to long-term employment, and no patient returned to work after 2 years of unemployment (Francel, Vander Kolk et al. 1992). Hence, these injuries result in substantial costs, both in terms of disability to the patients and socioeconomic burden of treatment, rehabilitation and unemployment to society.

1.1.4 Fragility fractures

Osteoporosis is a systemic disease characterized by the loss of bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and increased risk of fracture. Fractures in osteoporotic bone, or so-called 'fragility fractures', are an extremely common cause of morbidity and represent the major clinical burden that would benefit greatly from biological therapies that can accelerate fracture repair. Worldwide over 200 million people suffer from osteoporosis and 9 million fragility fractures occur every year (Holroyd, Cooper et al. 2008). The lifetime risk of sustaining a clinically significant fragility fracture is 40%, which is equivalent to cardiovascular disease, and cost the NHS £1.7 billion in the year 2000 alone (Harvey, Dennison

et al. 2010). The most common sites of fragility fractures are the hip, spine and distal forearm. Fragility fractures cause significant morbidity while both hip fractures and vertebral deformities are additionally associated with excess mortality. Fracture of the hip is the most severe type. It results in permanent disability in 50% of cases and has a mortality rate of 21-36% in the first year as these patients require prolonged hospitalisation, which in turn is associated with the risk of complications such as pneumonia and thromboembolic disease (Center, Nguyen et al. 1999; Harvey, Dennison et al. 2010). Osteoporosis also confers a dramatically increased failure rate of up to 50% of surgical fixation as the screws used to secure the supporting plate often fail to gain sufficient purchase and can either 'cut out' or 'pull through' the weakened bone structure (Barrios, Brostrom et al. 1993; Kim, Han et al. 2001; Cornell 2003). Rates of recovery and mobilisation are limited by the time required for fracture healing as premature loading leads to implant failure, accounting for the excessive morbidity and mortality seen in this vulnerable group of patients.

The global incidence of hip fractures is estimated to increase from 1.66 million per annum in 1990 to 6.26 million by 2025. In Europe, fragility fractures are associated with more disability-adjusted life years than many other chronic non-communicable diseases, including hypertension, breast cancer, and rheumatoid arthritis(Johnell and Kanis 2006). Therefore, there is an urgent unmet need to develop therapeutic strategies to accelerate the healing of fragility fractures.

1.2 Bone structure

1.2.1 Bone types and structure

The human adult skeleton comprises 213 bones, excluding the sesamoids. Bone is a metabolically active connective tissue that provides structural support as well as a means of movement. It also provides protection for vital organs, serves as a reservoir for minerals and growth factors, regulates mineral and acid-base homeostasis and is a site for haematopoeisis. Bones can be classified into flat intramembranous and long endochondral bones according to their structure and embryological origin. The former primarily make up the bones of the craniofacial skeleton as well as the scapulae, sternum and the pelvis. The long bones include the bones of the limbs and clavicles. Unlike flat bones, they consist of three regions: a central diaphysis and a metaphysis and epiphysis at either end. The diaphysis is a hollow shaft composed mainly of cortical bone, while the metaphyses (flared ends) and epiphyses (which contains the articular surface) are largely cancellous bone surrounded by a thin cortical shell.

The two general structural types of bone are cortical – the compact bone present in the shafts (diaphysis) of the long bones – and cancellous (also called trabecular) bone – the 'spongy' bone present in the vertebrae, pelvis and (ends) metaphyses of long bones. Overall the mature adult skeleton consists of 80% cortical bone by weight. Cortical bone has a porosity of 5-10% only, provides maximum resistance to torsion and bending and is responsible for the compressive strength of bone. By contrast, cancellous bone has a porosity of 50-90%, makes up only 20% of adult human skeleton and has much greater surface

area per unit volume. It also has a higher rate of metabolic activity and remodeling and responds rapidly to mechanical stimuli as the primary bone cells lie on the surface and are in close proximity to circulating growth factors and cytokines. The primary function of cancellous bone is to allow deformation and absorption of loads.

Histologically, there are two types of bone: primary or woven bone and secondary or lamellar bone. Woven bone forms the embryonic skeleton and is eventually resorbed and replaced as the skeleton matures. It is the initial bone formed at growth plates during development and growth, and also at the early stages of fracture callus formation. It comprises a disorganized and irregular pattern of collagen fibrils and mineralization. Therefore, it is flexible and weak.

By contrast, lamellar bone is highly organized with tightly packed collagen fibrils in concentric sheets with uniform distribution of osteocytes and bone matrix. Hence it is rigid and strong. It replaces woven bone during growth and during later stages of fracture healing. There are various forms of lamellar bone: osteons, trabecular, circumferential and interstitial. The first two are the most important.

Osteons represent the major structural unit of cortical bone. They consist of longitudinal cylinders running in parallel to the long axis of the bone. Each osteon consists of 4-20 concentric lamellae surrounding a central Haversian canal which is lined by endosteal cells and contain blood vessels, lymphatics and

occasionally nerves. Canaliculi, or cell processes, from osteocytes extend radially from the central canal allowing diffusion of nutrients through the bone matrix.

Cancellous bone also contains lamellar bone but this is of a different structure and is arranged as trabeculae. Such an arrangement provides a high surface area to volume ratio. Although they do not contain Haversian canals, trabeculae contain sinusoids that form an interface between bone structures and marrow. Trabeculae are more cellular with greater vascularity and higher metabolic activity than cortical osteons.

The periosteum is a bilayered fibrous cover over the external surface of bone, attaching to the external cortex. Ligaments, tendons and joint capsules attach to bones by crossing through the dense acellular outer fibrous layer of periosteum. The inner layer, the cambium, contains a number of cell types, including osteoprogenitor cells and osteoblasts, as well as a rich vascular plexus. Periosteal cells are directly connected to the endosteum and marrow space via oblique Volkman's canals that traverse the bone matrix. The periosteum provides the predominant blood supply to the outer third of the bony cortex. Depending on the prevailing microenvironmental cues, including the influence of hormones and cytokines, these cells resorb and form bone and thereby contribute to the stability and callus formation during fracture healing, particularly in an immature skeleton. In the skeletally mature animal, the contribution of the periosteum to fracture healing diminishes as the cambium layer regresses and loses it osteogenic activity(O'Driscoll, Saris et al. 2001). In our fracture model, the periosteum is stripped to ensure that fracture healing occurs largely by

endochondral healing as seen in the adult human, particularly in high energy fractures where the periosteum is often stripped and muscle is likely a major contributor of osteoprogenitors(Liu, Schindeler et al. 2010; Chan, Harry et al. 2012)

1.2.2 Osteoporosis

Ostoporosis is a common systemic disease characterized by a systemic impairment of bone mass and microarchitecture that results in fragility fractures. Osteoporosis is defined in terms of both bone mineral density (BMD) and history of fracture (Cooper and Harvey 2012). Thus osteoporosis is diagnosed if the BMD measured by dual X-ray absorptiometry is more than 2.5 standard deviations below the sex-matched young adult mean. If this criterion is met in a patient with a history of fragility fractures, the term 'established osteoporosis' is used.

By far the most common cause is oestrogen-deficiency in postmenopausal women, 40% of whom in the white population are affected (Rachner, Khosla et al. 2011). It is one of the major global health problems and is increasing in prevalence due to the ageing populations, particularly in the Western World. MicroCT analysis reveals marked trabecular thinning of osteoporotic bone compared to normal bone. Osteoporosis is now believed to be a clinical manifestation of impaired bone remodeling. Furthermore, impaired bone remodeling has also been found to affect the course of fracture healing in animal models of osteoporosis, leading to poorer histological, biomechanical and

radiological measures of union (Namkung-Matthai, Appleyard et al. 2001; Lill, Hesseln et al. 2003; McCann, Colleary et al. 2008). However, the clinical situation is less straightforward as delayed fracture healing in osteoporotic bone is not as readily apparent in humans. Nonetheless, a reduced healing capacity in the presence of osteoporosis can be inferred from the dramatically greater failure rate of implant fixation(Barrios, Brostrom et al. 1993). While the early occurrence of implant failure may be attributed to the inferior mechanical properties of cancellous bone and early full-load-bearing patterns post surgery, the late occurrence of the cut out or pull through phenomena is related to impaired fracture healing and overloading at the bone-implant interface. Furthermore, at the cellular level, bone marrow derived stromal cells from osteoporotic patients show reduced proliferation and osteogenic potential in vitro and react differently in response to growth factors when compared to healthy controls (Sterck, Klein-Nulend et al. 1998; Rodriguez, Garat et al. 1999; Rodriguez, Montecinos et al. 2000). Hence, the treatment of fragility fractures remains an unmet clinical need and would benefit from strategies that can enhance or accelerate fracture healing.

1.3 Bone Biology

1.3.1 Bone cells

Bone largely consists of bony matrix, which has organic and inorganic (or mineralized) components, and bone cells. Cells make up 10% of the total bone volume and are derived from the mesenchymal and haematopoietic lineages. Mesenchymal stem cells (MSC) give rise to bone forming osteoblasts and osteocytes while the haematopoetic stem cells of monocytic lineage give rise to bone resorbing osteoclasts.

1.3.1.1 Osteoblasts

Osteoblasts line the surfaces of bone and synthesize and secrete organic bone matrix. Osteoblasts produce extracellular matrix containing type-1 collagen and other non-collagenous proteins, including osteocalcin, osteonectin and osteopontin, which become mineralized. The unique stiffness of bone compared to other types of tissues is due to the process of mineralization by osteoblasts. Soluble calcium and phosphate ions crystallise to insoluble calcium phosphate within the organic matrix of bone. This typically occurs 10-15 days after the secretion of organic matrix.

Osteoblasts are characterized by the expression of a number of markers, including Runx2, osteocalcin, osterix, collagen 1 and alkaline phosphatase (ALP). They are derived from osteoprogenitor cells, which are mesenchymal in origin and found in bone canals, endosteum, periosteum and marrow space. They may also arise from vascular pericytes or from circulating cells. When stimulated by

the appropriate signals, osteoprogenitor cells migrate, proliferate and differentiate into osteoblasts. There are currently intense efforts to elucidate the signaling pathways that regulate MSC activity in translational bone biology. A number of factors that stimulate osteogenic differentiation have been identified, including members of the transforming growth factor-β (TGF-β) superfamily, most notably the bone morphogenetic proteins (BMPs), as well as various cytokines. Our group has recently found that the proinflammatory cytokines TNF and IL-6 are able to stimulate osteogenic differentiation in vitro in a dose dependent manner(Glass, Chan et al. 2011). The canonical Wnt/β-catenin signaling pathway has also recently been discovered to be a key determinant of the fate of MSCs, its activation leading to differentiation into osteoblasts rather than adipocytes(Baron and Rawadi 2007). This discovery has led to the promising development of agents designed to antagonize the natural inhibitors of this pathway, such as anti-sclerostin and anti-Dikkopf antibodies, and promote bone formation, particularly in osteoporosis.

1.3.1.2 Osteocytes

Osteocytes make up 90% of all bone cells. They are terminally differentiated osteoblasts that are surrounded by organic matrix and reside in lacunae. Osteocytes communicate with each other directly by their long dendritic processes which travel via canaliculi that traverse the bone matrix, thus forming an extensive cellular network within bone.

Osteocytes maintain the bony matrix and mediate calcium homeostasis and can be induced to resorb bone directly by parathyroid hormone (PTH), although paradoxically, intermittent PTH stimulates bone formation. They also express various factors that regulate phosphate metabolism, suggesting a role in matrix mineralization. Uniquely, they express and secrete a soluble glycoprotein named sclerostin, which inhibits osteoblast differentiation and bone formation (Poole, van Bezooijen et al. 2005). Thus, osteocytes form a mechanosensory network and function like an endocrine organ. They respond to mechanical loading and unloading as well as to hormones including PTH and parathyroid hormone-related protein, and affect distant organs such as the kidney. Thus, osteocytes integrate hormonal and growth factor signaling with mechanical signals to orchestrate bone remodeling by sending signals to osteoblasts and osteoclasts, thereby regulating bone mass in the postnatal skeleton (Bonewald 2011).

1.3.1.3 Osteoclasts

Osteoclasts are giant multinucleated cells whose primary function is to resorb bone matrix. They are found in shallow depressions, called Howship lacunae, on bone surfaces as well as deep resorption cavities and cutting cones, which are microscopic structures that are particularly prominent during fracture repair. They have a motile cytoskeleton and express adhesion molecules, including integrins, which enable them to attach to bone matrix. The contact surface resembles a brush border, which maximizes surface area and creates a sealing zone, thus providing a highly enriched acidic microenvironment to resorb mineralized matrix (Tartrate-Resistant Acid Phosphatase). Cathepsin K, a

cysteine proteinase, degrades collagen and breaks down bone. Thus, the inorganic component of bone is removed during bone remodelling and hole-like lacunae appear.

Osteoclasts are particularly important in the later stages of fracture healing in bone remodeling. Patients with defective cathepsin K activity suffer from pycnodysostosis, a rare disease characterized by osteosclerosis (Gelb, Shi et al. 1996). Osteoclast activity is influenced by a number of factors, including receptor activator of NF-κB ligand (RANKL), osteoprotegerin, IL-1, IL-6, macrophage colony-stimulating factor (M-CSF), PTH, 1,25-dihihydroxyvitamin D and calcitonin. High levels of TNF have been demonstrated to stimulate bone resorption and accounts for the bone resorption seen in rheumatoid arthritis (Dimitroulas, Nikas et al. 2013).

Osteoclasts originate from haemopoietic stem cells and are closely related to monocytes and macrophages, which can also serve as osteoclast precursors. Differentiation from osteoclast precursor to activate multinucleated osteoclasts is dependent on RANKL, a member of the TNF family, and the permissive role of macrophage-colony-stimulating factor (M-CSF). RANKL is highly expressed on osteoblasts, bone marrow stromal cells and T and B lymphocytes. Osteoclasts express its receptor RANK on the cell membrane. RANKL/RANK ligation induces the differentiation, proliferation, multinucleation, activation and survival of osteoclasts, and ultimately bone resorption. Osteoprotegerin (OPG) is a naturally occurring antagonist of RANKL (Gelb, Shi et al. 1996).

1.3.2 Bone modeling

Bone modeling occurs during development and is induced in adults by mechanical loading or injury, such as a fracture. It occurs via uncoupled anabolic osteoblast formation with or without catabolic osteoclast bone resorption at distinct anatomic locations. There are a number of similarities between bone healing and bone formation, including the two types of ossification processes, namely intramembranous and endochondral (Fig 1.1). Both are observed during embryonic skeletal development and are recapitulated during fracture healing.

1.3.2.1 Intramembranous bone formation

Intramembranous bone formation occurs in the flat bones of the skeleton, including the craniofacial bones and the scapula. Here, MSCs differentiate directly into bone forming osteoblasts which form and deposit bone matrix without a cartilaginous intermediary. Osteoblasts proliferate and synthesize osteoid (unmineralized matrix), which eventually mineralizes. Angiogenesis is a key concomitant process. Bones of intramembranous origin heal entirely through intramembranous bone formation. However, an endochondral bone that has been fixed such that the fracture is perfectly reduced and stable can also heal through a fracture callus formed by intramembranous bone formation. The new woven bone is subsequently remodeled to lamellar bone.

1.3.2.2 Endochondral bone formation

The long bones including limbs, ribs and vertebrae are formed by endochondral bone formation, which is characterized by the formation of a cartilaginous

intermediate. During bone growth, it occurs at the physeal plate and during fracture healing at the site of injury. MSCs differentiate into either cartilage-forming chondrocytes or bone-forming osteoblasts. The chondrocytes proliferate and form a hyaline cartilaginous template. Subsequently, they cease to proliferate in the avascular regions, hypertrophy and eventually apoptose, releasing a number of factors including angiogenic factors. The hypoxic environment and angiogenic factors produced by the hypertrophic and apoptotic chondrocytes stimulates angiogenesis. The ingrowth of blood capillaries then occurs to invade the callus matrix and osteoprogenitor cells are recruited locally and differentiate into osteoblasts to form bone. Thus, in contrast to intramembranous bone formation, endochondral bone formation occurs in hypoxic regions where blood vessels are reduced. During fracture repair, the cartilage is progressively replaced with woven bone to form a hard fracture callus, which is eventually remodeled to cortical lamellar bone.

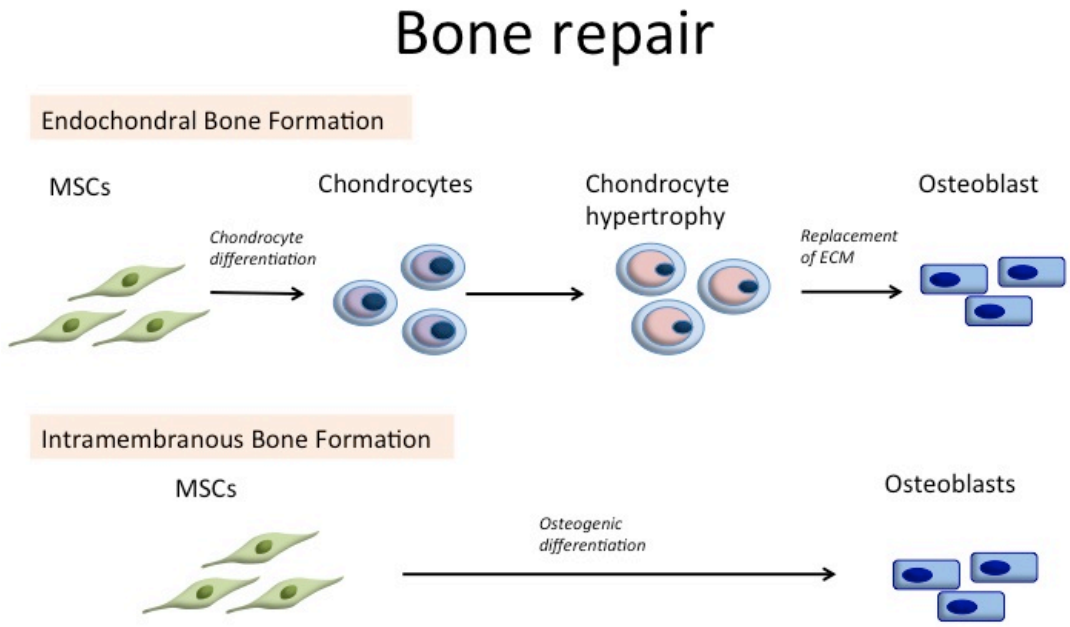

Fig 1.1. Endochondral and intramembranous bone formation

1.3.3 Bone remodeling

The post-natal skeleton provides a system of struts, which enables locomotion but must also sustain the forces exerted upon it on a daily basis. Hence, it undergoes a constant process of remodeling and repair of the microdamage to meet the demands of the body and maintain optimal bone density and skeletal integrity. Thus, bone is a dynamic organ that is continuously remodeled throughout life.

Bone remodeling is a physiological process that maintains skeletal integrity, homeostasis and mechanical strength. MSCs within the skeletal tissues contribute to bone modeling and remodeling by providing the pool of osteoblasts

to form the mineralized bone matrix (Bielby, Jones et al. 2007). Unlike bone modeling, bone remodeling is a coupled process that involves the sequential regulated resorption and replacement of bone through the coordinated activities of osteoclasts and osteoblasts, respectively. It occurs throughout life to continuously renew cortical and trabecular bone (of both intramembranous or endochondral origin) and repair microdamage, contributing to the adult cortical bone turnover rate of 2-3% per year. Bone remodeling can be seen histologically within defined units called bone remodeling compartments (BRC) (Khosla, Westendorf et al. 2008; Mosekilde, Torring et al. 2011).

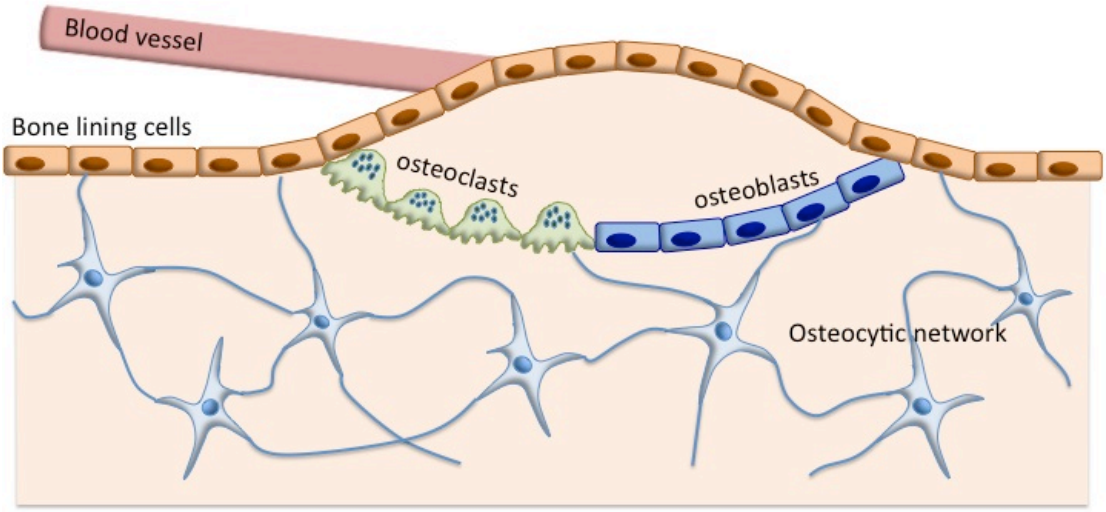

***Fig 1.2**. Bone remodeling compartment forms the multicellular remodeling unit of bone.*

Bone remodeling requires the functions of all three types of bone cells, which are brought together to form a so-called basic multicellular unit (BMU). Detailed histological studies have revealed that bone remodeling occurs within a bone

remodeling compartment (BRC) in both cancellous and cortical bone. The BRC comprises the basic multicellular unit (BMU), a canopy of bone-lining cells and associated capillaries (Fig 1.2) (Khosla, Westendorf et al. 2008). The prevailing notion is that the osteocytic network senses damage and mechanical strain as well as changes in the hormonal environment of the bone, and regulates the bone remodeling process via the bone lining cells. The bone lining cells attract osteoclasts to begin bone resorption, and form the roof of the BRC with adjoining bone lining cells. The ingrowth of a capillary into the BRC provides a supply for the cells of the BMU for bone remodeling. Once the osteoclastic bone resorption phase is completed, bone formation by osteoblasts occurs, thus completing the remodeling cycle.

1.4 Fracture biology

1.4.1 Types of Fracture healing

Fracture healing is characterized by a spatially and temporally coordinated cascade of events which involves the interaction between many cells types and the release of cytokines, chemokines and growth factors. This process is tightly regulated and any deviation will compromise the final outcome.

Fracture healing may be classified into primary and secondary healing. The former involves perfect anatomic reduction and rigid fixation. Healing occurs by osteonal healing which involves intramembranous ossification without the formation of a visible callus. However, this rarely occurs. Rather, most fractures heal by secondary fracture healing. This involves a combination of intramembranous and endochondral ossification and the subsequent formation of a callus. In contrast to primary healing, secondary healing is promoted by micromotion and inhibited by absolutely rigid fixation. Intramembraneous ossification occurs within the medullary canal and via osteoprogenitor cells in the periosteum adjacent to the fracture site while endochondral ossification occurs between the bone ends at the fracture site. A soft cartilaginous callus is formed locally and provides some stability. This is then replaced by a harder bony callus which is then remodeled to form a new bone structure that is similar to the original, provided the fracture has been adequately reduced and stabilized.

1.4.2 Sequence of events during endochondral healing:

1.4.2.1 Inflammation and haematoma formation

Cell injury and death as a result of skeletal trauma leads to acute local inflammation due to the release of cytokines and recruitment of phagocytes. Tissues trauma also leads to a disruption of blood vessels, bleeding and formation of a haematoma.

1.4.2.2 Recruitment and differentiation of osteoprogenitor cells

During fracture healing, precursor cell populations capable of reconstituting all the cellular elements that comprise bone are recruited to form new bone. The sources of these cells are multiple and include MSCs in local tissues comprising the bone marrow and adjacent soft tissues (periosteum, muscle, fat and fasciocutaneous tissues) (Bosch, Musgrave et al. 2000; Junker, Sommar et al. 2010). In the skeletally immature human, the periosteal cells make a substantial contribution, but this diminishes in the adult(O'Driscoll, Saris et al. 2001). Animal studies have also demonstrated systemic recruitment of MSCs (Shirley, Marsh et al. 2005; Granero-Molto, Weis et al. 2009). More recent immunohistological studies have demonstrated co-localization of pericyte and MSC-markers, suggesting that the local perivascular niche also contributes(Arthur, Zannettino et al. 2009). The direct transformation of endothelial cells into mesenchymal cells, named endothelial-to-mesenchymal transition, has also been shown both in vitro and in vivo and likely plays a role in

fracture repair and wound healing (Lewinson, Maor et al. 2001; Medici, Shore et al. 2010).

Despite the common stem-like behavior of these cell populations, they exhibit differences in morphology, growth rates, proliferation potential and differentiation capacity (Arthur, Zannettino et al. 2009; Glass, Chan et al. 2011). The stem-like cells isolated from these various tissues are heterogeneous and the definition of what constitutes a MSC remains controversial, in large part due to the absence of a consensus on the cellular markers(Horwitz, Le Blanc et al. 2005). The Mesenchymal and Tissue Stem Cell Committee of the International Society of Cellular Therapy have proposed three minimal criteria that define MSC. These include (1) the adherence of the isolated cells to plastic in culture, (2) the expression of CD105, CD73 and CD90 and the lack of expression of CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR in more than 95% of the cells, (3) the differentiation of the MSC into osteoblasts, adipocytes and chondrocytes in vitro (Dominici, Le Blanc et al. 2006).

Although MSCs are defined by their ability to exhibit tri-lineage differentiation, current evidence suggests that MSCs exhibit different osteogenic potential, depending on their origin. Our group has previously shown that human MSCs derived from muscle have equivalent osteogenic potential to those from bone marrow but superior to those from adipose and fasciocutaneous tissues (Glass, Chan et al. 2011). This is particularly pertinent in the surgical setting as it informs the type of soft tissue reconstruction that may be used to cover open fractures, particularly of the lower limb. Whilst there is insufficient clinical

evidence to advocate the use of muscle over fasciocutaneous tissue to cover open fractures, the overall experimental evidence clearly shows that muscle coverage leads to superior fracture healing compared to fasciocutaneous tissue (Harry, Sandison et al. 2008; Harry, Sandison et al. 2009).

1.4.2.3 Callus formation

Fracture healing in the long bones of a skeletally mature human occurs by endochondral ossification. This is characterized by the formation of a hyaline cartilaginous intermediary, which constitutes the soft callus. This eventually undergoes ossification whereby the cartilage is replaced by woven bone, thus forming the hard callus.

1.4.2.4 Remodeling

Bone remodeling occurs throughout life. However, it is particularly prominent at regions that require repair, including at fracture sites. It begins typically several weeks following injury and may take several years to achieve a bone structure that is similar to the original. The osteocytic network is believed to be responsible for sensing structural damage and coordinating the bone remodeling process.

The woven bone is structurally weak and is resorbed by osteoblasts. The osteoblasts deposit osteoid, which becomes mineralized and form stronger lamellar bone. Remodeling of a fracture callus maximizes the structural strength of the bone and is described by Wolff's law, which states that bones are

remodeled in such as way as to accommodate stresses placed upon them. Bone is reinforced at sites of stress, including across the fracture site, but undergoes resorption where it does not experience stress.

1.5 Strategies to enhance fracture healing

1.5.1 Currently approved biological treatments

Currently bone morphogenetic proteins (BMPs) are the only clinically approved agents to enhance bone formation and repair. Recombinant human BMP-2 is approved for treatment of spinal fusions and BMP-7 for treatment of non-union of long-bone fractures secondary to trauma. However, BMPs have not been approved to enhance healing of fragility fractures (Kanakaris, Petsatodis et al. 2009). Indeed, there is currently no approved therapy for accelerating healing of fragility fractures.

BMPs are a group of secreted signaling proteins belonging to the transforming growth factor-β superfamily (Wozney, Rosen et al. 1988). They were originally identified by their ability to induce ectopic bone formation (Urist 1965). BMPs are critical in skeletal development, postnatal growth and fracture healing (Hogan 1996; Zhang and Bradley 1996). BMP signaling is initiated by BMP binding to type 1 and type 2 serine/threonine kinase receptors. This leads to the formation of a heterodimeric receptor complex and phosphorylation of the type 1 receptor, activating signaling cascades that predominantly involve SMADs and resulting in the transcription of BMP responsive genes (Wu, Shi et al. 2007). Over 20 distinct BMP isoforms have been discovered of which three, BMP-2, -4 and -7 are associated with fracture healing. Immunohistological studies show that these isoforms are expressed by osteoblasts and chondrocytes in rat and human fracture calluses (Komatsu and Warden 2010). They are also critical in the differentiation of MSCs into bone forming osteoblasts. In particular, BMP2 is

critical in skeletal development, including cartilage and bone formation (Hogan 1996). During the early stages of embryogenesis, BMP-2 is expressed in specific areas of limb buds to form prechondrogenic condensations and it later promotes cellular differentiation into chondrocytes (Pizette and Niswander 2000). In the postnatal animal, BMP-2 is expressed and regulates cellular differentiation, proliferation and matrix production during bone healing (Bostrom, Lane et al. 1995; Si, Jin et al. 1997). Limb targeted deletion of BMP-2 resulted in abrogation of fracture healing (Tsuji, Cox et al. 2008) but similarly targeted deletion of BMP-4 did not affect fracture healing (Tsuji, Cox et al. 2008).

Although addition of exogenous BMPs results in improved healing in animal models, clinical trials of BMPs for tibial non-union (Friedlaender, Perry et al. 2001) and fracture healing (Govender, Csimma et al. 2002) have failed to achieve the efficacy anticipated (Lane 2001; Lieberman, Daluiski et al. 2002; Garrison, Shemilt et al. 2010). This is likely because a single supraphysiological dose of BMP, which has a short half life, is unlikely to induce the complex pattern of growth factor and cytokine production required for optimal fracture repair. The success of BMP in preclinical models to enhance endochondral healing usually requires high and sustained levels using adenoviral transduction (Kanakaris, Petsatodis et al. 2009). However, techniques to expand autologous MSCs ex vivo, including those transduced to overexpress BMPs, face significant translational hurdles and cost implications. Strategies whereby a drug is delivered at the fracture site to promote the recruitment of endogenous stem cells and their osteogenic differentiation would overcome these problems.

1.5.2 Therapeutic strategies to promote bone formation

Further understanding of the fracture healing process will continue to identify potential therapeutic targets and enable development of treatments to promote bone healing. The promotion of new bone formation would be beneficial in fracture healing as well as conditions characterized by bone loss, such as osteoporosis. The latest concepts of bone remodeling provide a rational basis for the development of mechanism-based therapies. Thus, the bone remodeling compartment (BRC) and elucidation of the factors involved in the tightly coupled cross talk between osteoblasts and osteoclasts, as well as the signaling pathways that promote osteoblast differentiation will reveal viable therapeutic targets. The current theoretical concept describes a coupled relationship between osteoclasts and osteoblasts that requires bidirectional communication. Classic anti-resorptives are thought to reduce osteoclast viability, thus suppressing osteoclastic signaling, which ultimately, in turn, suppresses osteoblast bone formation. The development of drugs that can inhibit osteoclast activity without compromising their viability offers an alternative strategy which maintains physiological communication between the two cell types and results in overall bone formation.

1.5.2.1 Osteoblast-Osteoclast Coupling

The concept of osteoclast-osteoblast coupling was first proposed in 1981. Using organ culture of chicken tibiae, it was observed that parathyroid hormone (PTH) stimulated the release of a soluble factor that induced bone formation in response to an acute increase in resorption (Howard, Bottemiller et al. 1981).

Numerous coupling factors have since been identified but their sources and how their release is controlled have been less well defined.

A number of key molecules coordinate activities of osteoblasts and osteoclasts during bone remodeling. The resorptive phase, which lasts approximately one month in humans (Eriksen 1986), involves the activation of pre-osteoclasts, derived from haematopoietic stem cells, at resting bone surfaces to differentiate into mature osteoclasts. The resorptive activity of osteoclasts is regulated by local osteoblastic cells which express RANK-L (Eriksen, Eghbali-Fatourechi et al. 2007). Thus, RANK-L activate RANK on pre-osteoclastic cells to promote osteoclastic proliferation, differentiation and resorptive activity, and prevent their apoptosis. During resorption, a number of putative coupling factors are produced by osteoclasts or released from the matrix, including TGF-β, activin A or Ca^{2+} to activate osteoblasts which are derived from MSCs (Lorenzo, Horowitz et al. 2008; Raggatt and Partridge 2010). The differentiation, proliferation and function of osteoblasts are regulated by a number of transcription factors including Runx2, osterix and the Wnt/β-catenin signaling pathway. The osteoblasts form osteoid that mineralizes after 10-40 days. Approximately 10% of these cells undergo terminal differentiation and become osteocytes (Khosla, Westendorf et al. 2008; Raggatt and Partridge 2010). Bone formation takes a period of 2-4 months during which osteoblasts are converted to flat lining cells (Eriksen 1986). The amount of bone formation balances the bone resorption in a healthy young adult to maintain bone mass (Eriksen 1986). The sensory mechanism that regulates the amount of bone formed in a bone structural unit (BSU) is thought to be regulated by sclerostin, an endogenous Wnt antagonist.

Sclerostin is released by neighbouring osteocytes and provides a negative feedback mechanism to prevent overfilling of the BSU (van Bezooijen, Roelen et al. 2004; Poole, van Bezooijen et al. 2005).

1.5.2.2 Anti-resorptive agents

A number of anti-resorptive and anabolic drugs have been developed against osteoporosis but have not yet been found to be effective in enhancing fracture healing. Most of these agents aim to increase bone mass by uncoupling bone resorption and formation of the remodeling process. However, their use are also limited by intolerable adverse effects in a subset of patients or increased risk of rare but serious complications including gastrointestinal intolerance and osteonecrosis of the jaw (Mosekilde, Torring et al. 2011).

The current FDA-approved drugs that are available for the prevention and treatment of osteoporosis include bisphosphonates, calcitonin and selective oestrogen receptor modulators (SERMS). These inhibit bone resorption by osteoclasts and have been shown to effectively reduce the risk of vertebral, non-vertebral and hip fractures (Rachner, Khosla et al. 2011). However, these have not been approved for enhancement of fracture healing.

By far the largest class is the bisphosphonates, which have a high affinity for bone and a long safety record. Bisphosphonates are structural analogues of pyrophosphate, a naturally occurring bone resorption inhibitor. They inhibit osteoclast resorption and attach strongly to hydroxyapatite binding sites on

bony surfaces, especially those undergoing active resorption. Thus bone impregnates with bisphosphonates. During resorption, bisphosphonates impairs osteoclast formation of ruffled border and production of protons and impairs osteoclastic development and recruitment while promoting osteoclast apoptosis(Sato, Grasser et al. 1991; Hughes, Wright et al. 1995; Rodan and Fleisch 1996; Colucci, Minielli et al. 1998). Bisphosphonates have the advantage of low cost and effectiveness over a range of osteoporosis subtypes, including postmenopausal, male and steroid-induced osteoporosis, as well as Paget's disease. However, their effectiveness is limited to the prevention of further bone loss and do not stimulate new bone formation.

1.5.2.3 Parathyroid Hormone

The only drug available that demonstrates an anabolic effect on the skeleton is parathyroid hormone (PTH). It is either given as the full-length peptide (PTH 1-84) or its N-terminal fragment, teriparatide (PTH 1-34). These must be given intermittently as continuous administration leads to bone resorption. However, its use is limited due to concerns about the development of osteosarcomas and side effects, which include hypercalcaemia and hypercalciuria. Hence, its use is limited to 18 months in Europe and 24 months in the USA.

PTH is approved for the treatment of osteoporosis but can only be prescribed for 18 – 24 months and is not approved to enhance repair of fragility fractures. While an excess of PTH, for example in severe hyperparathyroidism or continuous PTH treatment, leads to bone loss (Qin, Qiu et al. 2003), when given

intermittently, it leads to new bone formation and gain of bone mass in humans (Qin, Qiu et al. 2003). However, the precise mechnanism by which PTH exerts its anabolic effects on bone remain elusive. Current evidence suggest that it affects multiple pathways to promote bone formation. Microarray analyses of rodent bones treated with PTH show that around 1000 genes are modulated by PTH, including genes involved in Wnt signaling (Wnt4 and Sfrp4), transcription factors (cAMP responsive element modulator), growth factors (amphiregulin) and chemokines (CCL2).

PTH treatment has been promising in rodents, including young, aged, intact and ovariectomized rats, leading to improved bone regeneration and fracture repair (Kim and Jahng 1999; Barnes, Kakar et al. 2008; Warden, Komatsu et al. 2009). Similar studies using other species, including mice, rabbits and monkeys also showed that intermittent PTH treatment improves bone regeneration (Barnes, Kakar et al. 2008). However, the use of PTH doses required greatly exceeded the approved clinical dosage (Komatsu, Brune et al. 2009). There has been one prospective randomized double-blind clinical trial of PTH(1-34) to accelerate fracture healing (Aspenberg, Genant et al. 2010). 102 postmenopausal women with Colles fractures received placebo, 20 μg PTH(1-34) or 40 μg PTH(1-34) daily for 8 weeks. Although it failed to prove the primary hypothesis that 40 μg dose would reduce time to healing compared to placebo, a significant improvement was reported for the 20 μg dose. However, the authors advised that this observation should be interpreted with caution as the trial had a number of limitations, including the primary radiological outcome measure and large variation in fracture severity, which limited the power of the study.

1.5.2.4 Targeting Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway is a ubiquitous system for intercellular communication, playing key roles in development and homeostatic renewal of many tissues, including bone. Wnts are secreted soluble glycoproteins that stimulate a number of signaling pathways by binding a receptor complex that consists of LDL receptor-related protein (LRP) and co-receptors, including members of the frizzled (Fz) family. The canonical Wnt signaling pathway has been shown to be a key regulator of osteoblast lineage specification, proliferation and terminal differentiation (Khosla, Westendorf et al. 2008). It is activated by the binding of the appropriate Wnt protein to LRP5 and involves the stabilization of β-catenin and regulation of multiple transcription factors including lymphoid enhancer factor 1 (Lef1) and T cell factors. It is active in all cells of the osteoblast lineage including preosteoblasts, bone lining cells and osteocytes and is blocked by the binding of inhibitors including Dickkoph-1 (DKK1) and sclerostin. Mutations in SOST (encoding sclerostin) and DKK1 in humans and mice lead to increased bone mass. Wnt/β-catenin signaling is activated as a normal physiological response to mechanical loading (Robinson, Chatterjee-Kishore et al. 2006) and contributes to the fracture healing process (Chen, Whetstone et al. 2007; Komatsu, Mary et al. 2010). It determines osteoblastic differentiation of progenitor cells, stimulates osteoblast proliferation and enhances osteoblast and osteocyte survival. Activation of wnt signaling downregulates RANKL expression in osteoblasts, thus influencing osteoclast maturation and activity within the BMU (Spencer, Utting et al. 2006).

A number of possible approaches to manipulate Wnt signaling include the development of Wnt agonists, inhibition of endogenous Wnt antagonists and inhibition of glycogen synthase kinase-3 β (GSK-3β), a kinase that phosphorylates β-catenin and promotes its degradation. The purification of Wnt however is an extremely difficult and expensive process. Neutralising antibodies to endogenous inhibitors of the Wnt/β-catenin signaling pathway, including sclerostin and DKK1, have shown early promise but are limited to systemic administration, leading to non-specific bone deposition. Furthermore, these are associated with safety concerns, such as carcinogenesis, as Wnt signaling is activated by mutations in many cancers including osteosarcoma and excessive generalized bone formation leading to closure of osteal foramina and nerve entrapment (Baron and Hesse 2012; Rossini, Gatti et al. 2013).

Lithium chloride, commonly used as a mood stabilizer drug, and a number of small molecule inhibitors of GSK-3β have been demonstrated to reverse bone loss caused by aging, oestrogen deficiency and LRP5 mutations, increased sensitivity of osteoblasts and osteocytes to mechanical loading and improved fracture healing. There is limited clinical evidence that the relative risk of fracture is reduced amongst users of lithium chloride (Vestergaard, Rejnmark et al. 2005) but one study also found that the fracture incidence increased following discontinuation of the drug (Wilting, de Vries et al. 2007).

1.5.2.5 Strontium Ranelate

Strontium is a trace element that is closely related to calcium. It appears to have both antiresorptive and anabolic activity and have significant beneficial effects on bone balance in normal and osteopenic animals (Marie, Ammann et al. 2001). Various clinical trials have shown that it improves bone mineral density in osteoporotic women and reduces the incidence of vertebral and nonvertebral fractures (Reginster, Kaufman et al. 2012). However, while strontium appears to promote fracture repair in ovariectomized rats, no clinical trials has been conducted to demonstrate this effect.

1.5.2.6 Cellular therapy

Fracture repair requires the recruitment of osteoprogenitor cells. MSCs are, by definition, multipotent and can therefore serve as a source of osteoprogenitor cells. MSCs may originate from a variety of tissues including the bone marrow, periosteum, dermis, adipose tissue and muscle, as well as blood vessels and the circulation. In closed fractures, the main sources of osteoprogenitor cells are thought to be the bone marrow and periosteum (Jaiswal, Haynesworth et al. 1997; Bianco, Riminucci et al. 2001; Sekiya, Vuoristo et al. 2002; Gerstenfeld, Cullinane et al. 2003; Hutmacher and Sittinger 2003). However, high energy open fractures of long bones are characterized by loss of the periosteum and bone marrow, especially following insertion of an intramedullary rod. Under these circumstances the main osteoprogenitor cells must originate from the local soft tissues or the circulation (Shirley D 2005; Schindeler, Liu et al. 2009).

Treatment of non-unions with MSCs is currently an area of intense research

focus. Several clinical studies have demonstrated that transplantation of mesenchymal progenitors in aspirated bone marrow promotes bone repair in non-unions (Homma, Zimmermann et al. 2013). In preclinical studies, MSCs are amplified ex vivo and often transduced to overexpress osteogenic factors such as BMPs. However, the exact role of MSCs remains poorly understood. Furthermore, the concentration and quality of MSCs may vary significantly and there remain considerable challenges with quality control with respect to delivering the requisite number of MSCs, the timing and costs for in vitro expansion and risks, including contamination with bacteria or viruses and carcinogenesis.

While cellular therapy in fracture healing remains in its infancy, soft tissue reconstruction in open fractures may be considered as a means to deliver MSCs in addition to providing soft tissue coverage. Both fasciocutaneous tissue and muscle are used to cover open fractures and both are rich reservoirs of MSCs (Schindeler, Liu et al. 2009; Liu, Schindeler et al. 2010). The debate over which is superior remains an issue of contention. Recent data from our group and others have contributed to this topic (Harry, Sandison et al. 2008; Harry, Sandison et al. 2009; Glass, Chan et al. 2011; Chan, Harry et al. 2012).

The characteristics, including the osteogenic potential, of MSCs vary depending on their tissue origin. For example, human stromal cells derived from muscle exhibit a significantly greater potential for osteogenesis than those from fasiocutaneous tissue, including both skin and adipose, and are equivalent to those from bone marrow (Im, Shin et al. 2005; Niemeyer, Fechner et al. 2010;

Glass, Chan et al. 2011). Using a critical sized rat femoral diaphyseal defect model, muscle was found to be more effective in promoting bone repair than adipose tissue (Evans, Liu et al. 2009). Muscle-derived stem cells can be recruited from muscle and stimulated to undergo osteogenic differentiation by proinflammatory cytokines, especially TNF-α, released at the site of injury (Glass, Chan et al. 2011). It is well established that muscle provides a suitable environment for osteogenesis, although damaged muscle is less effective (Utvag, Grundnes et al. 2003). In 1965, Urist (Urist 1965) found that new bone formed readily when decalcified bone was implanted into muscle and deduced that the inductor cells were derived from the host bed. Furthermore, purified BMPs injected into muscle are capable of inducing ectopic bone formation (Iwata, Sakano et al. 2002; Jingushi, Urabe et al. 2002). Using a mouse model, Zacks et al. (Zacks and Sheff 1982) found that muscle (but not liver tissue) demonstrated a significant osteogenic effect. Extraskeletal ossification observed in patients with fibrodysplasia ossificans progressiva(Shore, Xu et al. 2006) and heterotopic ossification following either orthopaedic surgery or blast injuries tends to occur in muscle(Iorio R 2002; Forsberg JA 2009).

Unfortunately, most of the relevant clinical evidence comprises descriptive retrospective observational case series (Table 1) and all studies are categorized as Level 4 evidence according to the Oxford Centre for Evidence-based Medicine. Few of these specifically compared muscle with fasciocutaneous flaps and those that did were severely limited by the lack of power and case heterogeneity, including a wide variety of patients with clinical indications ranging from open fractures to burns or contour deficits. There were insufficient details in the

publications to allow us to separate the flaps used to cover open fractures. Furthermore, the outcome measures differed considerably between studies, for example, not all studies reported time to fracture union, rates of deep infection or even flap survival. Therefore, the current published literature precludes amalgamation of data from different studies and hence any meaningful meta-analysis or systematic review that can provide guidance for the use of different flap options in the management of open fractures of the lower limb.

Nonetheless, there is a growing body of experimental evidence that demonstrates that the biological characteristics of the tissues in a soft tissue flap can significantly influence fracture healing, thereby potentially reducing union time and the rate of delayed or non-union.

1.6 Innate immunity – a rational therapeutic target in fracture healing

Bone fracture triggers a cascade of events that lead to repair and bone formation. This involves a number of key molecular and cellular events that are temporally and spatially coordinated. However, the precise mechanisms underlying this process, particularly during the early inflammatory process, remain poorly defined. The inflammatory phase precedes the regenerative and remodeling stages of fracture healing. It is logical to hypothesize that this phase is critical to the final clinical outcome because the signals that are released during inflammation initiates the cascade of downstream events that culminate in skeletal repair. This section provides an overview of osteoimmunology and the evidence to support the role of inflammation in bone healing.

1.6.1 Osteoimmunology

The term 'osteoimmunology' was coined to describe studies that are related to the interactions between bone and the immune system (Lorenzo and Choi 2005). The discovery of RANKL as a potent osteoclast differentiation factor (Theoleyre, Wittrant et al. 2004) first brought together the fields of immunology and bone. Since then, the ongoing and dynamic interaction between the skeletal and immune systems has become fully appreciated (Arron and Choi 2000). Current efforts in this field largely focus on understanding the role of immune cells in normal bone homeostasis as well as inflammatory diseases and osteoporosis, where the balance between resorption and formation is lost.

1.6.2 Immunity

The mammalian immune system has evolved to protect the host organism against invading pathogens. It is classically subdivided into two types: the innate and adaptive immune systems.

1.6.3 Innate immunity

The innate immune system is a first-line host defense mechanism and comprises preventative measures, such as epithelial barriers and mucus secretion, against invasion as well as a swift, powerful yet non-specific response to any breach of these barriers through the release of cytokines, anti-microbial peptides and recruitment of phagocytic leukocytes. It features a recognition system involving pattern recognition receptors (PRRs), including toll-like receptors (TLRs), which recognize a variety of microbial components, termed Pathogen-Associated Molecular Patterns (PAMPs). PPRs are expressed by both immune and non-immune cells, including epithelial cells, fibroblasts, parenchymal cells and endothelial cells. Activation of the PPRs results in the production of cytokines and chemokines, ultimately leading to an innate immune and inflammatory response and the recruitment and activation of leukocytes and other immune cells. More recently, it has become clear that PPRs not only sense pathogen invasion, but also endogenous ligands called alarmins, which are released by damaged or dead cells in sterile injury, including trauma, burn or ischaemia. The term Damage Associated Molecular Patterns (DAMPs) is used to include PAMPs and alarmins.

Hence the innate immune system is critical in sensing cellular damage and death, clearing the cellular debris and activating the tissue healing process. However, the role of the innate immune response in initiating the healing response remains poorly defined.

1.6.4 Cells of the innate immune response

1.6.4.1 Neutrophils

Neutrophils are a type of polymorphonuclear leukocyte and one of the key participants during acute inflammation. They are derived from myeloid precursors in the bone marrow. In the circulation, they have an average diameter of 7-10 μm with a segmented nucleus and granular cytoplasm.

Following an insult, infective or sterile, neutrophils are typically the first leukocytes to be recruited as part of the innate inflammatory immune response. They are equipped to eliminate pathogens by a variety of mechanisms and clear cellular debris. However, although traditionally viewed as simple phagocytes with pro-inflammatory functions, recent data provide compelling evidence that neutrophil functions are much more extensive and that neutrophils play crucial roles in the resolution of inflammation as well as tissue healing (Kolaczkowska and Kubes 2013).

During inflammation, neutrophils are recruited into tissues and eventually apoptose before being removed by macrophages and dendritic cells. Neutrophils

contain secretory vesicles that are rapidly mobilized on activation, whereupon vesicle proteins such as β2 integrins incorporate into the cell membrane to enable cell adhesion and transmigration. The cells also contain granules, which consist of proinflammatory proteins, including myeloperoxidase (MPO), neutrophil elastase and lactoferrin. MPO and neutrophil elastase serve as common markers of neutrophils.

Neutrophils are able to eliminate pathogens via a number of mechanisms, including phagocytosis, degranulation and release of neutrophil extracellular traps (NETs). After phagocytosis, the pathogens are encapsulated in phagosomes and are killed by the release of NADPH oxygenase-dependent mechanisms, reactive oxygen species or antibacterial proteins such as cathepsins, defensins, lactoferrin and lysozyme. The antibacterial proteins can be released from the granules either into phagosomes or into the extracellular milieu. NETs consist of a core DNA element to which histone proteins and granular enzymes including MPO and neutrophil elastase are attached. NETs act by immobilizing pathogens, thus facilitating subsequent phagocytosis of trapped microorganisms, and kill pathogens by means of antimicrobial histones and proteases. The role of neutrophils in sterile inflammation is less well defined.

1.6.4.2 Monocytes

Monocytes originate in the bone marrow from a common haematopoietic stem cell. They undergo differentiation steps during which they commit to the myeloid and then to a monocyte lineage. In response to macrophage colony-stimulating

factor (M-CSF), haematopoietic stem cells divide and differentiate into monoblasts and subsequently pro-monocytes, before becoming monocytes. These exit the bone marrow and enter the bloodstream (Mosser and Edwards 2008). Monocytes constitute 2% to 10% of all leukocytes in the human body. They circulate within the blood and migrate to sites of infection or damage in response to inflammation, where they differentiate into macrophages or dendritic cells. Monocytes and macrophages are major producers of cytokines and macrophages also phagocytose dead neutrophils. The cytokines secreted by these cells determines whether there will be further inflammation or resolution.

Monocytes and macrophages accumulate at sites of bone injury and bone remodeling and have been found to contribute to the regulation of bone metabolism(Marcus 1987; Williams, Jiang et al. 1992; Rahimi, Wang et al. 1995). Activated monocytes and macrophages also produce proinflammatory cytokines including TNF and IL-1, which stimulate osteoclastic bone resorption, but can also promote bone formation through the release of growth factors including BMPs (Fukui, Ikeda et al. 2006; Hess, Ushmorov et al. 2009; Lu, Wang et al. 2013).

As mentioned above, monocytes can also serve as precursors for osteoclasts. In vitro, monocyte can be stimulated by a combination of M-CSF and RANKL to differentiate into osteoclasts and are hence associated with bone resorption. However, in recent years, a cell type named ‘osteomac’, also derived from the monocytic lineage, has been shown to be crucial for bone formation following skeletal injury in a murine model (Mizoguchi, Muto et al. 2009; Alexander, Chang

et al. 2011). Depletion of these F4/80+ cells in a transgenic murine model or by administration of clodronate liposomes suppressed woven bone deposition and mineralization during bone repair. Crucially, F4/80+ osteomacs are distinct from osteoclasts, which are F4/80 quiescent.

1.6.5 Recruitment of innate immune cells

Inflammation involves a complex series of events that results in the recruitment of specific leukocyte subsets at the sites of tissue damage. This process is regulated by a variety of cell-associated and soluble factors named chemotactic cytokines, or chemokines. Chemokines constitute a large family of peptides comprising between 60 and 100 amino acids whose main function is to regulate cell trafficking. Currently, around 50 human chemokines and 20 chemokine receptors have been identified. Chemokines are classified into four groups according to the number and relationship of the cysteine residues at the N-terminus of the molecule: CXC, CC, CX_3C and C.

Regulation of the migratory response of these cells is crucial in maintaining host-defense mechanisms (Geng 2001). Chemokines are secreted in response to signals including proinflammatory cytokines. Chemokines induce chemotaxis through the activation of G-protein-coupled receptors (GPCRs), which also involves adhesion molecules and glycosaminoglycans. Chemokines bind to specific cell surface seven transmembrane receptors which are coupled to heterotrimeric G proteins, the activation of which initiate intracellular signaling cascades, intracellular calcium release and ultimately cell migration up the

concentration gradient toward the chemokine source. The chemokine/chemokine receptor system shows a considerable amount of redundancy as proteins within each subfamily can competitively bind to the same receptor on target cells, and most chemokines bind to several different receptors. In an innate immune response, the main leukocyte subsets recruited are neutrophils and monocytes/macrophages. Here are some of the key chemokines involved in this process.

IL-8 is a major CXC chemokine for leukocytes. The homologue of IL-8 is Keratinocyte Chemoattractant (KC) in mice. IL-8 activates neutrophils via CXCR1 and CXCR2, the latter being the predominant receptor mediating its chemotactic response (Callard and Gearing 1994). IL-8 binds CXCR1 and CXCR2 with high affinity and is chemotactic for neutrophils as well as monocytes, which express both CXCR1 and CXCR2 (Gerszten, Garcia-Zepeda et al. 1999; Horuk 2001).

The monocyte chemoattractant protein-1, MCP-1, also known as CCL2, is a member of the CC chemokine subfamily. It is produced by a number of cell types including epithelial, endothelial, smooth muscle, fibroblasts and monocytes. It is a major mediator of monocyte/macrophage infiltration at inflammatory sites under both physiological and pathological conditions and also recruits memory T lymphocytes, dendritic cells and natural killer cells to sites of tissue injury and infection. CCL2 expression can be induced by mediators including platelet-derived growth factor, LPS, IL-1, IL-10 and TNF(Yadav, Saini et al. 2010). It mediates its effects via the CCR2 receptor, which is the specific receptor for CCL2 and is expressed by monocytes, macrophages and T-lymphocytes (de Boer, Sont

et al. 2000). CCR2 mediated CCL2 cellular effects including recruitment of monocytes and macrophages and in CCR2 deficient mice, the recruitment of macrophages to sites of injury is impaired (Kuziel, Morgan et al. 1997; Ma, Wei et al. 2002; Schober, Zernecke et al. 2004). The CCL2/CCR2 axis also controls the movement of monocytes from the bone marrow into the bloodstream and from the circulatory system into sites of inflammation after injury (Tsou, Peters et al. 2007). Monocyte differentiation and macrophage polarization are critical to the resolution of inflammation.

Aside from MCP-1, CCL3, also known as macrophage inflammatory protein 1 alpha (MIP-1α), and soluble IL-6 receptor (sIL-6R), are two of the key neutrophil-derived chemokines responsible for monocyte recruitment (Soehnlein and Lindbom 2010). CCL3 is a small inducible CC cytokine which plays a role in inflammatory responses through binding to receptors CCR1, CCR4 and CCR5. Soluble IL-6 receptor, sIL6R, is a soluble form of the IL6R and can bind IL6 with the same affinity as the membrane-bound form. The complex of IL6 and sIL6R induces IL6 transignaling. Recent studies in animals of inflammatory conditions including inflammatory bowel disease and rheumatoid arthritis found that IL6 transignaling serves as a major proinflammatory paradigm of IL6 signaling during pathophysiological inflammation in vivo (Rabe, Chalaris et al. 2008).

1.6.6 Acquired or adaptive immune system

This thesis focuses on the role of the innate inflammatory response on fracture healing. While a detailed description of the acquired immune system is beyond the scope of this thesis, it is mentioned here for the sake of completeness. In contrast to the innate immune system, the adaptive immune system provides a highly specific response against previously encountered pathogens through the acquisition of immunological memory, recognition of microbial antigens and production of cytokines and antibodies. The cells of the adaptive immune system include T lymphocytes, B lymphocytes, natural killer cells and dendritic cells.

Dendritic cells present microbe-derived peptides with a major histocompatibility antigen on the cell surface, migrate to a regional lymph node where they activate the cognate T cells and thereby induce adaptive immunity. Adaptive immunity confers the ability to recognize and remember specific pathogens and therefore allows a specific and efficient attack against future invasion by the same pathogens.

1.6.7 Interactions of immune and bone cells

It is well known that autoimmune disorders such as rheumatoid arthritis (RA) and ankylosing spondylitis (AS) are associated with systemic bone loss. Furthermore, RA is also associated with focal bone loss in synovial joints. The complex interactions between the immune cells of haematopoeitic origin and the bone cells and their precursors of mesenchymal origin continue to emerge. These involve the release of cytokines as well as direct cell contact. RANKL is

produced by activated T cells, dendritic cells, fibroblasts and NK cells, leading to osteoclastogenesis and bone resorption. In early menopause, during the acute phase of oestrogen deficiency, RANKL expression by bone marrow stromal cells and lymphocytes increases and is associated with enhanced bone loss (Eghbali-Fatourechi, Khosla et al. 2003).

Furthermore, the activation state of the immune cell influences its effect on bone metabolism. For example, resting T cells inhibit whereas activated T cells support osteoclast formation. The dissection of the immunological pathways that lead to bone resorption seen in rheumatoid arthritis has led to the development of drugs that target a number of components. Cytokine blockade e.g. anti-TNF (etanercept), anti-IL-1 (anakinra) and anti-IL-6 (tocilizumab) has been most successful. Antibodies that target subsets of immune cells including B cells e.g. rituximab, or T cells e.g. abatacept, have also shown some success in recent major clinical trials for rheumatoid arthritis, providing evidence that these immune cells play important roles in the maintenance of skeletal integrity (Paula and Alves 2014).

Because of the enormous public health problems of osteoporosis and rheumatoid arthritis, which are characterized by unbalanced bone loss, the field of osteoimmunology has focused mainly on the effects of the adaptive immune response on the regulation of bone turnover, specifically osteoclastic bone resorption. By contrast, there has been a relative paucity of understanding of how the immune system affects bone formation.

An intriguing macrophage-osteoblast interaction that stimulates new bone formation has recently been demonstrated. A population of macrophage-like cells lining the bone surface osteoblasts in both mice and humans, the so-called 'osteomacs', has been identified (Hume, Loutit et al. 1984; Chang, Raggatt et al. 2008; Alexander, Chang et al. 2011). These cells promote osteoblast mineralization in vitro and are critical in the maintenance of mature osteoblasts in vivo (Chang, Raggatt et al. 2008) as well as bone formation in a murine model of bone injury (Alexander, Chang et al. 2011). Depletion of osteomacs led to the concurrent depletion of osteoblasts, suggesting that the former are vital in the maintenance of the latter. Although osteomacs are derived from the myeloid lineage, they are distinct from multinucleated osteoclasts (Mizoguchi, Muto et al. 2009; Alexander, Chang et al. 2011). It is likely that osteomac and osteoblast precursor cells diverge from a common progenitor earlier in the myeloid lineage and mature along independent pathways to perform different functional roles. The former contributes to the regulation of bone formation that is unrelated to osteoclastic bone resorptive activity (Alexander, Chang et al. 2011).

Another recent study in our lab using primary human cells in vitro also demonstrated that cells of the monocytic lineage are involved in bone formation. Monocytes were found to potently induce MSC differentiation into osteoblasts via cell contact as well as soluble factors (Nicolaidou, Wong et al. 2012). Furthermore, cytokine activated natural killer T (NKT) cells were found to induce osteoblast differentiation (Wythe, Nicolaidou et al. 2013). Hence, there is now evidence that immune cells play a role in bone formation.

1.6.8 Inflammatory pathways and bone formation – a relatively unexplored territory

Inflammation has often been associated with impaired repair processes, including bone healing. This is due to the distinct bony erosions seen in patients with rheumatoid arthritis and other inflammatory conditions. However, it is also recognized that inflammation is a pre-requisite to initiate and orchestrate the downstream processes that ultimately lead to tissue repair (Chan, Roth et al. 2012). Currently, little is known about the role of the innate immune response in bone formation.

There is now some evidence to suggest that inflammatory pathways are implicated in bone formation. The observation that patients with ankylosing spondylitis, an autoimmune inflammatory condition that is associated with the formation of new bone particularly in the vertebral column, provides circumstantial evidence that inflammation induces new bone formation. There is also evidence that T cells are able to promote bone formation both indirectly through enhanced osteoclastogenesis and directly through the production of Wnt10b (Wythe, Nicolaidou et al. 2013). Another relevant clinical condition is fibrodysplasia ossificans progressive. This is a rare and disabling genetic condition which is characterized by heterotopic bone formation within soft tissues. Histological analysis shows that the ectopic ossifying lesions triggered by inflammation are a result of endochondral rather than direct intramembranous bone formation (Shore and Kaplan 2008). Heterotopic ossification following

trauma or blast injuries also tends to occur at sites of injury(Forsberg, Pepek et al. 2009).

The mechanism by which immune cells control osteoblastic differentiation remains to be elucidated, but current evidence from gene arrays have identified a number of inflammatory genes, including oncostatin M, that may be contributory (Nicolaidou, Wong et al. 2012). For example, the aforementioned study on the induction of osteogenic differentiation of MSCs by monocytes identified oncostatin M (OSM) production and STAT3 signaling to be a critical pathway (Nicolaidou, Wong et al. 2012). Interestingly, OSM is a member of the IL-6 family and a recognized proinflammatory cytokine. There is also some suggestion that T cells can influence osteoblast formation via Wnt signaling, but evidence is currently limited (Nicolaidou, Wong et al. 2012).

Studies of early fracture hematoma suggest that the inflammatory phase following fracture is critical to recruit the cells and orchestrate the events necessary for fracture healing (Grundnes and Reikeras 1993; Kolar, Schmidt-Bleek et al. 2010; Kolar, Gaber et al. 2011; Hoff, Maschmeyer et al. 2013). Multiple mediators are released during this phase, including by resident cells and immune cells. These mediators will exert autocrine or paracrine effects on resident and immune cells, as well as MSCs. Conversely anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs, dexamethasone or cytotoxic medications inhibit osteogenic differentiation and promote adipogenic differentiation in MSCs (Chang, Li et al. 2007; Ito, Suzuki et al. 2007; Kellinsalmi, Parikka et al. 2007; Oshina, Sotome et al. 2007). Furthermore, their use during

the early phases of fracture healing may impair the ultimate clinical outcome (Simon and O'Connor 2007; Dimmen, Nordsletten et al. 2008; Pountos, Georgouli et al. 2008).

1.6.9 TNF and bone formation

TNF is a prototypical proinflammatory cytokine that is released during the early innate inflammatory response. It is 212 amino acid long type II transmembrane homotrimeric cytokine synthesized as a membrane-bound protein. The soluble homotrimeric cytokine is released by proteolytic cleavage by the metalloprotease, TNF alpha converting enzyme (TACE or ADAM17). The secreted form of human TNF is a 17 kDa peptide that is secreted by immune cells including monocytes, macrophages and dendritic cells, as well as fibroblasts, adipocytes, keratinocytes, osteoblasts and many other cell types in response to tissue damage or infection(Bluml, Scheinecker et al. 2012). Both the transmembrane as well as soluble cytokine are biologically active. TNF signals via two distinct receptors, namely TNF receptor type 1 (TNFR1 or p55) and TNF receptor type 2 (TNFR2 or p75). Both of its receptors can also be cleaved off the cell surface and circulate in soluble forms, thus acting as decoy receptors for TNF.

TNFR1 is expressed on almost all nucleated cells. It is activated by binding of the soluble or membrane-bound cytokine although soluble TNF preferentially binds TNFR1. Activation of the TNFR1 pathway leads to the activation of nuclear factor κB (NFκB), which translocates into the nucleus and binds to the promoter region of target genes. NFκB activation upregulates the transcription of

proinflammatory mediators and the expression of a number of genes that are involved in the growth and division of cells as well as the differentiation of multi-potent cells. For example, activation of NFκB has been demonstrated to promote osteoblastic differentiation in MSCs derived from ligaments (Kosaka, Imakiire et al. 2000). A number of other factors including platelet-derived growth factor BB (PDGF-BB) and transforming growth factor β1 (TGF- β1) can also stimulate NFkB activity.

By contrast, the expression of TNFR2 is more restricted and is found on endothelial cells and immune cells, including monocytes, macrophages, T cells, B cells and NK cells. The primary ligand for TNFR2 is membrane-bound TNF. The signaling events mediated by TNFR2 are poorly understood compared to TNFR1. There is some evidence that TNFR2 confers anti-inflammatory and anti-arthritogenic effects in murine models of arthritis (Bluml, Scheinecker et al. 2012).

Human and mouse TNF share 79% amino acid sequence identity. Slight structural changes in the TNF sequence can lead to dramatic changes in its binding characteristics to TNF receptors. Murine TNF activates mouse TNFR1 and TNFR2 equally well whereas human TNF acts on mouse TNFR1 but does not bind TNFR2 (Lewis, Tartaglia et al. 1991).

It is well established that TNF modulates osteoclast biology by inducing the RANKL pathway and plays a pathogenic role in the focal bone loss found in the synovial joints of patients with rheumatoid arthritis (Hofbauer, Lacey et al.

1999). However, recent evidence has emerged to show that under certain circumstances, TNF can also paradoxically stimulate osteogenesis and is involved in bone repair. Thus, both TNF and IL-1β can induce BMP-2 expression in adult articular chondrocytes and a chondrosarcoma cell line via regulation at both the transcriptional and post-transcriptional levels (Fukui, Ikeda et al. 2006). Furthermore, during fracture healing in dual TNF receptor 1 and 2 deficient mice, osteoblasts which are normally recruited into the marrow space are replaced with granulation tissue cells and fracture healing becomes impaired (Gerstenfeld, Cho et al. 2001; Kon, Cho et al. 2001). Our group has previously shown that administration of low dose rhTNF, which activates the murine TNFR1 but not TNFR2 pathway, at the fracture site in a murine tibial fracture model of endochondral healing accelerated callus formation and remodeling (Glass, Chan et al. 2011). This was in part achieved through the recruitment and osteogenic differentiation of MSCs by TNF (Glass, Chan et al. 2011). These findings suggest that TNF plays a crucial role in promoting postnatal fracture healing.

There is also some evidence to show that TNF is able to usurp the Wnt signaling pathway, which is crucial in the determination of fate of MSC differentiation (Cawthorn, Heyd et al. 2007; Verjee, Verhoekx et al. 2013). The Wnt pathway induces bone formation both by diverting the differentiation of MSCs away from chondrocytes and adipocytes towards osteoblasts and by inhibiting osteoblast apoptosis and osteoclasogenesis. However, whilst this phenomenon has been demonstrated in adipocytes and myofibroblasts, it has not yet been shown to pertain in bone.

1.7 Summary

Fractures constitute a major public health problem, particularly fragility and high energy open fractures, which are associated with excessive morbidity and mortality. The ideal biological therapy for accelerating fracture healing would entail local application of pro-osteogenic factor(s) at the time of surgical treatment. Therefore, it is important to understand how the early inflammatory response initiates and orchestrates fracture repair, an area that remains poorly understood. A competent immune system is essential for effective wound healing, although excessive inflammation has been found to be deleterious to wound healing. For example, the absence of TNF or IL-6 signaling has been found to impair fracture healing (Gerstenfeld, Cho et al. 2003; Yang, Ricciardi et al. 2007) whereas persistent and elevated levels of TNF in rheumatoid arthritis are associated with bone destruction (Binder, Puchner et al. 2013) Therefore, a critical balance must be achieved to ensure an optimal healing environment (Chan, Roth et al. 2012).

To circumvent this issue, targeting upstream pathways may offer a strategy that promotes the expression of multiple factors and provides a more physiological environment that promotes bone formation.

Inflammation represents the earliest response following trauma and initiates a cascade of downstream events crucial for wound healing. However, the mechanism by which this occurs remains poorly defined. A detailed understanding of how the upstream events initiate fracture healing is a necessary step in the development of therapeutics to enhance this process.

Interventions targeting events during this early time window should allow effective clinical translation as the therapeutic would be administered at the time of reduction and surgical fixation of the fractures.

1.8 Hypothesis and aims

The hypothesis of the thesis is that controlled and targeted upregulation of the innate immune response leads to enhancement of fracture healing.

The aims of this thesis are to:

1. describe the early fracture healing pathway, and
2. elucidate the mechanism of action by which recombinant TNF administered in the first 24 hours after injury enhances fracture healing.

References

Alexander, K. A., M. K. Chang, et al. (2011). "Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model." J Bone Miner Res **26**(7): 1517-1532.

Arron, J. R. and Y. Choi (2000). "Bone versus immune system." Nature **408**(6812): 535-536.

Arthur, A., A. Zannettino, et al. (2009). "The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair." J Cell Physiol **218**(2): 237-245.

Aspenberg, P., H. K. Genant, et al. (2010). "Teriparatide for acceleration of fracture repair in humans: a prospective, randomized, double-blind study of 102 postmenopausal women with distal radial fractures." J Bone Miner Res **25**(2): 404-414.

Audige, L., D. Griffin, et al. (2005). "Path analysis of factors for delayed healing and nonunion in 416 operatively treated tibial shaft fractures." Clin Orthop Relat Res **438**: 221-232.

Barnes, G. L., S. Kakar, et al. (2008). "Stimulation of fracture-healing with systemic intermittent parathyroid hormone treatment." J Bone Joint Surg Am **90 Suppl 1**: 120-127.

Baron, R. and E. Hesse (2012). "Update on bone anabolics in osteoporosis treatment: rationale, current status, and perspectives." J Clin Endocrinol Metab **97**(2): 311-325.

Baron, R. and G. Rawadi (2007). "Targeting the Wnt/beta-catenin pathway to regulate bone formation in the adult skeleton." Endocrinology **148**(6): 2635-2643.

Barrios, C., L. A. Brostrom, et al. (1993). "Healing complications after internal fixation of trochanteric hip fractures: the prognostic value of osteoporosis." J Orthop Trauma **7**(5): 438-442.

Bianco, P., M. Riminucci, et al. (2001). "Bone marrow stromal stem cells: nature, biology, and potential applications." Stem Cells **19**(3): 180-192.

Bielby, R., E. Jones, et al. (2007). "The role of mesenchymal stem cells in maintenance and repair of bone." Injury **38 Suppl 1**: S26-32.

Binder, N. B., A. Puchner, et al. (2013). "Tumor necrosis factor-inhibiting therapy preferentially targets bone destruction but not synovial inflammation in a tumor necrosis factor-driven model of rheumatoid arthritis." Arthritis Rheum **65**(3): 608-617.

Bluml, S., C. Scheinecker, et al. (2012). "Targeting TNF receptors in rheumatoid arthritis." Int Immunol **24**(5): 275-281.

Bonewald, L. F. (2011). "The amazing osteocyte." J Bone Miner Res **26**(2): 229-238.

Bosch, P., D. S. Musgrave, et al. (2000). "Osteoprogenitor cells within skeletal muscle." J Orthop Res **18**(6): 933-944.

Bostrom, M. P., J. M. Lane, et al. (1995). "Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing." J Orthop Res **13**(3): 357-367.

Callard, R. E. and A. J. H. Gearing (1994). The Cytokine Factsbook, Academic Press.

Cawthorn, W. P., F. Heyd, et al. (2007). "Tumour necrosis factor-alpha inhibits adipogenesis via a beta-catenin/TCF4(TCF7L2)-dependent pathway." Cell Death Differ **14**(7): 1361-1373.

Center, J. R., T. V. Nguyen, et al. (1999). "Mortality after all major types of osteoporotic fracture in men and women: an observational study." Lancet **353**(9156): 878-882.

Chan, J. K., L. Harry, et al. (2012). "Soft-tissue reconstruction of open fractures of the lower limb: muscle versus fasciocutaneous flaps." Plast Reconstr Surg **130**(2): 284e-295e.

Chan, J. K., J. Roth, et al. (2012). "Alarmins: awaiting a clinical response." J Clin Invest **122**(8): 2711-2719.

Chang, J. K., C. J. Li, et al. (2007). "Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells." Biochem Pharmacol **74**(9): 1371-1382.

Chang, M. K., L. J. Raggatt, et al. (2008). "Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro and in vivo." J Immunol **181**(2): 1232-1244.

Chen, Y., H. C. Whetstone, et al. (2007). "Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing." PLoS Med **4**(7): e249.

Colucci, S., V. Minielli, et al. (1998). "Alendronate reduces adhesion of human osteoclast-like cells to bone and bone protein-coated surfaces." Calcif Tissue Int **63**(3): 230-235.

Cooper, C. and N. C. Harvey (2012). "Osteoporosis risk assessment." BMJ **344**: e4191.

Cornell, C. N. (2003). "Internal fracture fixation in patients with osteoporosis." J Am Acad Orthop Surg **11**(2): 109-119.

de Boer, W. I., J. K. Sont, et al. (2000). "Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD." J Pathol **190**(5): 619-626.

Dimitroulas, T., S. N. Nikas, et al. (2013). "Biologic therapies and systemic bone loss in rheumatoid arthritis." Autoimmun Rev **12**(10): 958-966.

Dimmen, S., L. Nordsletten, et al. (2008). "Negative effect of parecoxib on bone mineral during fracture healing in rats." Acta Orthop **79**(3): 438-444.

Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Donaldson, L. J., I. P. Reckless, et al. (2008). "The epidemiology of fractures in England." J Epidemiol Community Health **62**(2): 174-180.

Eghbali-Fatourechi, G., S. Khosla, et al. (2003). "Role of RANK ligand in mediating increased bone resorption in early postmenopausal women." J Clin Invest **111**(8): 1221-1230.

Einhorn, T. A. (1995). "Enhancement of fracture-healing." J Bone Joint Surg Am **77**(6): 940-956.

Eriksen, E. F. (1986). "Normal and pathological remodeling of human trabecular bone: three dimensional reconstruction of the remodeling sequence in normals and in metabolic bone disease." Endocr Rev **7**(4): 379-408.

Eriksen, E. F., G. Z. Eghbali-Fatourechi, et al. (2007). "Remodeling and vascular spaces in bone." J Bone Miner Res **22**(1): 1-6.
Evans, C. H. (2010). "Gene therapy for bone healing." Expert Rev Mol Med **12**: e18.
Evans, C. H., F. J. Liu, et al. (2009). "Use of genetically modified muscle and fat grafts to repair defects in bone and cartilage." Eur Cell Mater **18**: 96-111.
Forsberg, J. A., J. M. Pepek, et al. (2009). "Heterotopic ossification in high-energy wartime extremity injuries: prevalence and risk factors." J Bone Joint Surg Am **91**(5): 1084-1091.
Forsberg JA, P. J., Wagner S (2009). "Heterotopic Ossification in high energy wartime extremity injuries: prevalence and risk factors." J Bone Joint Surg Am **91**(5): 8.
Francel, T. J., C. A. Vander Kolk, et al. (1992). "Microvascular soft-tissue transplantation for reconstruction of acute open tibial fractures: timing of coverage and long-term functional results." Plast Reconstr Surg **89**(3): 478-487; discussion 488-479.
Friedlaender, G. E., C. R. Perry, et al. (2001). "Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions." J Bone Joint Surg Am **83-A Suppl 1**(Pt 2): S151-158.
Fukui, N., Y. Ikeda, et al. (2006). "Pro-inflammatory cytokine tumor necrosis factor-alpha induces bone morphogenetic protein-2 in chondrocytes via mRNA stabilization and transcriptional up-regulation." J Biol Chem **281**(37): 27229-27241.
Garrison, K. R., I. Shemilt, et al. (2010). "Bone morphogenetic protein (BMP) for fracture healing in adults." Cochrane Database Syst Rev(6): CD006950.
Gelb, B. D., G. P. Shi, et al. (1996). "Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency." Science **273**(5279): 1236-1238.
Geng, J. G. (2001). "Directional migration of leukocytes: their pathological roles in inflammation and strategies for development of anti-inflammatory therapies." Cell Res **11**(2): 85-88.
Gerstenfeld, L. C., T. J. Cho, et al. (2001). "Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling." Cells Tissues Organs **169**(3): 285-294.
Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.
Gerstenfeld, L. C., D. M. Cullinane, et al. (2003). "Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation." J Cell Biochem **88**(5): 873-884.
Gerszten, R. E., E. A. Garcia-Zepeda, et al. (1999). "MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions." Nature **398**(6729): 718-723.
Glass, G. E., S. P. Barrett, et al. (2011). "The microbiological basis for a revised antibiotic regimen in high-energy tibial fractures: preventing deep infections by nosocomial organisms." J Plast Reconstr Aesthet Surg **64**(3): 375-380.
Glass, G. E., J. K. Chan, et al. (2011). "TNF-{alpha} promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A **108**(4): 1585-1590.
Gopal, S., P. V. Giannoudis, et al. (2004). "The functional outcome of severe, open tibial fractures managed with early fixation and flap coverage." J Bone Joint Surg Br **86**(6): 861-867.
Govender, S., C. Csimma, et al. (2002). "Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients." J Bone Joint Surg Am **84-A**(12): 2123-2134.
Granero-Molto, F., J. A. Weis, et al. (2009). "Regenerative effects of transplanted mesenchymal stem cells in fracture healing." Stem Cells **27**(8): 1887-1898.
Grundnes, O. and O. Reikeras (1993). "The importance of the hematoma for fracture healing in rats." Acta Orthop Scand **64**(3): 340-342.
Harris, A. M., P. L. Althausen, et al. (2009). "Complications following limb-threatening lower extremity trauma." J Orthop Trauma **23**(1): 1-6.
Harry, L. E., A. Sandison, et al. (2008). "Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model." J Orthop Res **26**(9): 1238-1244.
Harry, L. E., A. Sandison, et al. (2009). "Comparison of the vascularity of fasciocutaneous tissue and muscle for coverage of open tibial fractures." Plast Reconstr Surg **124**(4): 1211-1219.
Harvey, N., E. Dennison, et al. (2010). "Osteoporosis: impact on health and economics." Nat Rev Rheumatol **6**(2): 99-105.
Hess, K., A. Ushmorov, et al. (2009). "TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway." Bone **45**(2): 367-376.
Hofbauer, L. C., D. L. Lacey, et al. (1999). "Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells." Bone **25**(3): 255-259.
Hoff, P., P. Maschmeyer, et al. (2013). "Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model." Cell Mol Immunol **10**(2): 151-158.
Hogan, B. L. (1996). "Bone morphogenetic proteins: multifunctional regulators of vertebrate development." Genes Dev **10**(13): 1580-1594.
Holroyd, C., C. Cooper, et al. (2008). "Epidemiology of osteoporosis." Best Pract Res Clin Endocrinol Metab **22**(5): 671-685.
Homma, Y., G. Zimmermann, et al. (2013). "Cellular therapies for the treatment of non-union: the past, present and future." Injury **44 Suppl 1**: S46-49.
Horuk, R. (2001). "Chemokine receptors." Cytokine Growth Factor Rev **12**(4): 313-335.
Horwitz, E. M., K. Le Blanc, et al. (2005). "Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement." Cytotherapy **7**(5): 393-395.
Howard, G. A., B. L. Bottemiller, et al. (1981). "Parathyroid hormone stimulates bone formation and resorption in organ culture: evidence for a coupling mechanism." Proc Natl Acad Sci U S A **78**(5): 3204-3208.

Hughes, D. E., K. R. Wright, et al. (1995). "Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo." J Bone Miner Res **10**(10): 1478-1487.

Hume, D. A., J. F. Loutit, et al. (1984). "The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of bone and associated connective tissue." J Cell Sci **66**: 189-194.

Hutmacher, D. W. and M. Sittinger (2003). "Periosteal cells in bone tissue engineering." Tissue Eng **9 Suppl 1**: S45-64.

Im, G. I., Y. W. Shin, et al. (2005). "Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells?" Osteoarthritis Cartilage **13**(10): 845-853.

Iorio R, H. W. (2002). "Heterotopic ossification after hip and knee arthroplasty: risk factors, prevention and treatment." J Am Acad Orthop Surg **10**(6): 8.

Ito, S., N. Suzuki, et al. (2007). "Glucocorticoids induce the differentiation of a mesenchymal progenitor cell line, ROB-C26 into adipocytes and osteoblasts, but fail to induce terminal osteoblast differentiation." Bone **40**(1): 84-92.

Iwata, H., S. Sakano, et al. (2002). "Demineralized bone matrix and native bone morphogenetic protein in orthopaedic surgery." Clin Orthop Relat Res(395): 99-109.

Jaiswal, N., S. E. Haynesworth, et al. (1997). "Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro." J Cell Biochem **64**(2): 295-312.

Jingushi, S., K. Urabe, et al. (2002). "Intramuscular bone induction by human recombinant bone morphogenetic protein-2 with beta-tricalcium phosphate as a carrier: in vivo bone banking for muscle-pedicle autograft." J Orthop Sci **7**(4): 490-494.

Johnell, O. and J. A. Kanis (2006). "An estimate of the worldwide prevalence and disability associated with osteoporotic fractures." Osteoporos Int **17**(12): 1726-1733.

Junker, J. P., P. Sommar, et al. (2010). "Adipogenic, chondrogenic and osteogenic differentiation of clonally derived human dermal fibroblasts." Cells Tissues Organs **191**(2): 105-118.

Kanakaris, N. K., G. Petsatodis, et al. (2009). "Is there a role for bone morphogenetic proteins in osteoporotic fractures?" Injury **40 Suppl 3**: S21-26.

Keating, J. F., P. A. Blachut, et al. (2000). "Reamed nailing of Gustilo grade-IIIB tibial fractures." J Bone Joint Surg Br **82**(8): 1113-1116.

Kellinsalmi, M., V. Parikka, et al. (2007). "Inhibition of cyclooxygenase-2 down-regulates osteoclast and osteoblast differentiation and favours adipocyte formation in vitro." Eur J Pharmacol **572**(2-3): 102-110.

Khosla, S., J. J. Westendorf, et al. (2008). "Building bone to reverse osteoporosis and repair fractures." J Clin Invest **118**(2): 421-428.

Kim, H. W. and J. S. Jahng (1999). "Effect of intermittent administration of parathyroid hormone on fracture healing in ovariectomized rats." Iowa Orthop J **19**: 71-77.

Kim, W. Y., C. H. Han, et al. (2001). "Failure of intertrochanteric fracture fixation with a dynamic hip screw in relation to pre-operative fracture stability and osteoporosis." Int Orthop **25**(6): 360-362.
Klamut, H. J., S. T. Chen, et al. (2004). "Progress toward skeletal gene therapy." Crit Rev Eukaryot Gene Expr **14**(1-2): 89-136.
Kolaczkowska, E. and P. Kubes (2013). "Neutrophil recruitment and function in health and inflammation." Nat Rev Immunol **13**(3): 159-175.
Kolar, P., T. Gaber, et al. (2011). "Human early fracture hematoma is characterized by inflammation and hypoxia." Clin Orthop Relat Res **469**(11): 3118-3126.
Kolar, P., K. Schmidt-Bleek, et al. (2010). "The early fracture hematoma and its potential role in fracture healing." Tissue Eng Part B Rev **16**(4): 427-434.
Komatsu, D. E., K. A. Brune, et al. (2009). "Longitudinal in vivo analysis of the region-specific efficacy of parathyroid hormone in a rat cortical defect model." Endocrinology **150**(4): 1570-1579.
Komatsu, D. E., M. N. Mary, et al. (2010). "Modulation of Wnt signaling influences fracture repair." J Orthop Res **28**(7): 928-936.
Komatsu, D. E. and S. J. Warden (2010). "The control of fracture healing and its therapeutic targeting: improving upon nature." J Cell Biochem **109**(2): 302-311.
Kon, T., T. J. Cho, et al. (2001). "Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing." J Bone Miner Res **16**(6): 1004-1014.
Kosaka, T., A. Imakiire, et al. (2000). "Activation of nuclear factor kappaB at the onset of ossification of the spinal ligaments." J Orthop Sci **5**(6): 572-578.
Kuziel, W. A., S. J. Morgan, et al. (1997). "Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2." Proc Natl Acad Sci U S A **94**(22): 12053-12058.
Lane, J. M. (2001). "BMPs: why are they not in everyday use?" J Bone Joint Surg Am **83-A Suppl 1**(Pt 2): S161-163.
Lewinson, D., G. Maor, et al. (2001). "Expression of vascular antigens by bone cells during bone regeneration in a membranous bone distraction system." Histochem Cell Biol **116**(5): 381-388.
Lewis, M., L. A. Tartaglia, et al. (1991). "Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific." Proc Natl Acad Sci U S A **88**(7): 2830-2834.
Lieberman, J. R., A. Daluiski, et al. (2002). "The role of growth factors in the repair of bone. Biology and clinical applications." J Bone Joint Surg Am **84-A**(6): 1032-1044.
Lill, C. A., J. Hesseln, et al. (2003). "Biomechanical evaluation of healing in a non-critical defect in a large animal model of osteoporosis." J Orthop Res **21**(5): 836-842.
Liu, R., A. Schindeler, et al. (2010). "The potential role of muscle in bone repair." J Musculoskelet Neuronal Interact **10**(1): 71-76.
Lorenzo, J. and Y. Choi (2005). "Osteoimmunology." Immunol Rev **208**: 5-6.
Lorenzo, J., M. Horowitz, et al. (2008). "Osteoimmunology: interactions of the bone and immune system." Endocr Rev **29**(4): 403-440.

Lu, Z., G. Wang, et al. (2013). "Activation and promotion of adipose stem cells by tumour necrosis factor-alpha preconditioning for bone regeneration." J Cell Physiol **228**(8): 1737-1744.
Ma, M., T. Wei, et al. (2002). "Monocyte recruitment and myelin removal are delayed following spinal cord injury in mice with CCR2 chemokine receptor deletion." J Neurosci Res **68**(6): 691-702.
Marcus, R. (1987). "Normal and abnormal bone remodeling in man." Annu Rev Med **38**: 129-141.
Marie, P. J., P. Ammann, et al. (2001). "Mechanisms of action and therapeutic potential of strontium in bone." Calcif Tissue Int **69**(3): 121-129.
McCann, R. M., G. Colleary, et al. (2008). "Effect of osteoporosis on bone mineral density and fracture repair in a rat femoral fracture model." J Orthop Res **26**(3): 384-393.
Medici, D., E. M. Shore, et al. (2010). "Conversion of vascular endothelial cells into multipotent stem-like cells." Nat Med **16**(12): 1400-1406.
Mizoguchi, T., A. Muto, et al. (2009). "Identification of cell cycle-arrested quiescent osteoclast precursors in vivo." J Cell Biol **184**(4): 541-554.
Mosekilde, L., O. Torring, et al. (2011). "Emerging anabolic treatments in osteoporosis." Curr Drug Saf **6**(2): 62-74.
Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-969.
Namkung-Matthai, H., R. Appleyard, et al. (2001). "Osteoporosis influences the early period of fracture healing in a rat osteoporotic model." Bone **28**(1): 80-86.
Nanchahal, N., J. Nayagam, et al. (2009). Standards for the management of open fractures of the lower limb. London, Royal Society of Medicine Press.
Nicolaidou, V., M. M. Wong, et al. (2012). "Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation." PLoS One **7**(7): e39871.
Niemeyer, P., K. Fechner, et al. (2010). "Comparison of mesenchymal stem cells from bone marrow and adipose tissue for bone regeneration in a critical size defect of the sheep tibia and the influence of platelet-rich plasma." Biomaterials **31**(13): 3572-3579.
O'Driscoll, S. W., D. B. Saris, et al. (2001). "The chondrogenic potential of periosteum decreases with age." J Orthop Res **19**(1): 95-103.
Oshina, H., S. Sotome, et al. (2007). "Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells." Bone **41**(4): 575-583.
Paula, F. S. and J. D. Alves (2014). "Non-tumor necrosis factor-based biologic therapies for rheumatoid arthritis: present, future, and insights into pathogenesis." Biologics **8**: 1-12.
Pelled, G., A. Ben-Arav, et al. (2010). "Direct gene therapy for bone regeneration: gene delivery, animal models, and outcome measures." Tissue Eng Part B Rev **16**(1): 13-20.
Pizette, S. and L. Niswander (2000). "BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes." Dev Biol **219**(2): 237-249.

Poole, K. E., R. L. van Bezooijen, et al. (2005). "Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation." FASEB J **19**(13): 1842-1844.
Pountos, I., T. Georgouli, et al. (2008). "Pharmacological agents and impairment of fracture healing: what is the evidence?" Injury **39**(4): 384-394.
Qin, L., P. Qiu, et al. (2003). "Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics." J Biol Chem **278**(22): 19723-19731.
Rabe, B., A. Chalaris, et al. (2008). "Transgenic blockade of interleukin 6 transsignaling abrogates inflammation." Blood **111**(3): 1021-1028.
Rachner, T. D., S. Khosla, et al. (2011). "Osteoporosis: now and the future." Lancet **377**(9773): 1276-1287.
Raggatt, L. J. and N. C. Partridge (2010). "Cellular and molecular mechanisms of bone remodeling." J Biol Chem **285**(33): 25103-25108.
Rahimi, P., C. Y. Wang, et al. (1995). "Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse." Endocrinology **136**(6): 2752-2759.
Reginster, J. Y., J. M. Kaufman, et al. (2012). "Maintenance of antifracture efficacy over 10 years with strontium ranelate in postmenopausal osteoporosis." Osteoporos Int **23**(3): 1115-1122.
Robinson, J. A., M. Chatterjee-Kishore, et al. (2006). "Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone." J Biol Chem **281**(42): 31720-31728.
Rodan, G. A. and H. A. Fleisch (1996). "Bisphosphonates: mechanisms of action." J Clin Invest **97**(12): 2692-2696.
Rodriguez, J. P., S. Garat, et al. (1999). "Abnormal osteogenesis in osteoporotic patients is reflected by altered mesenchymal stem cells dynamics." J Cell Biochem **75**(3): 414-423.
Rodriguez, J. P., L. Montecinos, et al. (2000). "Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation." J Cell Biochem **79**(4): 557-565.
Rossini, M., D. Gatti, et al. (2013). "Involvement of WNT/beta-catenin signaling in the treatment of osteoporosis." Calcif Tissue Int **93**(2): 121-132.
Sato, M., W. Grasser, et al. (1991). "Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure." J Clin Invest **88**(6): 2095-2105.
Schindeler, A., R. Liu, et al. (2009). "The contribution of different cell lineages to bone repair: exploring a role for muscle stem cells." Differentiation **77**(1): 12-18.
Schober, A., A. Zernecke, et al. (2004). "Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets." Circ Res **95**(11): 1125-1133.
Seekamp, A., G. Regel, et al. (1996). "Rehabilitation and reintegration of multiply injured patients: an outcome study with special reference to multiple lower limb fractures." Injury **27**(2): 133-138.
Sekiya, I., J. T. Vuoristo, et al. (2002). "In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and

molecular events during chondrogenesis." Proc Natl Acad Sci U S A **99**(7): 4397-4402.
Shirley, D., D. Marsh, et al. (2005). "Systemic recruitment of osteoblastic cells in fracture healing." J Orthop Res **23**(5): 1013-1021.
Shirley D, M. D., Jordan G, McQuaid S, Li G (2005). "Systemic recruitment of osteoblastic cells in fracture healing." J Orthop Res **23**(5): 9.
Shore, E. M. and F. S. Kaplan (2008). "Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP)." Bone **43**(3): 427-433.
Shore, E. M., M. Xu, et al. (2006). "A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva." Nat Genet **38**(5): 525-527.
Si, X., Y. Jin, et al. (1997). "Expression of BMP-2 and TGF-beta 1 mRNA during healing of the rabbit mandible." Eur J Oral Sci **105**(4): 325-330.
Simon, A. M. and J. P. O'Connor (2007). "Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing." J Bone Joint Surg Am **89**(3): 500-511.
Soehnlein, O. and L. Lindbom (2010). "Phagocyte partnership during the onset and resolution of inflammation." Nat Rev Immunol **10**(6): 427-439.
Spencer, G. J., J. C. Utting, et al. (2006). "Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro." J Cell Sci **119**(Pt 7): 1283-1296.
Sterck, J. G., J. Klein-Nulend, et al. (1998). "Response of normal and osteoporotic human bone cells to mechanical stress in vitro." Am J Physiol **274**(6 Pt 1): E1113-1120.
Theoleyre, S., Y. Wittrant, et al. (2004). "The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling." Cytokine Growth Factor Rev **15**(6): 457-475.
Tsou, C. L., W. Peters, et al. (2007). "Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites." J Clin Invest **117**(4): 902-909.
Tsuji, K., K. Cox, et al. (2008). "BMP4 is dispensable for skeletogenesis and fracture-healing in the limb." J Bone Joint Surg Am **90 Suppl 1**: 14-18.
Urist, M. R. (1965). "Bone: formation by autoinduction." Science **150**(3698): 893-899.
Utvag, S. E., O. Grundnes, et al. (2003). "Influence of extensive muscle injury on fracture healing in rat tibia." J Orthop Trauma **17**(6): 430-435.
van Bezooijen, R. L., B. A. Roelen, et al. (2004). "Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist." J Exp Med **199**(6): 805-814.
Verjee, L. S., J. S. Verhoekx, et al. (2013). "Unraveling the signaling pathways promoting fibrosis in Dupuytren's disease reveals TNF as a therapeutic target." Proc Natl Acad Sci U S A **110**(10): E928-937.
Vestergaard, P., L. Rejnmark, et al. (2005). "Reduced relative risk of fractures among users of lithium." Calcif Tissue Int **77**(1): 1-8.
Warden, S. J., D. E. Komatsu, et al. (2009). "Recombinant human parathyroid hormone (PTH 1-34) and low-intensity pulsed ultrasound have contrasting additive effects during fracture healing." Bone **44**(3): 485-494.

Williams, S. R., Y. Jiang, et al. (1992). "Regulated expression of monocyte chemoattractant protein-1 in normal human osteoblastic cells." Am J Physiol **263**(1 Pt 1): C194-199.
Wilting, I., F. de Vries, et al. (2007). "Lithium use and the risk of fractures." Bone **40**(5): 1252-1258.
Wozney, J. M., V. Rosen, et al. (1988). "Novel regulators of bone formation: molecular clones and activities." Science **242**(4885): 1528-1534.
Wu, X., W. Shi, et al. (2007). "Multiplicity of BMP signaling in skeletal development." Ann N Y Acad Sci **1116**: 29-49.
Wythe, S. E., V. Nicolaidou, et al. (2013). "Cells of the Immune System Orchestrate Changes in Bone Cell Function." Calcif Tissue Int.
Yadav, A., V. Saini, et al. (2010). "MCP-1: chemoattractant with a role beyond immunity: a review." Clin Chim Acta **411**(21-22): 1570-1579.
Yang, X., B. F. Ricciardi, et al. (2007). "Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice." Bone **41**(6): 928-936.
Zacks, S. I. and M. F. Sheff (1982). "Periosteal and metaplastic bone formation in mouse minced muscle regeneration." Lab Invest **46**(4): 405-412.
Zhang, H. and A. Bradley (1996). "Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development." Development **122**(10): 2977-2986.

Chapter 2

Materials and Methods

Methods are presented in order of appearance in thesis chapters.

Where techniques were performed by persons other than JKKC, this has been acknowledged in the appropriate sections in chapter 2 and subsequent chapters.

Chapter 2
Materials and Methods

2.1 WEHI assay

WEHI 164 mouse monocyte cell line (clone 13) was grown in DMEM with 5% FCS until confluent and TNF bioactivity determined(Espevik and Nissen-Meyer 1986). The absorbance of the blue formazan product was read at A_{620nm}.

2.2 Murine fracture model

Animal fracture model procedures were approved by the institutional ethics committee and the United Kingdom Home Office (PLL 71/7161). Skeletally mature (12-14 week old) female C57/BL6 mice (non-ovariactomised and ovariectomised) were obtained from Charles River, UK. Normal chow and drinking water were provided at libitum. The murine fracture procedure was performed as previously described (Harry, Sandison et al. 2008). Briefly, an incision is made over the tibia. The periosteum is stripped circumferentially, the medullary canal is reamed and a 0.38-mm intramedullary fixation pin is inserted. An osteotomy is created at the junction of the middle and distal thirds of the tibia and the fixation pin cut flush with the tibial cortex and skin closed directly with non-absorbable sutures. Each experimental group comprised at least 6 animals.

The periosteum contains a cellular cambium layer, which consists of osteoprogenitor cells and osteoblasts. It contributes substantially to fracture healing in the skeletally immature human and adult mice. The osteoprogenitor cells contribute to both intramembranous and endochondral healing and the contribution of the periosteum can be demonstrated radiographically by a 'periosteal reaction'. However, in skeletally mature humans, the cambium layer

regresses and becomes sparsely cellular. In order to mimic the fracture healing process in the skeletally mature human as closely as possible, the periosteum of the mouse is stripped.

Anti-TNF: TN3-19.12, a neutralizing hamster IgG1 anti-TNF-α/β mAb (Leinco Technologies) or IgG control was given (1 mg) via intraperitoneal injections immediately and on days 2, 5, 8 after operation.

rmIL10 (R&D) was injected locally at the fractures site at 10 ng in 20 μL PBS immediately and 24 hours after operation; PBS was used as vehicle control.

Anti-IL10R or IgG isotype control (R&D) was given (2 μg) via intraperitoneal injections – timing as described in Chapter 3.

KC 30 ug/ml * 20 uL locally at fractures site immediately and at 24 hours after operation; PBS control

Neutralizing anti-CXCR2 antibody (R&D) 50 ug/mouse i.p. 1 hour pre-fracture and day 3 post fracture
Control: IgG2A

Anti-Ly6G-specific monoclonal antibody, 1A8, or IgG_{2A} isotype control, 2A3 (BioXCell) (Daley, Thomay et al. 2008) was given (0.5 mg) via intraperitoneal injections on days 1 and 3 before and after operation.

The CCR2 antagonist, INCB3344 (Brodmerkel, Huber et al. 2005; Shin, Baribaud et al. 2009; Xue, Wang et al. 2010; Xie, Kamei et al. 2011; Chan, Moore et al. 2012), was administered at 30 mg/kg/day via daily intraperitoneal injections starting 3 hours before operation and continuing until and including day 8 after operation (Haoyuan Chemexpress Co Ltd). Vehicle control was 10% DMSO/0.9% carboxymethylcellulose.

rmCCL2 (R&D) was injected locally at the fracture site 10 ng or 100 ng in 20 μL PBS immediately and 24 hours after operation; PBS was used as vehicle control.

TACE/ADAM17 (R&D) was injected locally at the fracture site 1 ng in 20 μL 30 minutes before operation, immediately and 24 hours after operation.

2.3 MicroCT scanning and analysis

Mouse tibiae were harvested at 14 or 28 days post fracture and fixed in 70% ethanol. Each bone was scanned with a Skyscan 1174 scanner (SkyScan, Kontich, Belgium), 50kV, 800μA, 8.3μm isometric voxel resolution, 0.7 degree rotation step. Bones exhibiting angulation, rotation or hypertrophic non-union were excluded. Scans were analyzed using SkyScan CTAnalyser software, version 1.9.3.0 (SkyScan, Kontich, Belgium). Each bone was analyzed by selecting a volume of interest commencing at the fracture site and proceeding proximally for 363 slices (3mm in height). This region was selected to avoid potential confounding effects of the fibula. Total callus volume was delineated from surrounding tissues and the original bone of the tibia using hand-contoured

regions of interest. Mineralized callus volume was determined using global thresholding at a density range calibrated to bone mineral density of 350 gm/cm^3, as previously described(Harry, Sandison et al. 2008). Percent callus mineralization was calculated as mineralized callus volume divided by total callus volume and normalized against vehicle (PBS) or IgG isotype control.

2.4 Bend testing

3 point flexural bend testing was performed using Instron 5942 single column table top system for low-force universal testing at the Kennedy Institute of Rheumatology. A specifically designed 3 point bending jig was used with a load cell of 40 newtons specificity. The procedure was performed on bones that had been dehydrated in 70% ethanol at 4^0C for storage and then rehydrated for a minimum of 3 hours in PBS at room temperature prior to testing. All soft tissues were dissected from the bone so that any callus formed is left intact at the fracture site. The limb was disarticulated at the knee and hind foot, leaving the tibia, fibula and tibiofibular joint distally. The specimen was placed in a medio-lateral orientation on the support span of the jib with the fracture callus within the support points, a distance of 8 mm apart. The specimen was loaded so that the medial side of the fracture was under compression whereas the lateral aspect on the undersurface would be under tension. The load cell is applied directly onto the callus. The sample is preloaded to 0.01 N and re-zeroed at top surface. Load was applied at a rate of 1 mm/minute until failure. The force-extension profiles are recorded and analysed using BlueHill® Software.

2.5 Assessing weight distribution using a Linton incapacitance tester.

Changes in weight distribution between the right (ipsilateral/injected) and left (contralateral/uninjected) were measured to indicate the level of joint discomfort. The force exerted by each hind limb was averaged over 3-5 seconds. This measurement was taken 3 times, converted into a percentage and averaged over the 3 measurements. Weight bearing was calculated as follows: Right x 100/Left = weight bearing %. Equal weight distribution is 100%, 70% or lower is classified as leaning

2.6 Luminex

The multiplex bead immunoassay is a solid phase 'sandwich' immunoassay designed to be read using a Luminex xMAP system (Luminex Corp, Austin TX.). Polystyrene microsphere measuring 5.6 μm in diameter are pre-coated with antibodies specific to the cytokines tested. The coated beads were added to each well of a pre-wetted filter bottom 96-well micro plate with the sample or standard, and incubated for 2 hours to permit binding of the analytes to the beads. After washing, a solution containing a mixture of biotinylated, analyte-specific antibodies was added and incubated for a further 1 hour. This stage permits binding of the biotinylated antibodies to the bound analytes on

polystyrene beads. After further washing, streptavidin, conjugated to R-Phycoerythrin (a fluorescent protein) was added and incubated for a further 30 minutes. During this stage, the fluorescent protein bound streptavidin binds to the biotinylated antibody forming a solid-phase sandwich. After repeated washing the plate is read using a Luminex 100™ instrument. The fluidics system of the Luminex 100™ permits a stream of suspended microspheres to line up in a single file prior to passing through the detection chamber. As each microsphere passes through the detection chamber, a red laser excites both the red and infra-red dyes, permitting a unique signature of fluorescence corresponding to a 'set' on the bead, which in turn represents the site of residence of a named analyte antibody. Simultaneously, a green laser excites the bound fluorescent protein (associated with binding of the analyte). Precision is ensured as each particle is read individually and a mean value is generated using the Luminex software,

2.7 Electrochemiluminescence

Electrochemiluminescence is an immunoassay that uses labels designed to emit light when electrochemically stimulated and detects binding events on patterned arrays. It provides a highly sensitive and specific method of quantification of analytes. MSD® SECTOR Imager 2400 was used to read multiplex 96-well plates purchased from MSD®. Immunoassay protocol was performed as per manufacturer's instructions.

2.8 Murine fracture supernatant

C57BL/6 mice were anaesthetized and closed fractures were created in the lower limbs. Mice are culled at 3 hours post-operatively and the fractured bone segments are harvested and incubated in serum-free DMEM + 1% penicillin/streptomycin for 12 hours. Cytokine levels were measured using a proinflammatory 7-plex plate and a SECTOR Imager 2400 reader (Mesoscale Discovery).

2.9 RT-PCR

Real-time reverse transcription PCR Inventoried TaqMan® Gene expression Assays for TNF, IL-6, IL-1b, IL-10 were used (Applied Biosystems) with Reverse Transcriptase qPCR™ Mastermix No ROX (Eurogentec). All other reagents were purchased from Qiagen.

2.10 *In situ* hybridisation

Murine lower limbs were fixed in 4% paraformaldehyde overnight and decalcified for 6 weeks in a 50% mixture of 20% EDTA and 4% paraformaldehyde. Care was taken during processing and section cutting to avoid RNAse contamination.

Specific localization of *Tnf* mRNA was accomplished by *in situ* hybridisation using an antisense riboprobe synthesised with T7 RNA polymerase using ^{35}S-UTP (~800 Ci/mmol; Perkin Elmer UK) IMAGE clone 40126376 (Source BioScience UK) containing the sequence of interest. A clone was grown in the

presence of kanamycin, purified using a Qiagen maxi prep kit and the resultant plasmid linearised with SpeI to prepare a template that made a 768 base antisense riboprobe that was used without hydrolysis. The region of sequence used to produce the riboprobe did not show significant homology to any other known mouse mRNA sequences in the RefSeq.

The methods for pre-treatment, hybridisation, washing and dipping of slides in Ilford K5 for autoradiography were performed as previously described(Steddon and Cunningham 2005) for formalin- fixed paraffin-embedded tissue with some modifications (Fromigue, Hay et al. 2009).

The presence of hybridisable mRNA in all compartments of the tissues studied was established in near serial sections using an antisense β-actin probe. Autoradiography was carried out at 4°C (two exposures per section at 8 and 15 days) before developing in Kodak D19 and counterstaining by Giemsa's method. Sections were examined under conventional or reflected light dark-field conditions (Nikon Eclipse ME600 epi-illumination microscope with Q imaging MicroPublisher 5.0 camera) that allowed individual autoradiographic silver grains to be seen as bright objects on a dark background.

2.11 Immunohistochemistry

Monocytes/macrophages: Limbs were fixed in 4% paraformaldehyde overnight and decalcified for 8-10 weeks in 15% EDTA. The limbs were bisected longitudinally and paraffin-embedded and 4 μm sections were cut.

Immunohistochemistry (IHC) was performed on deparaffinised and rehydrated sections, with specific primary antibody against F4/80 (rat anti-mouse (Biolegends), as previously described (Alexander, Chang et al. 2011), or anti-neutrophil elastase (Abcam), or anti-Ly6G (Abcam). All sections were counterstained using Mayer's haematoxylin (Leica Biosystems, UK). Masson's Trichrome staining was also used. Tissue staining was viewed using an Olympus BX51 microscope with an Olympus DP71 camera and DP Manager (Olympus) imaging software.

2.12 Quantification of circulating neutrophils

Mice were anaesthetized with pentobarbital until unresponsive to stimuli and a cardiac puncture was performed. Blood was collected into a syringe containing 100 µl sodium citrate. Blood smears were stained with DiffQuik for the enumeration of neutrophils and mononuclear cells. 6 animals per time point.

2.13 Air pouch model and FACS

10-12 week C57BL/6 female mice (Charles River, UK) were used. Air pouch model procedures were approved by the institutional ethics committee and the United Kingdom Home Office (PLL 70/7335). Air pouches were produced as previously described (Romano, Faggioni et al. 1997; Vestergaard, Rejnmark et al. 2005; Wilting, de Vries et al. 2007; Mosekilde, Torring et al. 2011) on the dorsum of the mice by subcutaneous injection of 5 ml sterile air on day 0 and 2 ml on day 4. On day 5, the animals were randomized into groups (minimum 6 animals per group) and the experiment was conducted in a double-blinded manner. Air

pouches were injected with 400 µl of media, fracture supernatant or fracture supernatant and 1 ng of rhTNF. The animals were sacrificed and air pouches lavaged with 2 ml of PBS at 4 hours post injection. The exudate was centrifuged at 1500 for 5 minutes and pellets containing the migrated cells were re-suspended in fresh PBS for counting using a haematocytometer following red cell lysis treatment. Characterization of leukocyte subpopulations migrating into the pouch space was determined by flow-cytometry staining with Ly-6G PE (clone 1A8, Miltenyi Biotec, UK), CD11b FITC (clone M1/70, eBioscience, UK) and CD115 APC (clone AFS98, eBioscience, UK) antibodies. PMNs were identified as Ly-6G$^+$, CD11b$^+$ cells, while the monocytic cells were identified as Ly-6G$^-$, CD11b$^+$ and CD115$^+$ cells as described previously(Vestergaard, Rejnmark et al. 2005). The levels of mCCL2 cytokine in the exudates were analysed with a SECTOR Imager 2400 reader (Meso Scale Discovery®).

All flow cytometry and analysis was performed by Dr. Adel Ersek (postdoctoral scientist, Kennedy Institute of Rheumatology). Cells from the air pouch exudate were suspended in PBS and 2% FBS at a concentration of 1x10^6 cells per ml and were divided into aliquots of 1x10^6 cells per ml and per FACS tube. Cell aliquots were stained with Ly-6G PE (clone 1A8, Miltenyi Biotec, UK), CD11b FITC (clone M1/70, eBioscience, UK) and CD115 APC (clone AFS98, eBioscience, UK) anti-mouse antibodies for 30 minutes at room temperature. After washing with PBS, typically a minimal of 20,000 events were acquired by BD-LSR II bench-top flow cytometer (BD Biosciences, NJ, USA) and the data analysed using FlowJo software for windows (Tree Star Inc.). Appropriate isotype controls were also

included. The total numbers of subsets of cells were derived by multiplying the percentage of cells with the total number of cells counted.

2.14 Transwell assay

5 μm transwell membranes (VWR International Ltd) were placed into wells of a 24-well plate, each containing 500 μl of test media enabling immersion of the transwell membranes. Triplicates were used for each test condition. 200 * 10^3 monocytes were added to the upper chamber of each transwell and incubated at 5% CO_2 and 37 ^oC for 14 hours.

The membranes were then removed from the wells and the upper chambers cleared of cells and cell debris using a cotton bud. The membrane was then washed gently using PBS and added to a well of another 24 well plate, each well containing 500 μl of 10% neutral buffered formalin. After 1 hour, the fixed transwell membrane was removed and washed gently in tap water. The membrane was then transferred to another well of a new 24 well plate containing 500 μl 1% crystal violet solution for 1 hour. Finally, the membrane was washed gently in tap water.

Using light microscopy, the number of cells present on the transwell membrane in any random field was counted three times and the mean calculated. Cell migration was expressed as a percentage relative to the control.

2.15 In vitro neutrophil experiment

Neutrophils are enriched to 82.7% purity (Suppl Fig 1) using a neutrophil isolation kit according to manufacturer's instructions (Stem Cell Technology) and incubated in 180 µl murine fracture supernatant + 10% FCS + 1% penicillin/streptomycin at 37^0C, 4% CO_2 and a density of 275,000 cells per well in a 96 well plate. rhTNF or PBS (in 20 µl) is added at 30 minutes. Cells and supernatants are harvested at 1 hour. Protein levels of mCCL2 cytokine in the supernatants were analysed as described above. Experiment performed in triplicates.

2.16 Immunocytochemistry

Bone marrow cells are extracted from murine tibiae under sterile conditions. The cells are washed and centrifuged and suspended at 200,000 cells per ml. They were plated on glass coverslips and then exposed to either media + rhTNF at 1 ng/ml (control) or fracture supernatant + rhTNF at 1 ng/ml (treatment). After 4 hours, cells were fixed with 4% paraformaldehyde in PBS for 15 min at 37°C, quenched with 50 mM NH_4Cl/PBS for 10 min, and permeabilised with 0.1% Triton X-100 in PBS for 5 min. Samples were blocked with 3% BSA in PBS for 30 min at room temperature followed by incubation with rabbit anti-neutrophil elastase and rat anti-CCL2 diluted in 3% BSA in PBS for 1 hour at room temperature. After washing steps, staining with the appropriate secondary antibody was performed. Neutrophil elastase staining was performed with Alexa Fluor 488, CCL2 was stained with Alexa Fluor 594 and the nucleus was stained

with DAPI. Samples were mounted with ProGold antifade mounting media and imaged with a fluorescence microscope.

2.17 Human Fracture supernatants

Fracture supernatants were produced to study the influence of the fracture cytokine environment on cells. Fracture fragments were obtained following debridement and surgical reconstruction of open tibial fractures or following amputation for un-reconstructable severe open tibial fractures under the terms of the ethical approval granted by the combined office of research ethics committee (COREC No: 07/Q0411/30).

The following criteria were used to define the donor population:

1. Patients less than 50 years old
2. Delay less than 72 hours
3. No sign of infection or gross contamination at debridement
4. No prior washout procedure performed

Following surgical debridement, fragments of devitalized bone produced by the high energy impact were collected. Where the limb was amputated, bone adjacent to the fracture was cut using a surgical saw (Stryker). These samples were used to produce the fracture supernatant. In patients who underwent amputation for severe ankle or foot trauma, proximal tibia was harvested and divided into segments using a surgical saw. This ensures that bone away from the site of local inflammation is harvested. This non-traumatically injured bone

was used to generate control supernatant. Serum free media was added to fractured or control bone at 5 ml per gram and incubated for 12 hours. The supernatant is then centrifuged at 6000 rpm for 15 minutes to remove all cells and debris before filter sterilization using a 0.2 μm filter (VWR International Ltd, Lutterworth, Leicestershire, UK). The supernatants are divided into aliquots to minimize repeated freeze-thaw cycles and stored at -80^{o}C prior to use.

2.18 MDSCs

Skeletal muscle was harvested from human subjects following surgery at Charing Cross Hospital (COREC No: 07/Q0411/30). All subsequent procedures were performed under sterile conditions in a laminar flow cabinet. The muscle is washed briefly in iodine solution (Videne) and rinsed three times in sterile PBS. The muscle was then finely chopped using sterile instruments and placed in 20 ml of pre-heated digestive medium (dispase) at 37^{o}C. The suspension is periodically agitated in a water bath at 37^{o}C for 30 minutes. It is then passed through a cell strainer and the collected digestate was centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 12 ml culture medium and added to a 10 cm culture plate. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37^{o}C. The culture media (DMEM + 10% FCS + 1% pen/strep) was changed at 24 hours and replaced twice weekly thereafter. Cells were split using trypsin solution when there is 70-80% confluence.

2.19 Alkaline phosphatase (ALP) quantification assay

Alkaline phosphatase is a marker of osteogenic differentiation and measurement of its activity is used to detect osteogenic differentiation of mesenchymal stem cells in vitro. $1x10^4$ human MDSCs (200 µl $5x10^4$ cells per ml in media) were added to wells of a 96 well plate in triplicate. At 24 hours, the media was pipetted off and replaced by 200 µl of test media. The media was replaced at day 3. At day 7, the media was removed and cells lysed in 20 µl NP-40 lysis buffer. An ALP quantification assay (WAKO pure chemical, Japan) was used according to manufacturer's instructions. Calibration solution from the assay (equating to 0.5 mmol/L ALP) was serially diluted to make concentrations of 0.25, 0.125 and 0.0625 mmol/L. 20 µl of calibration solution was added to each well. dH_20 acted as blank control. 100 µl reagent (1 tablet per 5 ml buffer solution) at room temperature was added to each well and incubated at 37°C for 30 minutes. 80 µl cold (4 °C) stop solution was then added to each well and the plate was read using an ELISA spectrophotometer at a wavelength of 405 nm. The concentration of ALP in each test well is determined according to the standard curve.

2.20 Enzyme-Linked ImmunoSorbant Assay (ELISA)

ELISA was used to measure the concentrations of HMGB1 (IBL international), S100A8/9 and TNF (BD Biosciences). These were standard, two antibody 'sandwich' ELISAs. This technique uses a purified antibody specific for the antigen in question, which is pre-coated onto a 96-well polystyrene plate. The sample is added to the well and specific antibody-antigen binding in the presence of antigen. A standard curve is obtained by adding serial dilutions of a standard protein of known concentration. After washing, an enzyme-linked

polyclonal specific to the antigen is added to the well. The well is washed again to remove unbound any antibody-enzyme reagent. A substrate solution in then added to the wells. The extent of the resultant colour change is proportional to the amount of bound antigen present. This reaction is then stopped and the absorbance measured using a spectrophotometer. The concentration of antigen is determined according to the standard curve. The S100A8/9 ELISAs were performed by the laboratory of Prof Thomas Vogl, Institute of Immunology, University of Muenster using their own in-house antibodies and protocol.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad software, San Diego, CA. One-way analyses of variance (ANOVA) with Bonferroni post-test analysis for multiple comparisons or 2-sided student t-test with Welch correction were used to compare different groups of data. Results were considered statistically significant when $P<0.05$. Significant results were expressed using asterisks, where *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. This convention was used throughout. Pearson product-moment correlation was used to measure the strength of a linear relationship between the level of alarmins and ALP activity.

References

Alexander, K. A., M. K. Chang, et al. (2011). "Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model." J Bone Miner Res **26**(7): 1517-1532.

Brodmerkel, C. M., R. Huber, et al. (2005). "Discovery and pharmacological characterization of a novel rodent-active CCR2 antagonist, INCB3344." J Immunol **175**(8): 5370-5378.

Chan, C. T., J. P. Moore, et al. (2012). "Reversal of vascular macrophage accumulation and hypertension by a CCR2 antagonist in deoxycorticosterone/salt-treated mice." Hypertension **60**(5): 1207-1212.

Daley, J. M., A. A. Thomay, et al. (2008). "Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice." J Leukoc Biol **83**(1): 64-70.

Espevik, T. and J. Nissen-Meyer (1986). "A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes." J Immunol Methods **95**(1): 99-105.

Fromigue, O., E. Hay, et al. (2009). "Calcium sensing receptor-dependent and receptor-independent activation of osteoblast replication and survival by strontium ranelate." J Cell Mol Med **13**(8B): 2189-2199.

Harry, L. E., A. Sandison, et al. (2008). "Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model." J Orthop Res **26**(9): 1238-1244.

Mosekilde, L., O. Torring, et al. (2011). "Emerging anabolic treatments in osteoporosis." Curr Drug Saf **6**(2): 62-74.

Romano, M., R. Faggioni, et al. (1997). "Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor." Mediators Inflamm **6**(1): 32-38.

Shin, N., F. Baribaud, et al. (2009). "Pharmacological characterization of INCB3344, a small molecule antagonist of human CCR2." Biochem Biophys Res Commun **387**(2): 251-255.

Steddon, S. J. and J. Cunningham (2005). "Calcimimetics and calcilytics--fooling the calcium receptor." Lancet **365**(9478): 2237-2239.

Vestergaard, P., L. Rejnmark, et al. (2005). "Reduced relative risk of fractures among users of lithium." Calcif Tissue Int **77**(1): 1-8.

Wilting, I., F. de Vries, et al. (2007). "Lithium use and the risk of fractures." Bone **40**(5): 1252-1258.

Xie, P., M. Kamei, et al. (2011). "Suppression and regression of choroidal neovascularization in mice by a novel CCR2 antagonist, INCB3344." PLoS One **6**(12): e28933.

Xue, C. B., A. Wang, et al. (2010). "Discovery of INCB3344, a potent, selective and orally bioavailable antagonist of human and murine CCR2." Bioorg Med Chem Lett **20**(24): 7473-7478.

Chapter 3

TNF is an important mediator during early fracture healing in vivo.

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3.1 Introduction

Proinflammatory cytokines are released as part of the innate immune response following injury and their effects depend on the timing of release, dose and context. Excess and chronic persistence of inflammation exacerbates injury but when present in a transient and self-limited manner, inflammation is a prerequisite for tissue repair. This dual role is exemplified by the pro-inflammatory cytokine, tumor necrosis factor (TNF). Sustained up-regulation of TNF has a destructive role in many inflammatory conditions including stimulating excessive osteoclastic bone resorption in the affected joints of patients with rheumatoid arthritis. However, TNF also acts as a growth factor for myelin-producing cells (Arnett, Mason et al. 2001) and differentiation factor for mesenchymal stem cells (Hess, Ushmorov et al. 2009), and has been shown to enhance the healing processes in the infarcted myocardium (Kim, Park et al. 2009) or fractured bone(Glass, Chan et al. 2011).

Targeting the acute local inflammatory response following skeletal injury would provide a practical therapeutic strategy to accelerate fracture healing as the biological therapy can be administered at the time of fracture fixation. Previous work by our group found that low levels of TNF in the fracture environment promoted the recruitment and osteogenic differentiation of mesenchymal stromal cells (MSCs) (Glass, Chan et al. 2011). Furthermore, local addition of 1 ng of rhTNF at the fracture site in the murine model of fracture healing led to accelerated repair as determined by histology and microCT analysis (Glass, Chan et al. 2011). A previous report found that endochondral fracture healing was

delayed in TNFR-deficient mice (Gerstenfeld, Cho et al. 2003). However, one of the limitations of studying genetically modified animals is the presence of compensatory pathways. This is particularly true in animals that do not exhibit an obvious bony phenotype as adults since genetic modification has not affected skeletal development. There is considerable redundancy in the regulation of fracture repair, which can cause the predicted phenotype to be masked by the expression of genes with redundant functions. For example, although the osteoinductive activity of BMP-7 was well established, study of its role in bone formation and fracture healing was hampered by perinatal death of BMP-7 deficient mice. A recent study using conditional deletion of BMP-7 found that the absence of locally produced BMP-7 had no effect on postnatal limb growth or fracture healing, suggesting the effective masking influence of compensatory pathways, such as BMP-4 (Tsuji, Cox et al. 2010). Therefore, it would be more relevant to conduct alternative gain and loss studies in which exogenous TNF is administered and endogenous TNF expression is depleted in wild type animals to confirm the role of TNF in fracture healing. Furthermore, for the purposes of clinical translation, it is important to define the therapeutic window regarding both dose and timing of this potential treatment strategy. The role of inflammation on fracture repair may be confirmed by administering an anti-inflammatory cytokine, such as interleukin 10, a central anti-inflammatory cytokine with pleiotropic effects on inflammation and immunoregulation (Moore, de Waal Malefyt et al. 2001), while specific depletion of endogenous TNF may be achieved by the administration of a neutralizing anti-TNF antibody.

3.2 Addition of recombinant TNF at the fracture site during early inflammatory phase accelerates fracture repair in vivo.

3.2.1 Confirmation of bioactivity of recombinant human (rh)TNF

It has previously been reported that 1 ng rhTNF injected at the fracture site on days 0 and 1 accelerated fracture repair (Glass, Chan et al. 2011). The biological activity of recombinant TNF can be measured based on specific biological activities of cytokines on various cell lines, including the cytotoxic effect of TNF on the murine fibrosarcoma cell line WEHI 164 clone 13 (Espevik and Nissen-Meyer 1986). The principle behind the cytotoxic TNF bioassay involves binding of TNF to membrane-bound cell surface TNF receptors, which results in killing of the TNF-sensitive cell line. The rhTNF used in the subsequent experiments was tested using a cytotoxic TNF bioassay to confirm bioactivity (Fig 3.1).

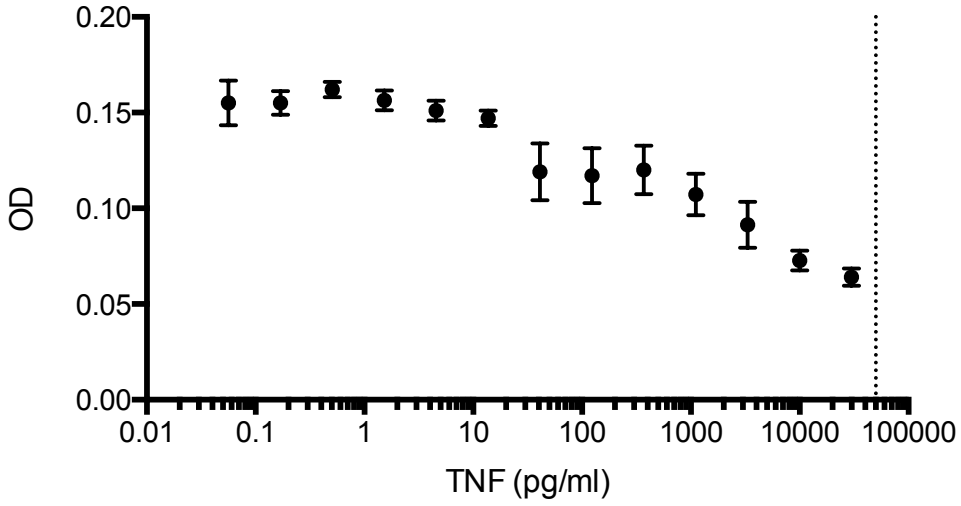


***Fig 3.1**. Cytotoxic TNF bioassay: dose-dependent cell killing by TNF confirms bioactivity of the recombinant protein (Pearson r = -0.7325, p=0.0014). Dotted line at 50 ng/ml – the concentration of rhTNF that led to enhanced fracture healing in vivo.*

3.2.2 Dose response of local rhTNF in vivo

To identify the optimal in vivo dose of rhTNF to accelerate fracture healing, a dose range of rhTNF was injected at the fracture site immediately after fracture and repeated at 24 hours (days 0 and 1) following osteotomy. rhTNF was effective in accelerating fracture healing, as indicated by increased % callus mineralization, at day 28 after injury at 1 ng only (Fig 3.2). As shown by the micro-CT reconstructions, treatment with 1 ng rhTNF led to enhanced healing and accelerated remodeling as evidenced by a more compact and mineralized fracture callus at day 28 (Fig 3.2).

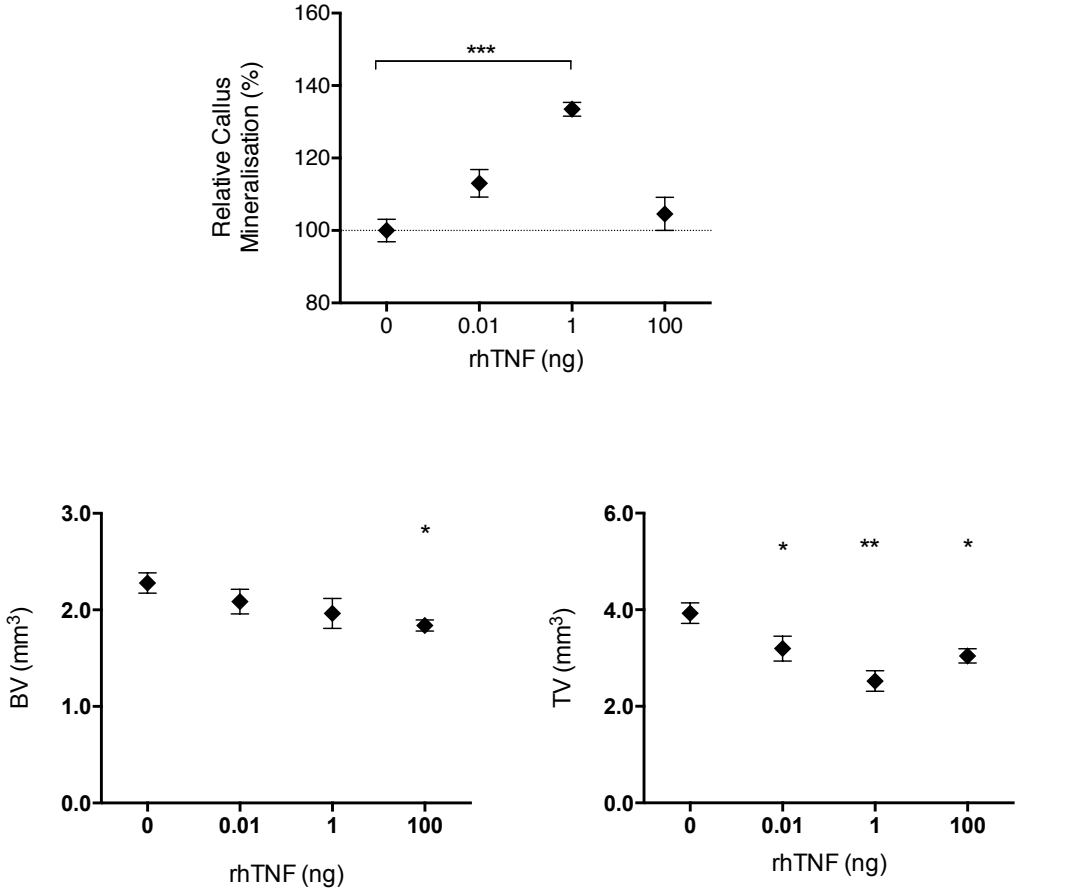


Fig 3.2. *Above: Addition of rhTNF at the fracture site on Days 0 and 1 led to accelerated healing, indicated by increased percentage callus mineralization, at day 28 after operation in a dose dependent manner. At least 6 mice were used per treatment group. Figures represent mean ± SEM. Relative % callus mineralization: PBS control, 100 ± 3.09 %; TNF 0.01ng (0.5 ng/ml), 113 ± 3.81 %; TNF 1 ng (50 ng/ml), 133 ± 1.91 %; TNF 100 ng (5*

*ug/ml), 104.6 ± 4.58 %). BV: PBS control, 2.28 ± 0.11 mm³; TNF 0.01 ng 2.09 ± 0.13 mm³; TNF 1 ng 1.96 ± 1.91 mm³; TNF 100 ng 1.84 ± 0.06 mm³. TV: PBS control, 3.93 ± 0.21 mm³; TNF 0.01ng 3.20 ± 0.26 mm³; TNF 1 ng 2.52 ± 0.21 mm³; TNF 100 ng 3.04 ± 0.15 mm³. *p<0.05, **p<0.01, ***p<0.001, 1-way ANOVA with Bonferroni's post-test correction.*
Below: Representative micro-CT images at the fracture site showing from left to right: lateral view of tibia, cross-section, cross and longitudinal sections with color overlay and 3D reconstruction. In the color overlays, the shade of blue corresponds to percentage mineralization: light blue denotes soft immature callus whereas dark blue denotes hard mineralized callus. Scale bar 2 mm.

3.2.3 Timing of rhTNF addition in vivo

To determine the efficacy of TNF with respect to timing of administration, rhTNF was injected at the fracture site on different days following surgery. Accelerated fracture healing was only evident when rhTNF at 1 ng was administered at the fracture site twice in the first 24h: immediately post injury and again at 24 hours post-fracture (Fig 3.3). This would be particularly relevant for clinical translation as it suggests that a treatment that enables release of TNF over the first 24 hours at the time of reduction and fixation of the fracture may be appropriate. The early window of opportunity coincides with the early inflammatory phase of fracture healing, suggesting that the mechanism of action of exogenous rhTNF occurs via the early innate immune response.

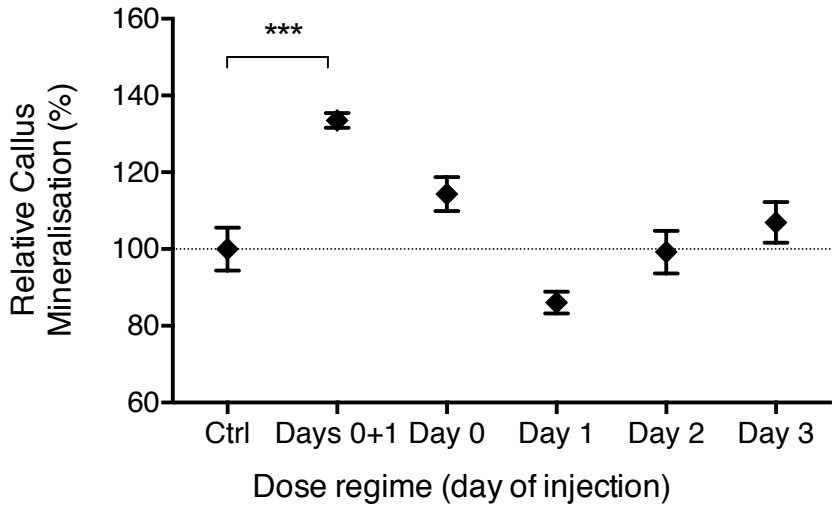


Fig 3.3*. rhTNF must be given twice within the first 24 hours to accelerate healing. At least 6 animals were used per group. Figures represent mean ± SEM. PBS control, 100 ± 5.57 %; TNF Day 0, Day1, 133.5 ± 1.91 %; TNF Day 0, 114.3 ± 4.40 %; TNF Day 1, 86.07 ± 2.83 %; TNF Day 2, 99.2 ± 5.58 %; TNF Day 3; 107 ± 5.28 %). * p ≤ 0.05, *** p ≤ 0.0001, 1-way ANOVA with Bonferroni's post-test correction. Day 0 = immediately post-skin closure, D1 = 24 h post-fracture, D2 = 48 h post-fracture, D3 = 72 h post fracture.*

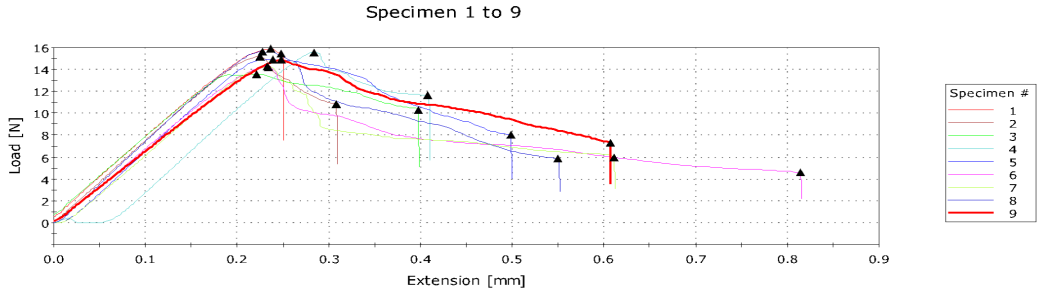
3.3 Alternative methods to assess fracture healing in murine model

Biomechanical testing is often considered as a gold standard in the assessment of bone quality in intact bone and fracture healing. Bending using 3-point bend testing was attempted to investigate whether this may be a reliable method to detect changes in fracture healing as seen in microCT analysis.

3.3.1 Three-point bend testing

Consistent force-extension profiles were generated when intact tibiae were bend-tested by three-point loading (Fig 3.4). However, due to the small size of the mouse tibia (< 25 mm in length) and the nature of the osteotomy, the fracture callus morphologies are heterogeneous and have different points of maximal weakness. The exact placement of the upper loading point on the callus is critical but it was not practically possible to be exactly consistent for every sample. This accounted for the wide variation in force-extension profiles generated and precluded detection of any effect in the samples tested (Fig 3.5).

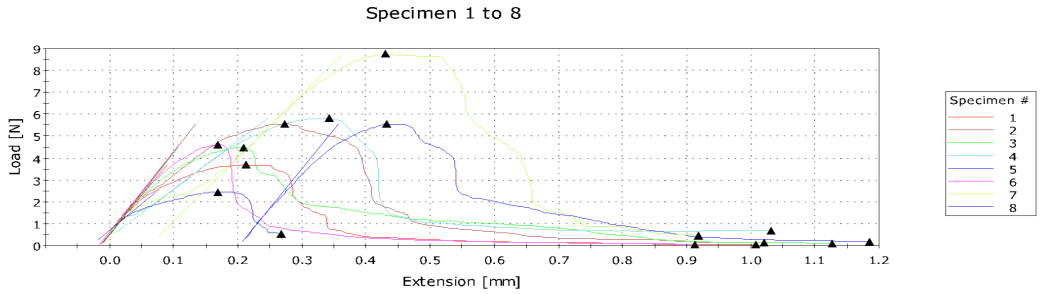
Intact tibia (no fracture):



Specimen	Max Load (N)	Extension at Max Load (mm)
1	15.9224	0.23606
2	15.19043	0.22491
3	13.58116	0.22125
4	15.56135	0.28341
5	14.93222	0.2385
6	14.32246	0.23213
7	14.22504	0.23391
8	15.64852	0.22753
9	14.92238	0.23769
Mean	**14.92289**	**0.23838**
SD	**0.76**	**0.02**

Fig 3.4*. Force-extension profiles for non-fractured tibiae of C57BL6 mice by 3 point bend testing.*

Fracture model (day 28 post-fracture)



Specimen	Max Load (N)	Extension at Max Load (mm)
1	3.6882	0.21178
2	5.55021	0.27291
3	4.48329	0.20831
4	5.81304	0.34209
5	5.55125	0.43219
6	4.62364	0.16791
7	8.75901	0.42975
8	2.45767	0.16847
Mean	**5.11579**	**0.27918**
SD	**1.85**	**0.11**

Fig 3.5*. Force-extension profiles for tibiae of C57BL6 mice at day 28 post-fracture by 3 point bend testing.*

Power calculation:

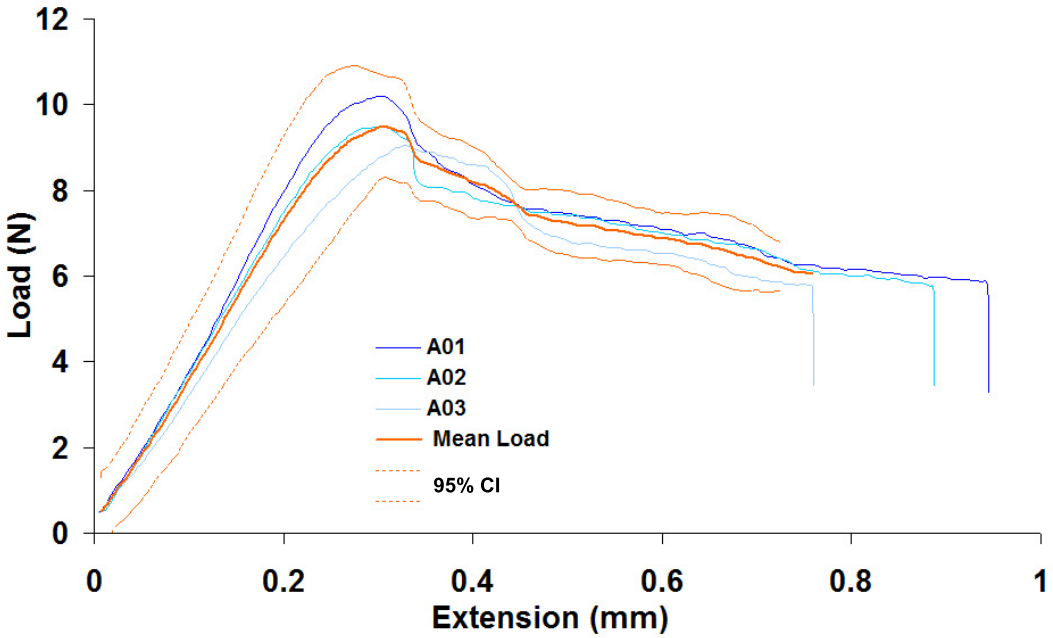
$$n = 2 \text{ X } SD^2 \text{ X } (z_\alpha + z_\beta)^2/\Delta^2$$

n = 103 in the treatment group is necessary to detect a 10% difference where alpha is 0.05, power of test is 80%, SD 1.85 and two sided test is used. Therefore it is not realistic to use three point bend testing for tibial fracture model to assess the efficacy of rhTNF.

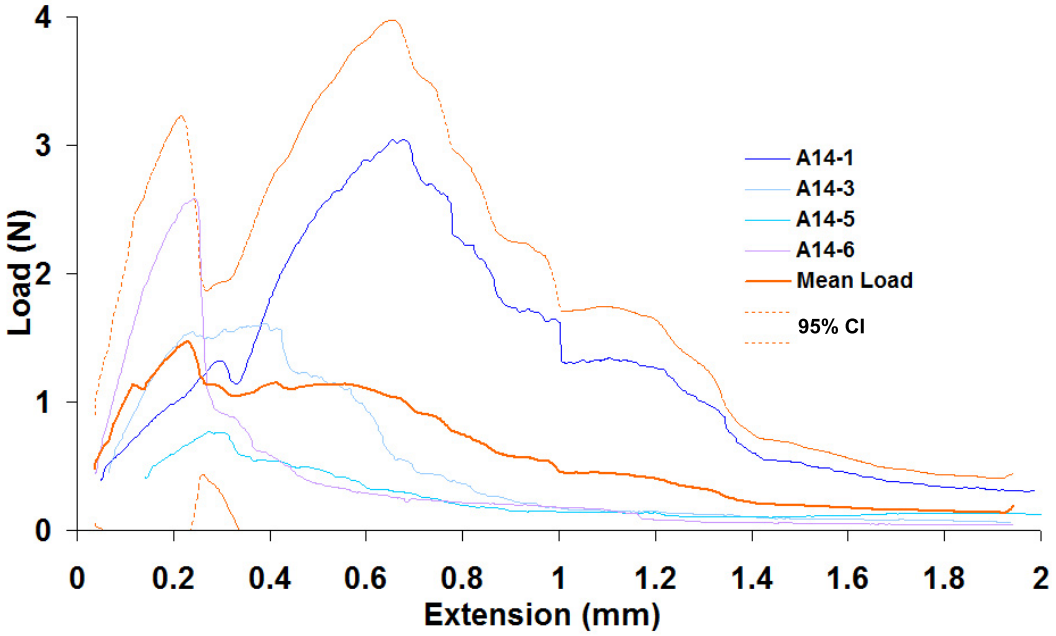
Three-point bend testing was also performed on non-fractured and fractured tibiae at days 14 and 28 post-fracture by collaborators Dr. Duncan Bassett and Dr. Graham Williams at Hammersmith Hospital. They found that the force-

extension profiles of fractured bones at day 14, like the day 28 specimens, are also too heterogeneous to allow comparisons between treatment and control groups (Fig 3.6).

Non-fractured tibiae control:



Day 14 post-fracture:



Day 28 post-fracture:

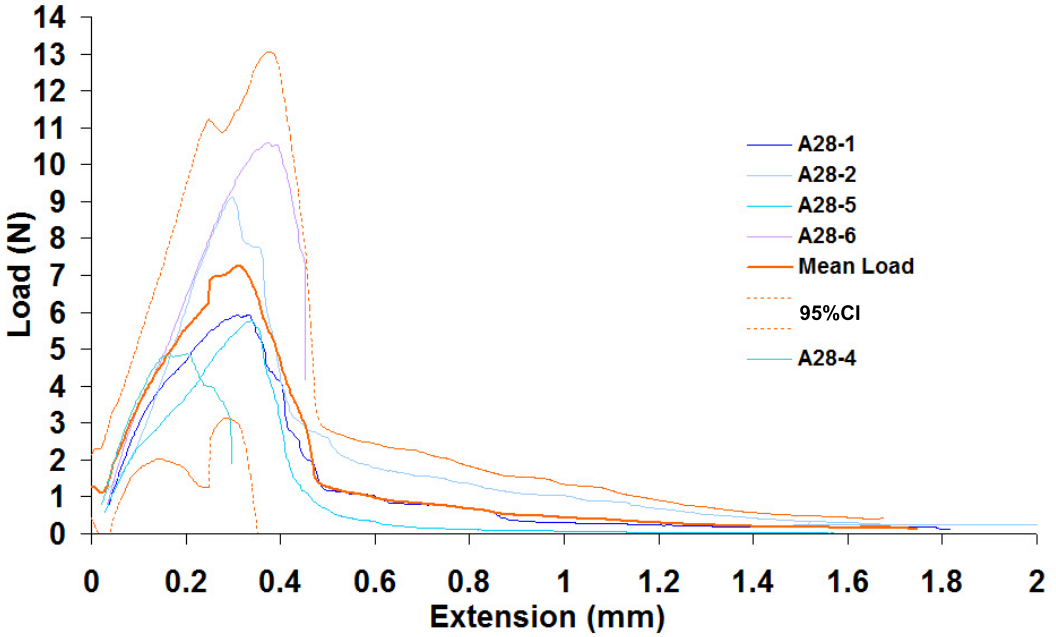


***Fig 3.6**. Force-extension profiles for non-fractured tibiae of C57BL6 mice and at days 14 and 28 post-fracture (performed at Hammersmith Hospital by Dr. Duncan Bassett and Dr. Graham Williams)*

3.3.3 Load-bearing

An important functional measure of the rate of fracture healing in patients is their ability to transmit weight through the injured limb (Goldhahn, Mitlak et al. 2008). Furthermore, the expression of TNF has been associated with inflammatory pain and shown to induce acute hyperalgesia in mice (McNamee, Alzabin et al. 2011). The Linton incapacitance meter can be used to assess weight bearing by measuring the weight transmitted by the mice through their operated limbs compared to the contralateral uninjured limbs. During the early phase of fracture healing at day 3, TNF-treated animals transmitted more weight through the fractured hind limb compared to PBS-treated controls, indicating that TNF administration did not exacerbate pain and actually allowed earlier load bearing (Fig 3.7). However, there was no difference on days 9 and 14.

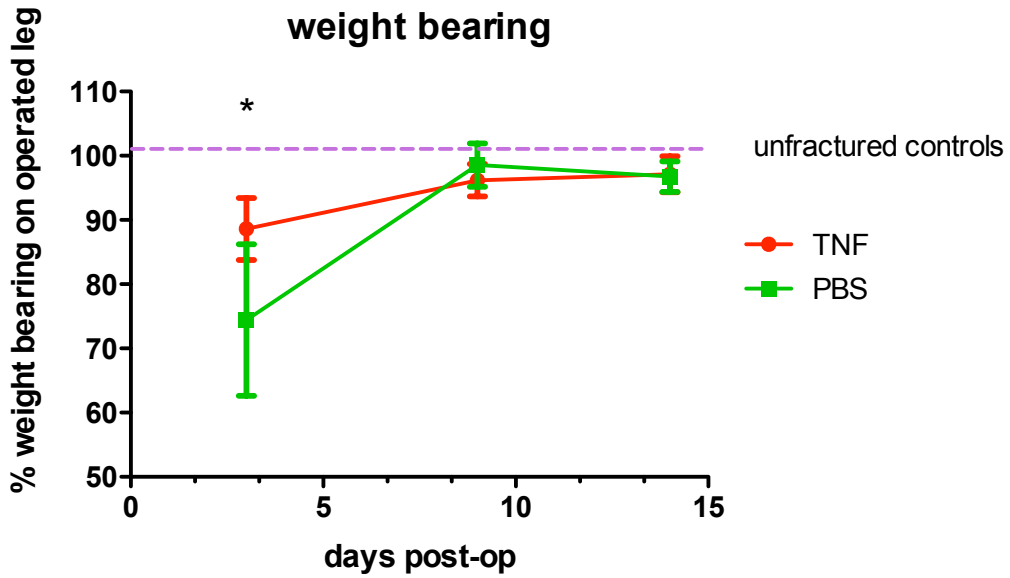


Fig 3.7*. Loading through the operated limbs was significantly higher in the TNF-treated group compared to PBS controls during the early stages of fracture healing. At least 6 animals were used per group. Figures represent mean ± SEM. *p<0.05, 1-way ANOVA with Bonferonni's post-test correction.*

3.4 Manipulation of local inflammation affects fracture healing.

3.4.1 Local cleavage of bound TNF using rTACE enhanced fracture healing.

Human TNF is initially expressed as a 26 kDa non-glycosylated type II transmembrane protein (*mem*TNF). *Mem*TNF undergoes cleavage by TNF convertase enzyme (TACE), also known as ADAM17, to release a 17 kDa soluble protein (*sol*TNF) which forms a homotrimer and is responsible for the major biological activity of TNF(Tang, Hung et al. 1996; Black, Rauch et al. 1997; Moss, Jin et al. 1997). TACE is a membrane bound enzyme and a member of the family of ADAM (a disintegrin and metalloprotease) proteins (Black and White 1998). Mice lacking TACE are not viable (Peschon, Slack et al. 1998). In order to test whether increased local TNF cleavage would affect fracture repair in vivo, recombinant (r)TACE was injected locally at the fracture site. The regimen used was 1 ng in 20 uL PBS (50 ng/ml) injected at the fracture site 30 mins pre-operatively, immediately after operation and again 24 hours later. rTACE treatment led to increased % callus mineralization at day 28 and microCT images showed denser fracture calluses that are bridged across the fracture site with advanced remodeling (Fig 3.8).

3.4.2 Downregulation of local inflammation impairs fracture repair.

Mice deficient in both TNF receptors TNFR1 (p55) and TNFR2 (p75) exhibited delayed endochondral fracture healing in mice (Gerstenfeld, Cho et al. 2003).

However, it is not possible to exclude the role of compensatory pathways in genetically modified mice.

To assess the role of the general inflammatory response on fracture healing in vivo, recombinant murine IL-10 (rm-IL10) was administered either systemically via intraperitoneal injection or locally by injection at the fracture site. The specific role of TNF was assessed by systemic administration of a neutralizing antibody, TN3. As shown in Fig 3.8, the relative callus mineralization was reduced on TNF inhibition. The micro-CT reconstructions show that systemic anti-TNF led to poor bridging across the fracture site and an incomplete, poorly mineralized and atrophic callus whereas local rm-IL10 treatment led to a large, poorly mineralized and immature callus. These observations are consistent with the accelerated callus maturation seen in mice treated with 1 ng of rhTNF.

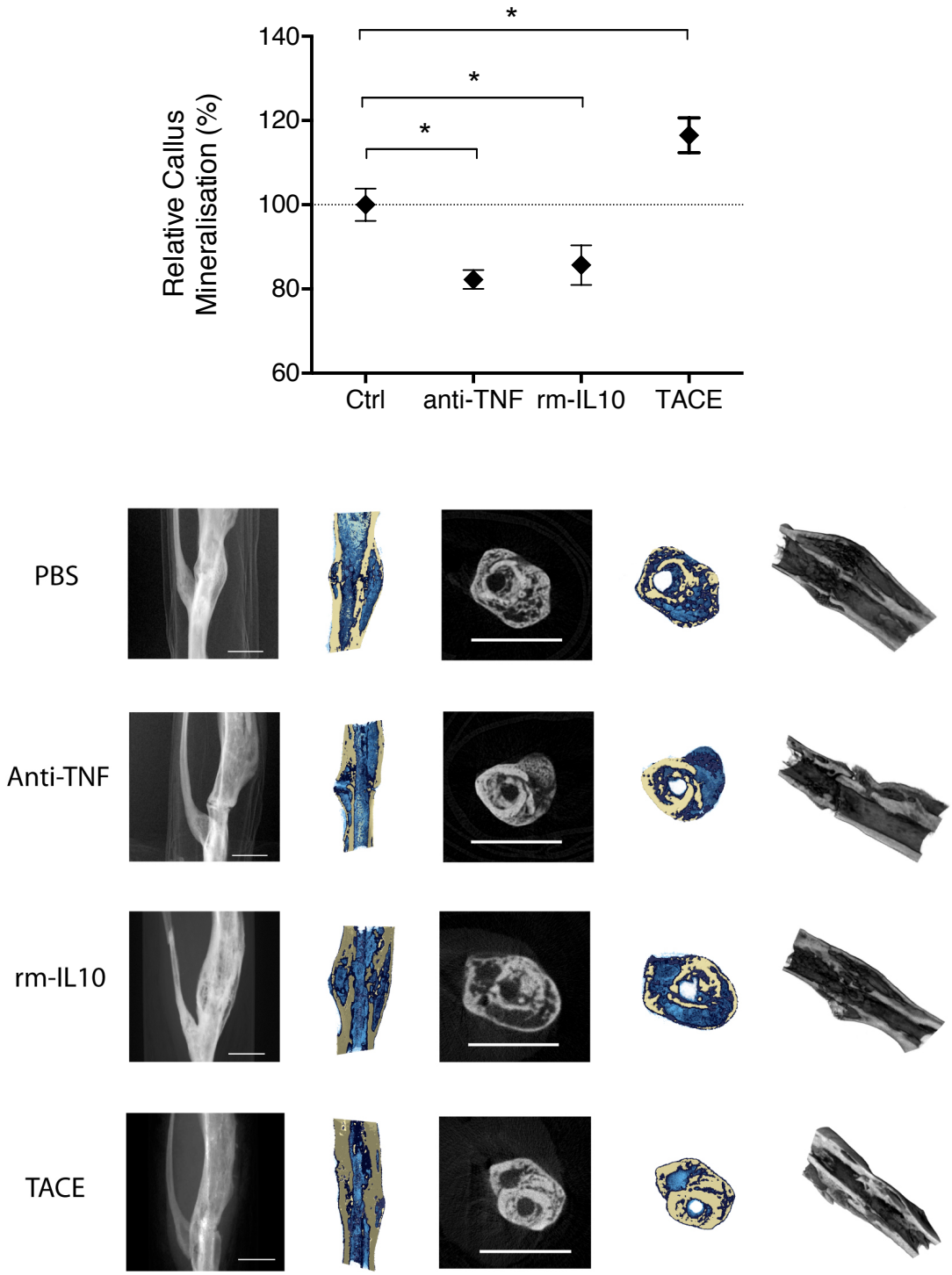


Fig 3.8. *Downregulation of inflammation affects fracture healing in vivo. At least 6 animals were used per group.*

*Above: Figures represent mean ± SEM. Relative % callus mineralization: PBS control, 100 ± 3.83 %; anti-TNF, 82.24 ± 2.23 %; rmIL10, 85.68 ± 4.70 %; TACE, 116.5 ± 4.14 %. * p ≤ 0.05, 1-way ANOVA with Bonferroni's post-test correction.*

Below: Representative CT images at day 28 post-fracture; treatment by 20 µL PBS injection at fracture site served as control. Anti-TNF treatment led to poor bridging across fracture site and poor mineralization of callus. rmIL-10 treatment led to a large unmineralized fracture callus, hallmarks of immature callus formation. TACE treatment led to a highly mineralized callus that bridged the fracture gap.

To assess the specificity of the effect of local addition of rhTNF as opposed to generalized upregulation of inflammation, mice were treated systemically with various dose regimes of anti-IL-10 receptor (anti-IL-10R) antibody. These included pre-emptive treatment pre-fracture as well as repeated dosing post-fracture. No effect on fracture healing was observed (Fig 3.9).

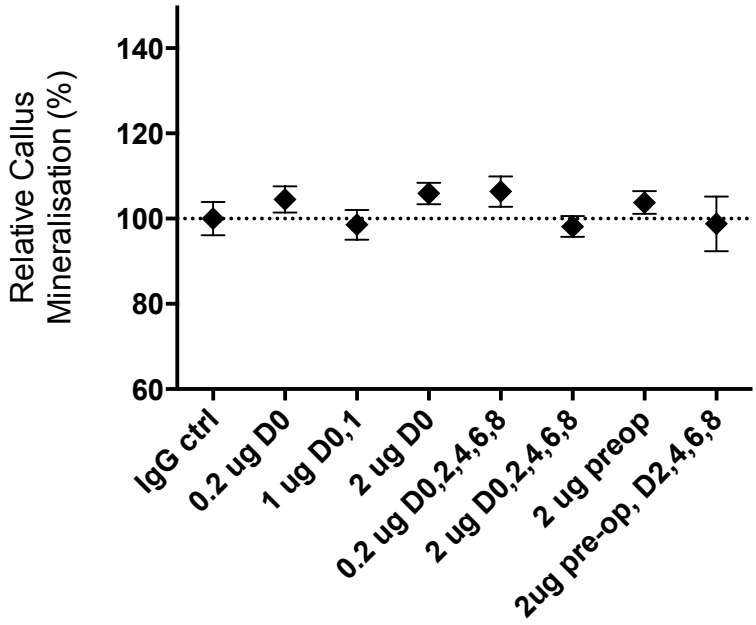


***Fig 3.9**. Anti-IL-10R antibody treatment did not affect fracture healing in vivo. At least 6 animals were used per group. Figures represent mean ± SEM. Relative % callus mineralization: IgG control, 100 ± 3.90 %; anti-IL-10 0.2 ug D0, 104.5 ± 3.05 %; anti-IL10 1 ug D0,1, 98.6 ± 3.49 %; anti-IL10 2 ug D0, 105.9 ± 2.52%; anti-IL10 0.2 ug D0,2,4,6,8, 106.4 ± 3.57%; anti-IL10 2 ug D0,2,4,6,8, 98.16 ± 2.41 %; anti-IL10 2 ug pre-op, 103.8 ± 2.65%;*

anti-IL10 pre-op, D2,4,6,8, 98.89 ± 6.41 % p ≤ 0.05, 1-way ANOVA with Bonferroni's post-test correction. Pre-op = 1 hour pre-fracture, D0 = immediately post-skin closure, D1 = 24 h post-fracture, D2 = 48 h post-fracture etc.*

3.5 Local addition of rhTNF accelerates fracture repair in osteopenic mice.

3.5.1 Ovariectomy induces osteopenia in C57BL6 mice.

Osteoporotic fractures represent a huge unmet clinical need. Ovariectomy in mice consistently replicates both the alterations in oestrogen and follicle stimulating hormone levels and the osteoporosis observed in postmenopausal women(Pacifici 2007). C57BL6 mice were rendered osteopenic by ovariectomy (Fig 3.10) as previously validated (He, Zhang et al. 2011).

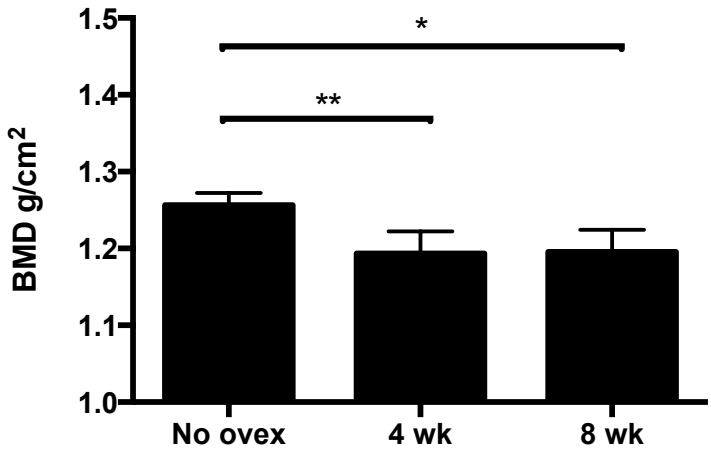


***Fig 3.10**. Ovariectomy induces osteoporosis by 4 weeks post-surgery. BMD measured by microCT analysis of tibiae of C57BL6 mice. At least 8 animals were used per group. Control 1.256 ± 0.01 g/cm²; 4 wk 1.193 ± 0.01 g/cm²; 8 wk 1.196 ± 0.01 g/cm². * p<0.01, ** p<0.01. Ovex = ovariectomy. Scans performed by Mr. Kevin McKenzie, University of Aberdeen.*

3.5.2 Local addition of rhTNF led to accelerated early phase fracture healing.

Subsequently, the effect of local addition of 1 ng rhTNF on fracture healing in these osteopenic animals was assessed at days 14 and 28 post-fracture. As shown in Fig 3b, treatment with 1 ng TNF at the fracture site on days 0 and 1 led to an increased relative callus mineralization at day 14 after surgery but by day 28 healing was equivalent to PBS treated controls (Fig 3.11). This indicates that local administration of rhTNF resulted in initial acceleration of healing to achieve the same final result over a shorter period. The microCT images show that rhTNF treatment led to mature callus bridging across the fracture, which was absent in the PBS-treated control group, at day 14.

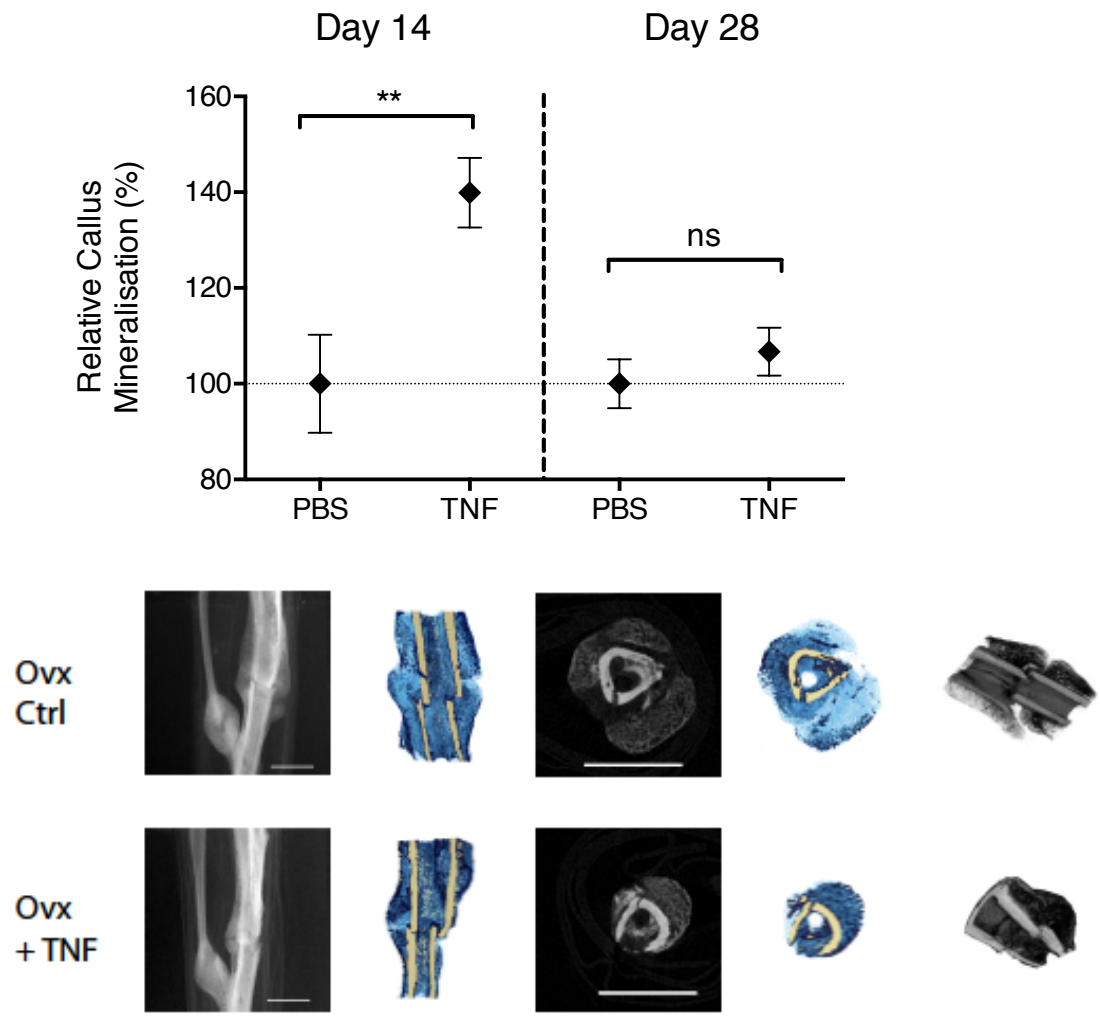


Fig 3.11*: rhTNF treatment augmented early phase fracture healing in osteopenic mice. Treatment with 1 ng rhTNF at the fracture site immediately and 24 hours post-fracture led to increased callus mineralization at day 14 but no difference at day 28, indicating accelerated early healing but the same final result at day 28. At least 6 animals were used per group.*

*Above: Figures represent mean ± SEM. Relative % callus mineralization: Day 14 PBS control, 100 ± 10.25%; Day 14 TNF, 139 ± 7.27%%; Day 28 PBS, 100 ± 5.09%, day 28 TNF, 106 ± 4.98%. *p<0.01, 2 sided t-test.*

Below: Representative CT images at day 14: rhTNF treatment led to mature callus bridging across the fracture which was absent in the PBS-treated control group.

3.3 Discussion

Fracture repair involves a complex cascade of events involving numerous cell types and the spatially and temporally coordinated release of multiple factors (Dimitriou, Tsiridis et al. 2005). Studies in humans and mice of the fracture hematoma, which are rich in these cells and factors, indicate that these early events following fracture are critical to the outcome of fracture healing (Grundnes and Reikeras 1993; Chung, Cool et al. 2006; Kolar, Schmidt-Bleek et al. 2010; Kolar, Gaber et al. 2011; Hoff, Maschmeyer et al. 2013). The immune cells found in human fracture hematoma promoted genes associated with inflammation and osteogenesis (Kolar, Gaber et al. 2011). The importance of the fracture hematoma was confirmed by a study that showed removal of the fracture haematoma impaired callus production and biomechanical parameters in a rat femoral fracture model (Grundnes and Reikeras 1993). Furthermore, the use of anti-inflammatory or cytotoxic medications, including corticosteroids, non-steroidal anti-inflammatory drugs or chemotherapeutic agents, during early fracture healing have been shown to be deleterious (Sudmann, Dregelid et al. 1979; Engesaeter, Sudmann et al. 1992; Altman, Latta et al. 1995; Gerstenfeld, Thiede et al. 2003; Simon and O'Connor 2007; Dimmen, Nordsletten et al. 2008; Pountos, Georgouli et al. 2008). These findings support the importance of the early inflammatory events in determining the final outcome of fracture healing.

Inflammation is necessary to initiate the reparative response following injury(Nathan 2006; Soehnlein and Lindbom 2010). Our group has previously found that proinflammatory cytokines, in particular TNF, play an important role

in fracture healing through the recruitment and osteogenic differentiation of MSCs (Glass, Chan et al. 2011). Others have confirmed that TNF also promotes osteogenic differentiation in a time- and dose-dependent manner(Mountziaris, Tzouanas et al. 2010; Lu, Wang et al. 2012). I found that local TNF treatment was only effective in accelerating fracture healing when given twice in the first 24 hours after surgery (Fig 3.3). A single dose of TNF on days 0 or 1 did not affect fracture repair. This may be due to the short half-life of less than 20 minutes of TNF in the circulation (Ferraiolo, Moore et al. 1988). Furthermore, neutralizing antibody to TNF impaired fracture healing. Therefore, taken together, these findings indicate that the early inflammatory phase in the first 24 hours represents a key rate-controlling step in fracture healing and may therefore be amenable to biological intervention. Furthermore, this early phase typically coincides with the timing of clinical treatment, which would be convenient for any potential biological treatment.

Fracture healing has been reported to be delayed during the early phase in TNFR-deficient mice (Gerstenfeld, Cho et al. 2003). Here, I have assessed the role of early inflammation and TNF in fracture healing in a number of different ways. TACE was added locally to maximize the bioactivity specifically at the fracture site. Addition of TACE immediate after surgery and at 24 hours post-fracture led to enhanced fracture healing (Fig 3.8). However, TACE has pleiotropic effects. Although it is most well known as the major sheddase for TNF, it also acts on at least 76 different substrates, including ligands of the EGF-R, adhesion protein L-selectin and the chemokine IL6-R(Rose-John 2013). Furthermore, TACE activity has been implicated in endochondral bone formation (Saito, Horiuchi et al. 2013)

and maintenance of bone mass (Horiuchi, Kimura et al. 2009). Local addition, however, is highly specific and led to enhanced healing at day 28 (Fig 3.8).

IL-10 is a potent anti-inflammatory cytokine with pleiotropic effects on inflammation and immunoregulation (Moore, de Waal Malefyt et al. 2001). IL-10 deficient mice spontaneously develop inflammatory bowel disease (Kuhn, Lohler et al. 1993) and patients with homozygous mutations in the IL-10 receptor subunits present with early onset colitis (Glocker, Kotlarz et al. 2009). Both of these diseases are associated with hyper-inflammatory immune responses, including increased TNF expression. IL-10 is produced by numerous types of immune cells including Th1, Th2, Th17, T reg, CD8+ T cells, B cells, and myeloid cells (Saraiva and O'Garra 2010). Of note, IL-10 downregulates the expression of selected inflammatory genes including TNF at a level of transcription (Smallie, Ricchetti et al. 2010). However, it also inhibits the synthesis of other cytokines including IFN-gamma, IL-2, IL-3 and GM-CSF, chemokines, inflammatory enzymes (including COX2) and the antigen presentation activity of antigen presenting cells. Local IL-10 treatment led to a large, poorly mineralized callus (Fig 3.8). By contrast, anti-TNF treatment using TN3 is highly specific, leading to the development of an atrophic callus (Fig 3.8).

In summary, this series of gain and loss in vivo experiments are consistent with previous reports and indicate that early TNF and local inflammation do play pivotal roles in the normal fracture repair process.

Biomechanical testing is often considered as a gold standard in the assessment of bone quality in both intact and fractured bones. However, this is most reliable in larger bones, such as femora of rats. Furthermore, some groups have found that analysis of μCT parameters to be a more sensitive method of evaluating murine fracture callus properties than biomechanical studies in part due to the greater amount of information that can be obtained to describe the structural and compositional properties of the callus with this non-destructive method (O'Neill, Stutz et al. 2012). I attempted three-point bend testing to investigate whether this may be a reliable tool to assess changes in fracture healing as seen in microCT analysis. Three-point loading allowed consistent force-extension profiles to be generated when intact tibiae were bend-tested (Figs 3.4, 3.6). However, due to the small size with a length of less than 25 mm and irregular shape of the mouse tibia, three-point loading created high shear stresses at the site of the callus and pure bending could not be achieved. Furthermore, the size of the mouse tibia and nature of the osteotomy meant that the fracture callus morphologies are heterogeneous and have different points of maximal weakness along both the longitudinal and transverse callus profiles. Hence the exact placement of the upper loading point on the callus becomes the main factor that determines the force-extension profile obtained, rather than the mineralization or size of the whole fracture callus. This accounted for the widely different force-extension profiles generated and precluded detection of any effect of the TNF in the samples tested (Fig 3.5). My calculations show that over 100 samples will be needed to detect a 10% difference with a power of 80%. Hence, bend testing as a tool to assess fracture healing was abandoned for the remainder of this thesis.

Functional outcomes such as load bearing and gait analysis would provide useful and tangible measures of fracture healing, particularly for translational purposes (Goldhahn, Mitlak et al. 2008). Although TNF treated mice were able to weight bear at least as well as the control group during the early phase of repair (Fig 3.7), this finding is perhaps surprising given that TNF has been implicated in inflammatory pain (McNamee, Alzabin et al. 2011). Although there was statistical significance in % load bearing at day 3 post-fracture, the confidence intervals overlapped and hence the observed effect should be verified. This could be done by conducting a longitudinal study using gait analysis at weekly intervals post-fracture. Our laboratory has now obtained an Exergait machine, a treadmill designed to study multiple parameters of gait in mice.

Of the methods investigated, microCT analysis provided the most informative and sensitive method for assessing fracture healing in mice, providing both qualitative and quantitative analyses of the fracture callus. Therefore, for the remainder of this thesis, microCT analysis will serve as the main outcome of fracture healing assessment.

Whilst there is a requirement for therapies to accelerate fracture healing in patients with high-energy open fractures, the greatest unmet need is for the rapidly growing number of individuals who sustain fragility fractures. Osteoporosis is characterized by low bone mass and weakened bone structure, leading to 1.5 million fragility fractures in the USA every year (Holroyd, Cooper et al. 2008). Half of all patients who sustain fragility fractures involving the femoral neck are permanently disabled and the mortality rate is 21-36% within

the first year (Eisman, Bogoch et al. 2012). Hence, there remains a pressing need to develop effective strategies to accelerate healing of fragility fractures. Therefore I examined whether TNF treatment would also be effective in mice that have been rendered osteopenic by oophorectomy. There was a 40% improvement in healing at 2 weeks and equivalent mineralization to PBS controls at 4 weeks (Fig 3.11). Rates of recovery and mobilization in patients with fragility fractures are critically dependent on fracture healing as premature loading leads to implant failure, accounting for the excessive morbidity and mortality seen in this vulnerable group of patients. If translated to the clinical setting, these findings would be of substantial benefit to patients.

In summary, the early inflammatory events following induction of fracture are crucial in the final outcome of fracture healing. Upregulation of inflammation by local addition of rhTNF or TACE promoted fracture healing whereas inhibition using rmIL-10 or a neutralizing antibody to TNF impaired fracture healing. The observation that addition of recombinant TNF was only effective if administered repeatedly within 24 hours of injury suggests that its mechanism of action involves the innate immune response that occurs immediately after injury. The subsequent chapter explores the innate immune response that occurs following skeletal injury.

References

Altman, R. D., L. L. Latta, et al. (1995). "Effect of nonsteroidal antiinflammatory drugs on fracture healing: a laboratory study in rats." J Orthop Trauma **9**(5): 392-400.

Arnett, H. A., J. Mason, et al. (2001). "TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination." Nat Neurosci **4**(11): 1116-1122.

Black, R. A., C. T. Rauch, et al. (1997). "A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells." Nature **385**(6618): 729-733.

Black, R. A. and J. M. White (1998). "ADAMs: focus on the protease domain." Curr Opin Cell Biol **10**(5): 654-659.

Chung, R., J. C. Cool, et al. (2006). "Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats." J Leukoc Biol **80**(6): 1272-1280.

Dimitriou, R., E. Tsiridis, et al. (2005). "Current concepts of molecular aspects of bone healing." Injury **36**(12): 1392-1404.

Dimmen, S., L. Nordsletten, et al. (2008). "Negative effect of parecoxib on bone mineral during fracture healing in rats." Acta Orthop **79**(3): 438-444.

Eisman, J. A., E. R. Bogoch, et al. (2012). "Making the first fracture the last fracture: ASBMR task force report on secondary fracture prevention." J Bone Miner Res **27**(10): 2039-2046.

Engesaeter, L. B., B. Sudmann, et al. (1992). "Fracture healing in rats inhibited by locally administered indomethacin." Acta Orthop Scand **63**(3): 330-333.

Espevik, T. and J. Nissen-Meyer (1986). "A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes." J Immunol Methods **95**(1): 99-105.

Ferraiolo, B. L., J. A. Moore, et al. (1988). "Pharmacokinetics and tissue distribution of recombinant human tumor necrosis factor-alpha in mice." Drug Metab Dispos **16**(2): 270-275.

Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.

Gerstenfeld, L. C., M. Thiede, et al. (2003). "Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs." J Orthop Res **21**(4): 670-675.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-{alpha} promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A **108**(4): 1585-1590.

Glocker, E. O., D. Kotlarz, et al. (2009). "Inflammatory bowel disease and mutations affecting the interleukin-10 receptor." N Engl J Med **361**(21): 2033-2045.

Goldhahn, J., B. Mitlak, et al. (2008). "Critical issues in translational and clinical research for the study of new technologies to enhance bone repair." J Bone Joint Surg Am **90 Suppl 1**: 43-47.

Grundnes, O. and O. Reikeras (1993). "The importance of the hematoma for fracture healing in rats." Acta Orthop Scand **64**(3): 340-342.

He, Y. X., G. Zhang, et al. (2011). "Impaired bone healing pattern in mice with ovariectomy-induced osteoporosis: A drill-hole defect model." Bone **48**(6): 1388-1400.

Hess, K., A. Ushmorov, et al. (2009). "TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway." Bone **45**(2): 367-376.

Hoff, P., P. Maschmeyer, et al. (2013). "Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model." Cell Mol Immunol **10**(2): 151-158.

Holroyd, C., C. Cooper, et al. (2008). "Epidemiology of osteoporosis." Best Pract Res Clin Endocrinol Metab **22**(5): 671-685.

Horiuchi, K., T. Kimura, et al. (2009). "Conditional inactivation of TACE by a Sox9 promoter leads to osteoporosis and increased granulopoiesis via dysregulation of IL-17 and G-CSF." J Immunol **182**(4): 2093-2101.

Kim, Y. S., H. J. Park, et al. (2009). "TNF-alpha enhances engraftment of mesenchymal stem cells into infarcted myocardium." Front Biosci **14**: 2845-2856.

Kolar, P., T. Gaber, et al. (2011). "Human early fracture hematoma is characterized by inflammation and hypoxia." Clin Orthop Relat Res **469**(11): 3118-3126.

Kolar, P., K. Schmidt-Bleek, et al. (2010). "The early fracture hematoma and its potential role in fracture healing." Tissue Eng Part B Rev **16**(4): 427-434.

Kuhn, R., J. Lohler, et al. (1993). "Interleukin-10-deficient mice develop chronic enterocolitis." Cell **75**(2): 263-274.

Lu, Z., G. Wang, et al. (2012). "Short-term exposure to tumor necrosis factor-alpha enables human osteoblasts to direct adipose tissue-derived mesenchymal stem cells into osteogenic differentiation." Stem Cells Dev **21**(13): 2420-2429.

McNamee, K. E., S. Alzabin, et al. (2011). "IL-17 induces hyperalgesia via TNF-dependent neutrophil infiltration." Pain **152**(8): 1838-1845.

Moore, K. W., R. de Waal Malefyt, et al. (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Moss, M. L., S. L. Jin, et al. (1997). "Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha." Nature **385**(6618): 733-736.

Mountziaris, P. M., S. N. Tzouanas, et al. (2010). "Dose effect of tumor necrosis factor-alpha on in vitro osteogenic differentiation of mesenchymal stem cells on biodegradable polymeric microfiber scaffolds." Biomaterials **31**(7): 1666-1675.

Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nat Rev Immunol **6**(3): 173-182.

O'Neill, K. R., C. M. Stutz, et al. (2012). "Micro-computed tomography assessment of the progression of fracture healing in mice." Bone **50**(6): 1357-1367.

Pacifici, R. (2007). "T cells and post menopausal osteoporosis in murine models." Arthritis Res Ther **9**(2): 102.
Peschon, J. J., J. L. Slack, et al. (1998). "An essential role for ectodomain shedding in mammalian development." Science **282**(5392): 1281-1284.
Pountos, I., T. Georgouli, et al. (2008). "Pharmacological agents and impairment of fracture healing: what is the evidence?" Injury **39**(4): 384-394.
Rose-John, S. (2013). "ADAM17, shedding, TACE as therapeutic targets." Pharmacol Res **71**: 19-22.
Saito, K., K. Horiuchi, et al. (2013). "Conditional inactivation of TNFalpha-converting enzyme in chondrocytes results in an elongated growth plate and shorter long bones." PLoS One **8**(1): e54853.
Saraiva, M. and A. O'Garra (2010). "The regulation of IL-10 production by immune cells." Nat Rev Immunol **10**(3): 170-181.
Simon, A. M. and J. P. O'Connor (2007). "Dose and time-dependent effects of cyclooxygenase-2 inhibition on fracture-healing." J Bone Joint Surg Am **89**(3): 500-511.
Smallie, T., G. Ricchetti, et al. (2010). "IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages." J Exp Med **207**(10): 2081-2088.
Soehnlein, O. and L. Lindbom (2010). "Phagocyte partnership during the onset and resolution of inflammation." Nat Rev Immunol **10**(6): 427-439.
Sudmann, E., E. Dregelid, et al. (1979). "Inhibition of fracture healing by indomethacin in rats." Eur J Clin Invest **9**(5): 333-339.
Tang, P., M. C. Hung, et al. (1996). "Human pro-tumor necrosis factor is a homotrimer." Biochemistry **35**(25): 8216-8225.
Tsuji, K., K. Cox, et al. (2010). "Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair." J Orthop Res **28**(3): 384-389.

Chapter 4

The innate immune response following skeletal injury: The role of neutrophils in fracture healing

Chapter 4
The innate immune response following skeletal injury:
The role of neutrophils in fracture healing

4.1 Introduction

Injury activates the innate immune response, which involves the recruitment and activation of immune cells, culminating in acute inflammation. In the previous chapter, I showed that TNF plays an important role in fracture healing in vivo and that local addition of rhTNF at the fracture site accelerates healing. However, addition of rhTNF was only effective when given within 24 hours of skeletal injury. This observation suggests that rhTNF may accelerate fracture repair by influencing the innate immune response. Whilst it is recognised that proinflammatory cytokines, including TNF, are released as part of the early inflammatory response to fracture injury (Kon, Cho et al. 2001), the precise quantities, spatial and temporal sequences of release and its origin(s) have not been defined. A description of the expression of endogenous TNF following fracture with regards to its timing of expression and cellular origin would provide further insight into its role in fracture repair and potentially the mechanism of action for exogenously administered recombinant TNF.

Sterile tissue injury activates the innate immune response, which is characterized by the swift recruitment of leukocytes including neutrophils and monocytes, and the release of proinflammatory cytokines. Neutrophils are the most abundant leukocyte subset in the blood of humans and mice. Following recruitment and activation, they initiate a host inflammatory defensive response, including phagocytosis and the release of reactive oxygen species, antimicrobial peptides and serine proteases (Soehnlein and Lindbom 2010). While neutrophils have traditionally been regarded as professional phagocytes

which clear debris and bacterial pathogens and delay healing, evidence has recently emerged to support a much wider role in orchestrating downstream events (Weiss 1989; Nathan 2006; Bastian, Pillay et al. 2011). It is now understood that the partnership between the phagocytes of the inflammatory response, controlled in large part by chemokine networks, is key to establishing an effective healing response (Nathan 2006; Soehnlein and Lindbom 2010). This is supported by evidence that wounds heal poorly in patients with insufficient neutrophils (Lekstrom-Himes and Gallin 2000) or whose neutrophils are functionally impaired such that they are unable to adhere to the endothelium or extracellular matrix and hence fail to accumulate in infected sites (Kong, Lee et al. 1992; Roos and Law 2001), or in whom neutrophils are not delivered to the damaged tissues in cases of full-thickness burns or sepsis (Rico, Ripamonti et al. 2002).

The role of neutrophils in bone healing is poorly understood at present. During postnatal tissue repair, including in bone, neutrophils are recruited locally soon after injury followed by macrophages (Andrew, Andrew et al. 1994; Chung, Cool et al. 2006). Previous studies have reported that neutrophils are able to express TNF and that murine neutrophils contain pre-formed TNF that is rapidly (within 15 min) mobilized to the cell surface upon cell activation (Bennouna and Denkers 2005). The depletion of neutrophils achieved using a neutralizing antiserum led to a reduction in the proportion of mesenchymal repair tissue within the injured growth plate cartilage in a rat model of bone repair (Chung, Cool et al. 2006). Furthermore, the role of neutrophil-derived TNF in the inflammatory response remains unknown (Bennouna, Bliss et al. 2003; Tsuda,

Takahashi et al. 2004; van Gisbergen, Sanchez-Hernandez et al. 2005). Local expression of TNF has been shown to be essential for the recruitment of leukocytes to extravascular sites (Tessier, Naccache et al. 1997). In this chapter, I describe the timing of endogenous TNF release and its cellular origin(s) during the early inflammatory response post-fracture and explore the role of neutrophils on fracture healing.

4.2 TNF is expressed locally during the early inflammatory response post fracture by innate immune cells.

4.2.1 Systemic release of cytokines post-fracture

TNF is released as part of the early inflammatory response to the bone injury (Kon, Cho et al. 2001) and is an important mediator in fracture repair (Gerstenfeld, Cho et al. 2003; Glass, Chan et al. 2011). I set out to detect the systemic release of TNF post-injury using the mouse fracture model. Mouse serum was collected at different time points following fracture using cardiac puncture and frozen down until analysis.

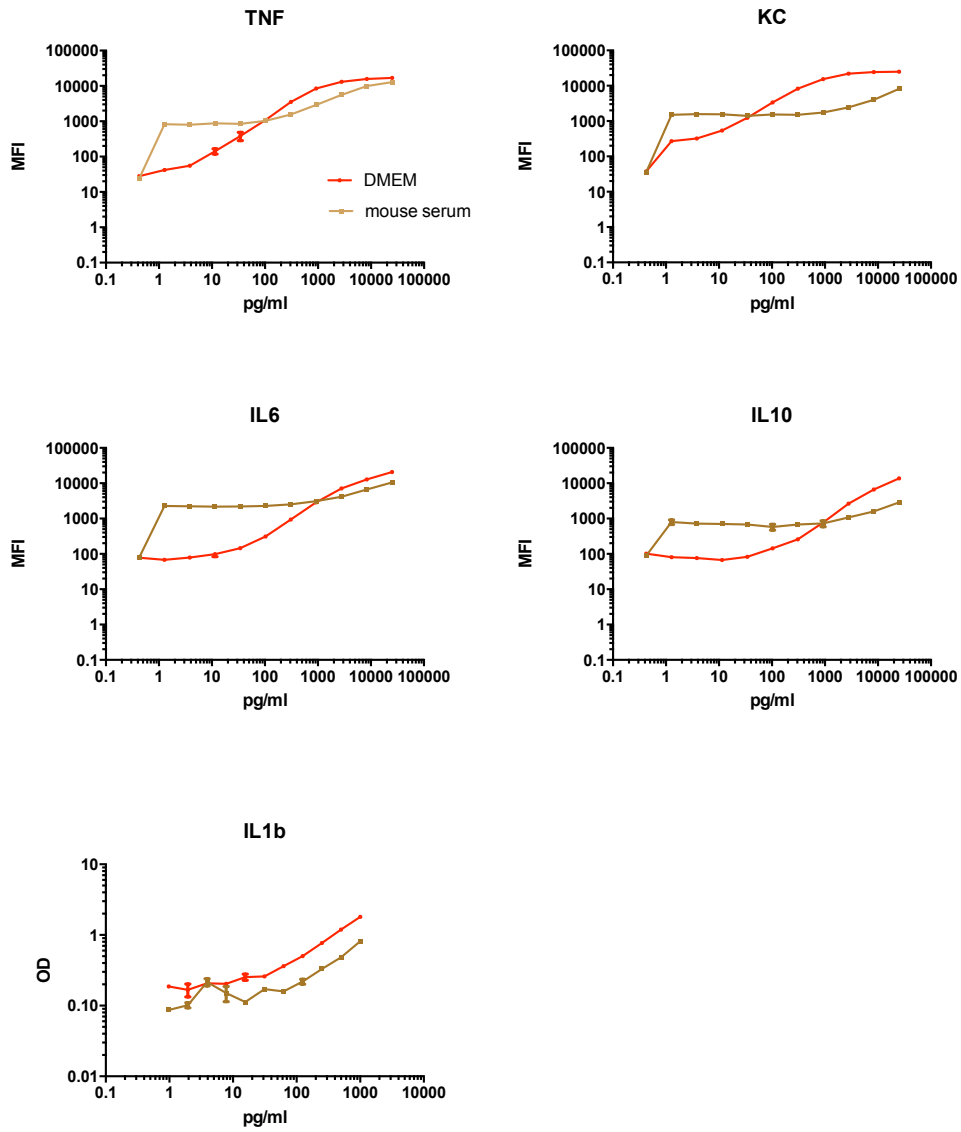


***Fig 4.1**. Calibration curves comparing serum free media and mouse serum for the detection of cytokines on the Luminex system. The presence of mouse serum reduced the sensitivity of this assay.*

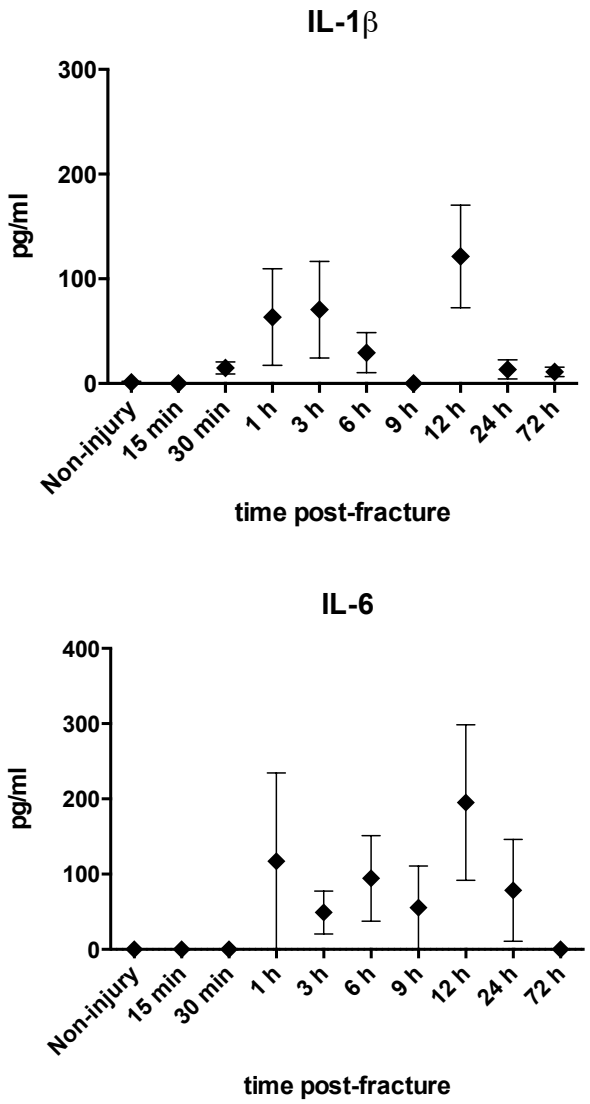


***Fig 4.2**. Systemic levels of IL-6 and IL-1b using Luminex. TNF and IL-10 were not detectable on luminex system. n ≥ 6 per time point. No significant differences using ANOVA.*

A number of detection systems were attempted. Luminex is a bioassay that uses microspheres that are coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. I found that the presence of mouse serum reduced the sensitivity of the Luminex assay to preclude the detection of cytokines in mouse serum samples (Figs 4.1, 4.2).

Meso Scale Discovery offers a more sensitive electrochemiluminescence system, which is based on the sandwich ELISA principle. The detection limits of the selected cytokines were less than 3.5 pg/ml in serum/plasma (MSD). I used a multiplex panel designed to simultaneously detect a selected number of cytokines associated with innate inflammation. The serum levels of TNF did not rise above baseline over the first 72 hours post-fracture (Fig 4.3) indicating that the level of trauma induced by the isolated tibial fracture in this murine model is insufficient to lead to a systemic inflammatory response. KC, a neutrophil chemokine, also peaked at 9 hours post-fracture. There was no detectable change in the systemic levels of IL-1β, IFN-γ or IL-10 (Fig 4.3).

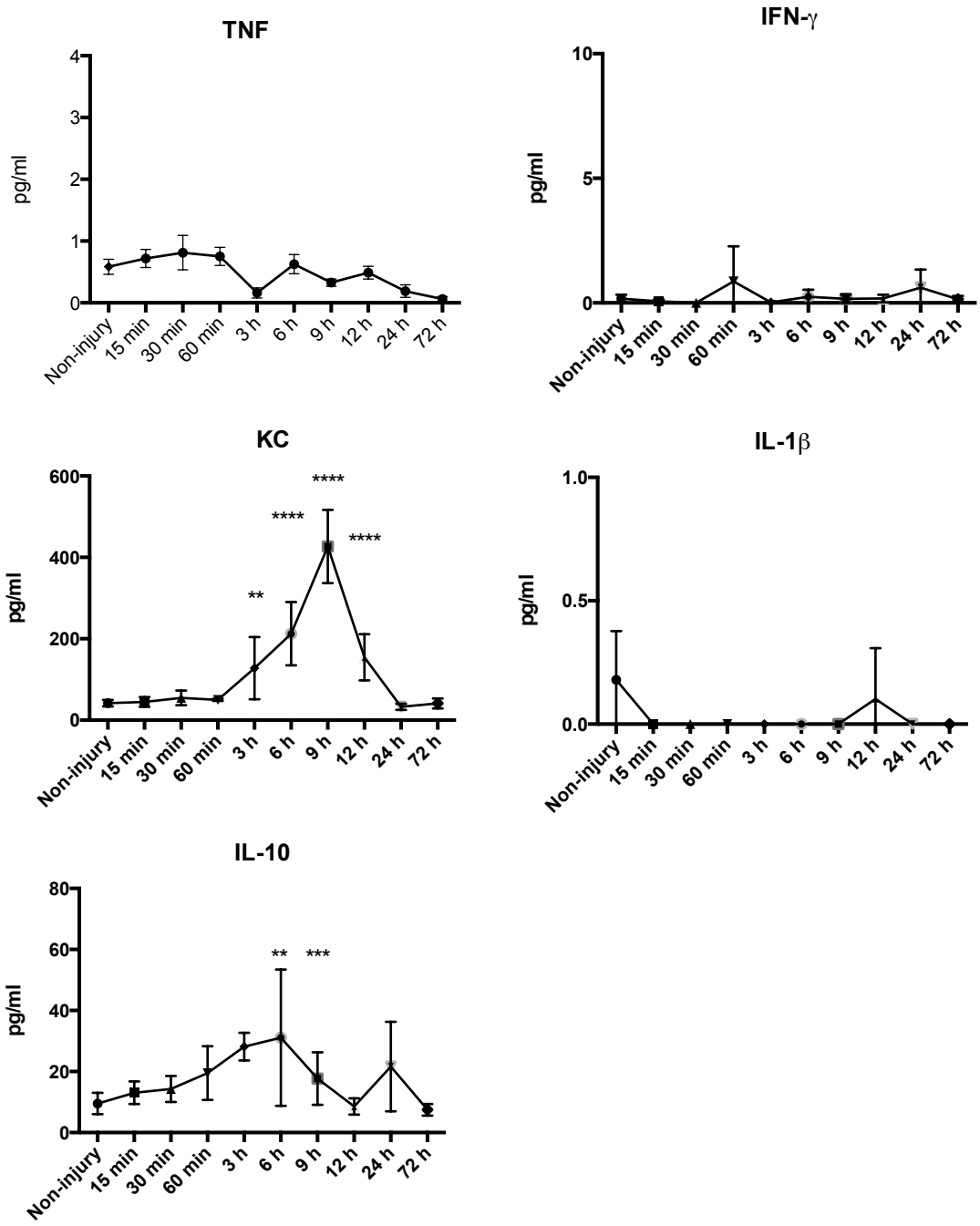


Fig 4.3*. Systemic levels of TNF and other cytokines over time course following fracture by chemiluminescence. 6 mice were used per time point. 1-way ANOVA with Bonferroni multiple comparison's test compared to non-injury control, * $p \leq 0.05$, ** $p \leq 0.001$, ***$p \leq 0.0001$, ****$p \leq 0.00001$.*

4.2.2 Murine fracture supernatants contain low levels of inflammatory cytokines.

To assess the local cytokine environment at the fracture site, murine fracture supernatants were produced by incubating fractured tibiae in media as described previously (Glass, Chan et al. 2011). Briefly, fracture fragments were harvested from murine tibiae 3 hours post-injury and cultured in serum-free media for 12 hours. The media was then filter-sterilized. Consistent with our published findings in human fracture supernatants (Glass, Chan et al. 2011), the levels of proinflammatory cytokines, including TNF, were low (Fig 4.4). The levels of TNF were below the level of detection by MSD (< 3.5 pg/ml).

Cytokine	pg/ml
TNF	0.0 $\pm$ 0.0
IL6	305.3 $\pm$ 135.1
KC	103.6 $\pm$ 37.6
IL1β	25.6 $\pm$ 7.9
IL10	6.52 $\pm$ 3.9

***Fig 4.4**. Levels of cytokines in the murine supernatants measured using chemoluminescence. 30 mice were harvested. Figures represent mean $\pm$ SEM.*

4.2.3 Low levels of cytokine expression detected at the fracture site using RT-PCR

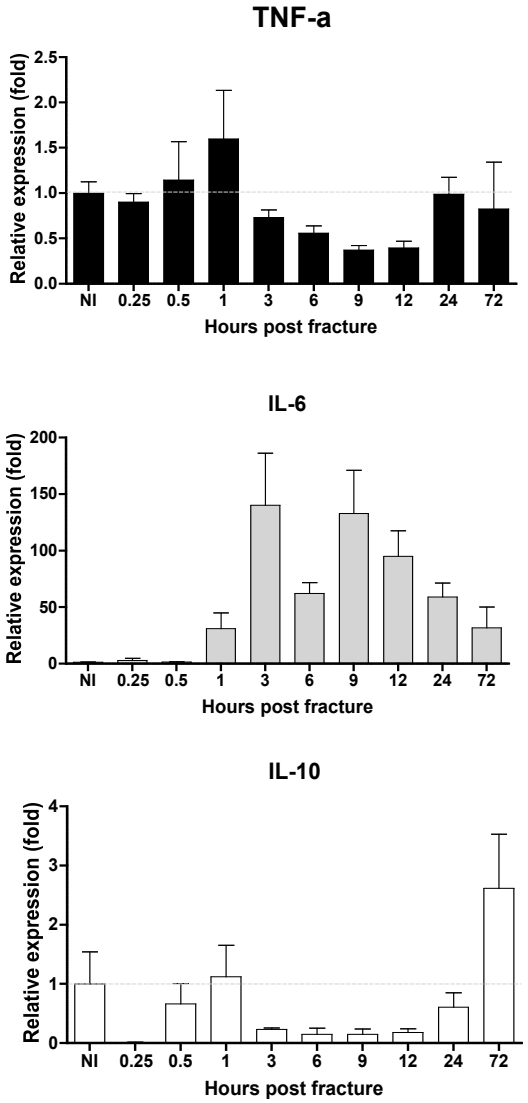


Fig 4.5*. Local levels of expression of TNF, IL-6 and IL-10 following fracture. No significant differences were observed using ANOVA. NI = no injury control*
(Experiments performed in collaboration with Dr. Garry Williams, post-doctoral scientist, Kennedy Institute of Rheumatology)

The observation that TNF inhibition impaired fracture healing in vivo suggests that endogenous TNF is expressed locally, albeit at a low level. To assess the level of cytokine production locally, the adjacent soft tissues at the fracture site were harvested for RT-PCR. However, the levels of cytokine expression were low as they were not detectable until cycle 25-30 (Fig 4.5).

To assess TNF expression at the cellular levels, in situ hybridization on histological sections of the murine fracture site was performed.

4.2.4 Non-isotopic in situ hybridization is not a suitable technique to detect TNF expression in local murine tissues.

Non-isotopic in situ hybridization was attempted but the level of autofluorescence precluded sensitive and specific detection of TNF expression with this technique (Fig 4.6).

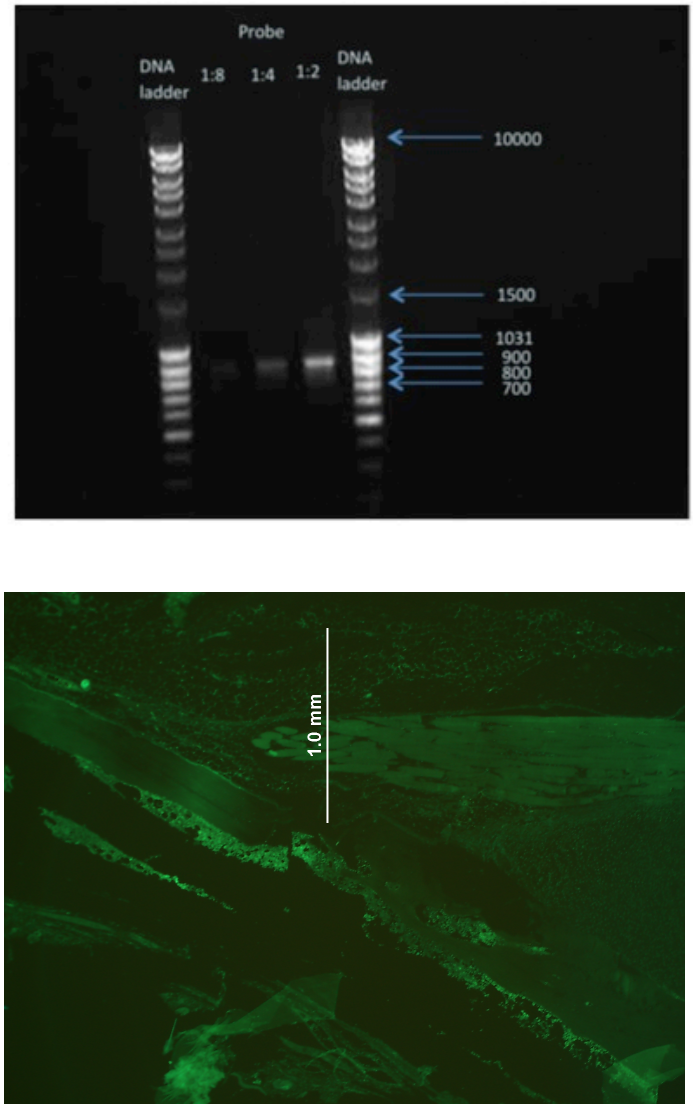


Fig 4.6*. Non-isotopic In-situ hybridization using mTNF template. Above: confirmation of probe size. Below: the level of background auto-fluorescence was too high to enable sensitive and specific detection of TNF expression using this technique.*

4.2.5 TNF is expressed by neutrophils within 15 minutes of bone injury at the fracture site, followed by monocytes/macrophages at day 3 using isotopic in situ hybridization and immunohistochemical staining.

TNF was expressed within 15 minutes of injury, co-localizing first with endothelial cells and neutrophils (Fig 4.7). Neutrophils were identified by their polymorphonuclear morphology as well as positive staining with anti-neutrophil elastase. From day 3 onwards, TNF expression co-localized with cells of the monocyte/macrophage lineage (F4/80). Neutrophils were the predominant cell type present before day 3 while F4/80+ cells were the predominant cell type on days 5-7.

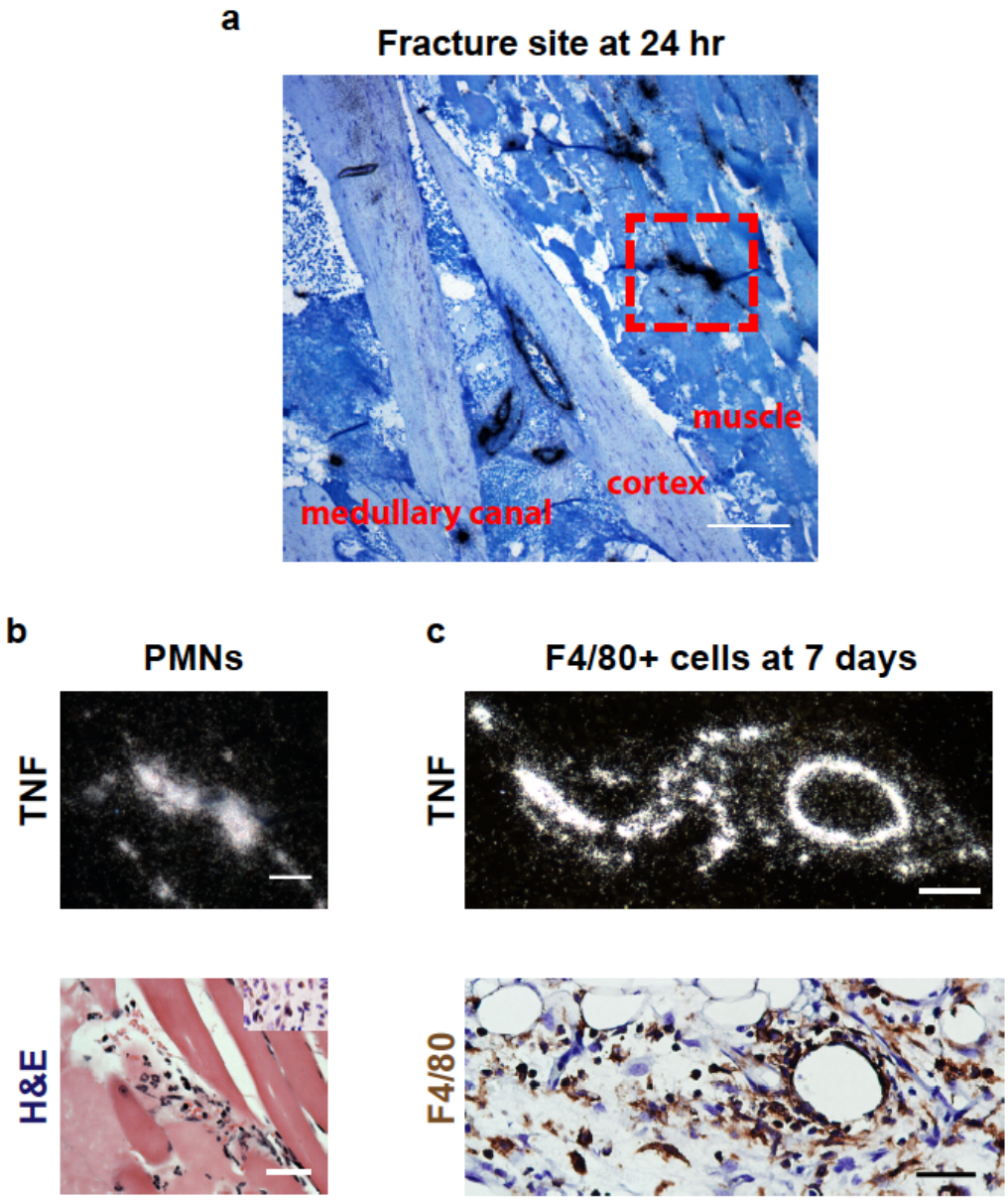


Fig 4.7*. 4.7a Representative ISH image (light field) showing TNF expression at the murine fracture site at 24 hours after operation. Scale bar 250 µm. Red box indicates region of interest. 4.7b. High power micrographs of region of interest: at 24 hours after operation, mTNF expression (dark field, above) co-localized with polymorphonuclear cells found on the adjacent H&E section (below). Scale bar 25 µm. Neutrophils were identified by their polymorphonuclear morphology as well as positive staining with anti-neutrophil elastase. 4.7c. High power micrographs: at 7 days, TNF expression (dark field, above) co-localized with F4/80 positive cells extravasating from a blood vessel on the adjacent H&E section (below). Scale bar 25 µm. (In situ hybridization performed by Ms. Rosemary Jeffery, Dr. William Otto and Professor Richard Poulsom at the Cancer Research UK, London)*

4.3 The role of neutrophils in fracture repair

4.3.1 Neutrophils are swiftly mobilized in the systemic circulation post-fracture.

As neutrophils were the first cells to express TNF at the fracture site, and recruitment of neutrophils represents a key early event during the inflammatory response (Nathan 2006), their role in fracture healing was investigated. First, the systemic mobilization of neutrophils in our murine fracture model was assessed. As shown in Fig 4.8, blood neutrophil count increased within 30 minutes of injury and peaked at 3 hours following fracture.

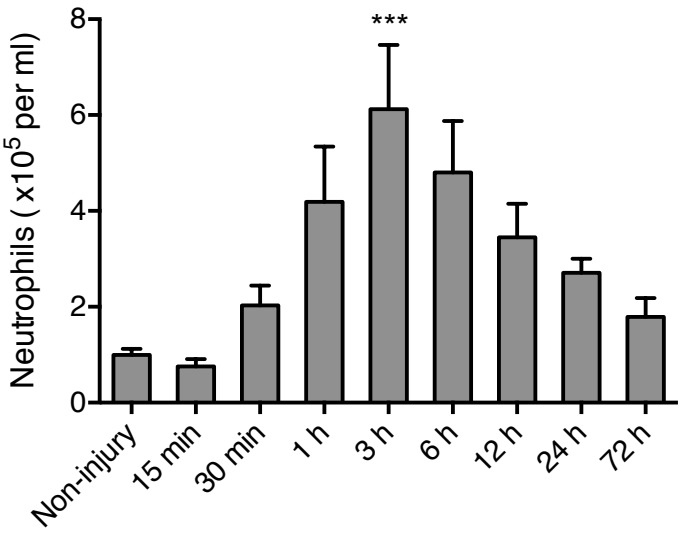


***Fig 4.8**. Neutrophils were mobilized into the murine systemic circulation within 30 minutes of injury in our murine fracture model. *** p ≤ 0.0001, ANOVA with Bonferroni correction compared to non-injury control. Performed in collaboration with Dr. Kate Gowers of Professor Sara Rankin's Group, National Institute of Heart and Lung, Imperial College London.*

4.3.2 Anti-CXCR2 inhibits systemic mobilization of neutrophils and reduces the size of fracture callus.

As KC is a major neutrophil chemokine and was found to be significantly elevated post-fracture (Fig 4.3), I investigated whether its expression is important in fracture repair in vivo. KC is a CXC chemokine and a functional homologue of human IL-8. Its expression can be stimulated by the presence of TNF and it signals via CXCR2 to mediate neutrophil chemotaxis (Scapini, Lapinet-Vera et al. 2000; Singer and Sansonetti 2004). To promote local neutrophil recruitment, KC or PBS was injected at the fracture site immediately and at 24 hours post-fracture. To inhibit systemic neutrophil mobilization, anti-CXCR2 or IgG isotype control was given intraperitoneally 1 hour pre-fracture and at 72 hours post-fracture. The number of neutrophils in the systemic circulation was counted at 3 hours, the time point at which the neutrophil count peaked in the time course experiment post-fracture (Fig 4.9), and on day 28 post-fracture. Local KC treatment did not affect neutrophil count while anti-CXCR2 significantly depleted neutrophil count at 3 hours post-fracture.

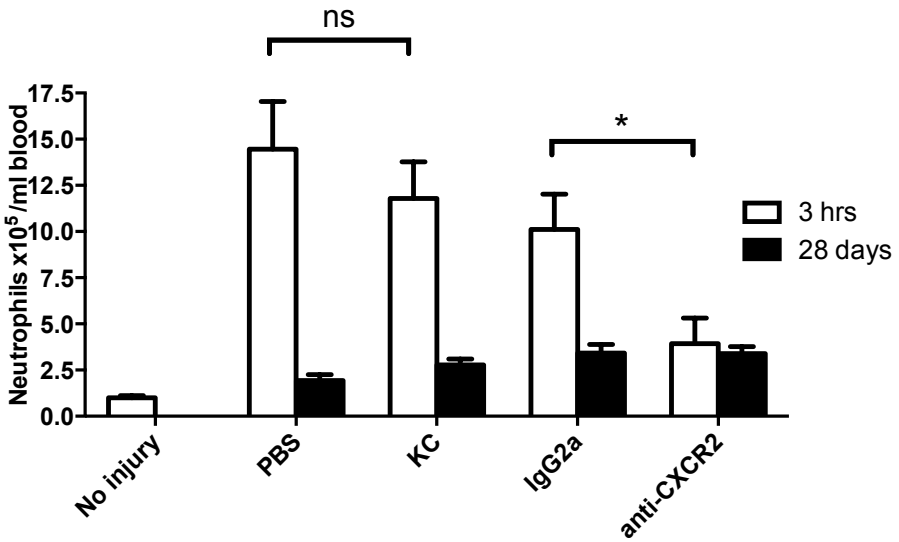
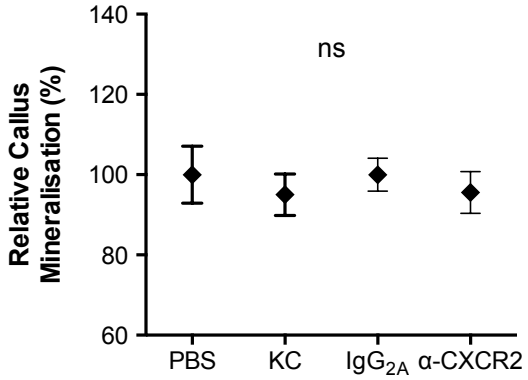


***Fig 4.9**. anti-CXCR2 treatment reduced the blood neutrophil count at 3 hours post-fracture compared to IgG control. KC treatment did not affect neutrophil count. Neutrophil counts returned to baseline levels in all treatment groups. At least 6 animals were used per group. Figures represent mean ± SEM. *p<0.05, 2 sided t-test.*

Although systemic treatment with anti-CXCR2 reduced neutrophil mobilization during the early post-fracture period, it did not affect relative % callus mineralization at day 28 although the amount of bone volume was reduced (Fig 4.10). Local injection of KC at the fracture site did not affect fracture healing at day 28.



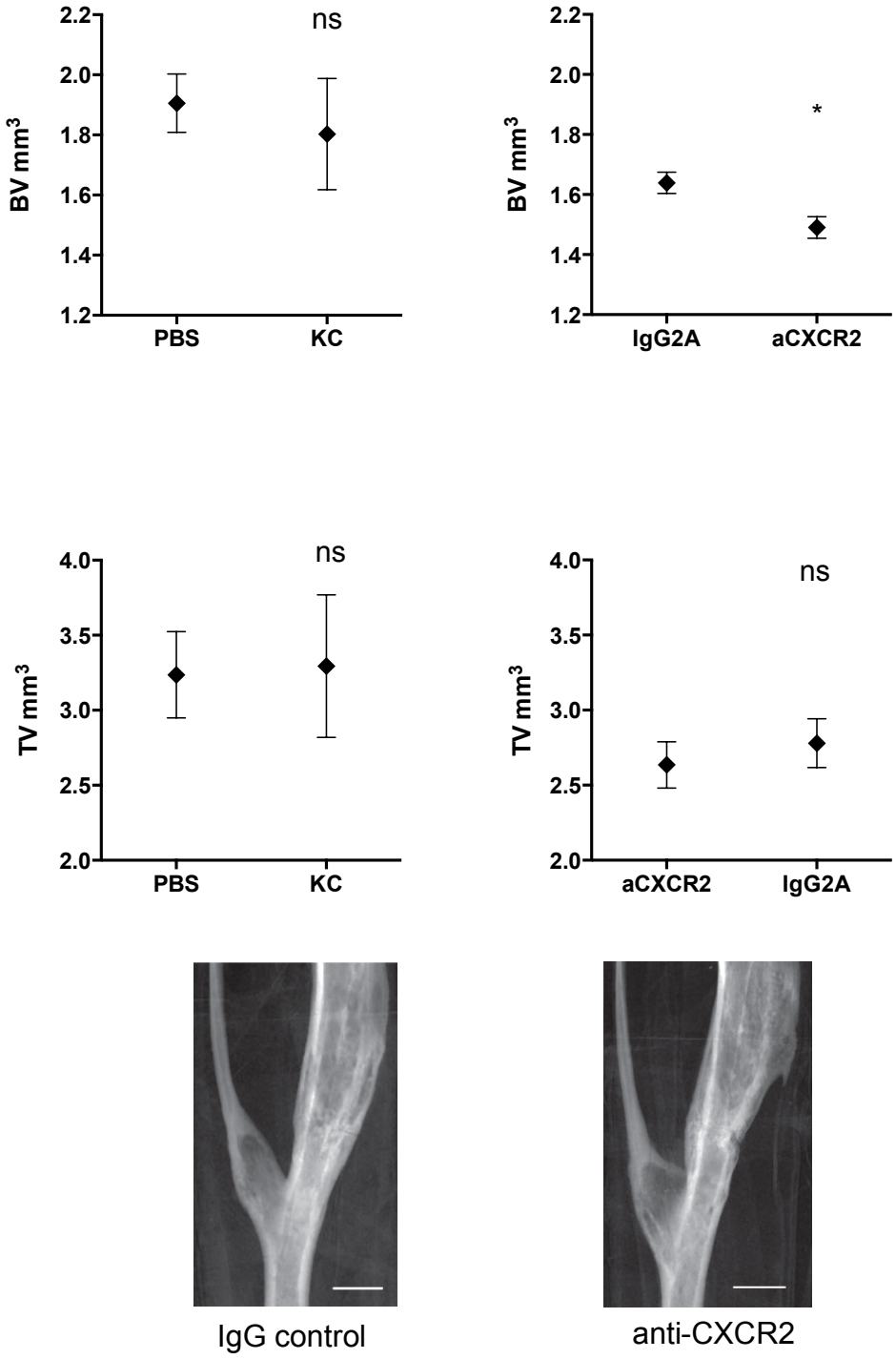
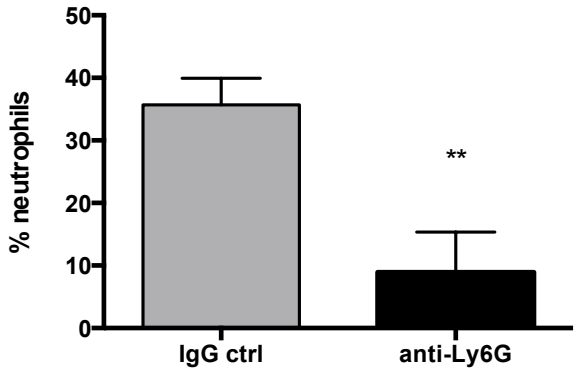
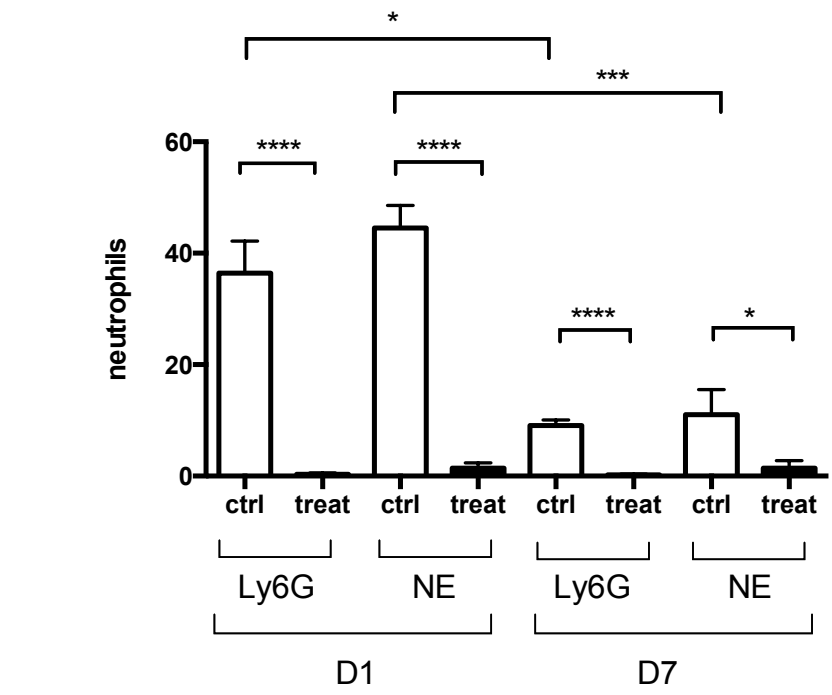


***Fig 4.10**. Row 1: KC and anti-CXCR2 treatment did not affect % callus mineralization at day 28 post-fracture. Rows 2&3: However, mineralized callus volume was reduced with anti-CXCR2 treatment but total volume was not affected. Row 4: Representative X-rays showing anti-CXCR2 treatment reduced callus volume, particularly the anterior cortex, but not relative % callus mineralization. (Advice on experimental design provided by Professor Sara Rankin, National Institute of Heart and Lung, Imperial College London)*

4.3.3 Anti-Ly6G antibody depletes neutrophils systemically, inhibits local recruitment at the fracture site and impairs fracture repair.

Next, mice were treated with a validated and specific Ly6G-blocking antibody (Daley, Thomay et al. 2008). This effectively inhibited the systemic mobilization and local recruitment of neutrophils to the fracture site (Fig 4.11) and was associated with significant impairment of fracture healing at day 28 after surgery. Representative histological sections stained with Masson's Trichrome at day 14 showed anti-Ly6G treatment led to formation of a large immature and unmineralized callus compared to IgG control treatment (Fig 4.12). MicroCT analysis and representative micro-CT images showed that anti-Ly6G treatment led to delayed mineralization and remodeling of the fracture callus at day 28 (Fig 4.13).





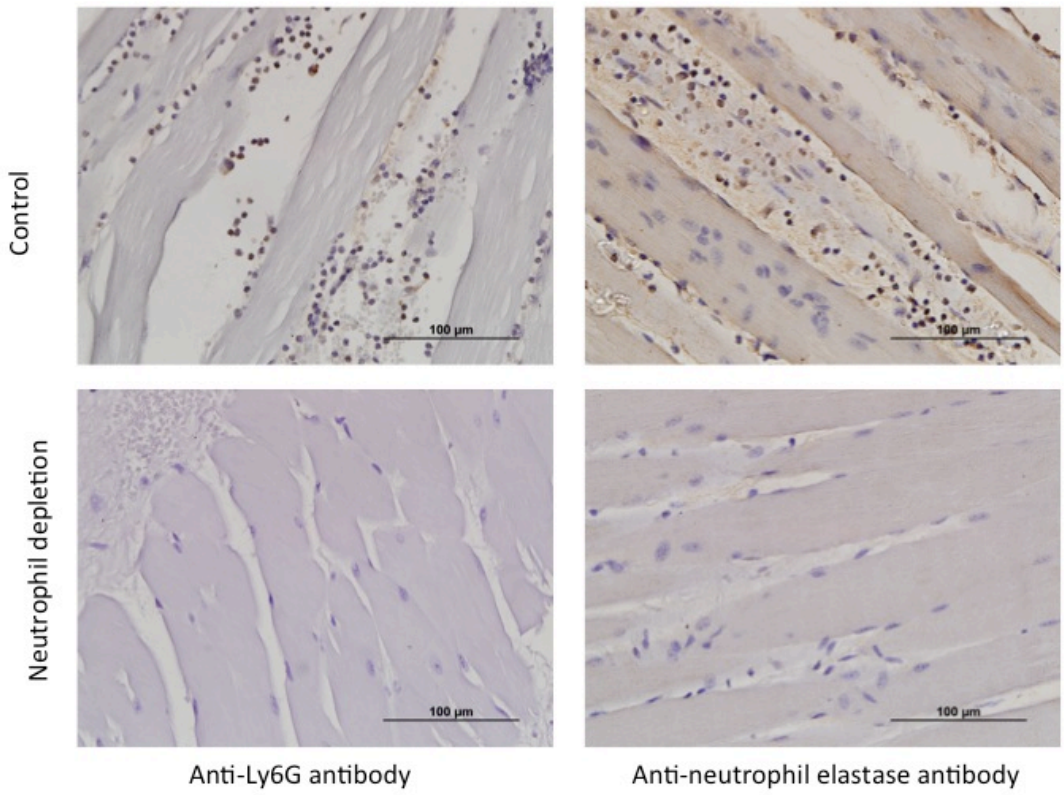


Fig 4.11. *Above: Anti-Ly6G antibody depletes neutrophils in systemic circulation. Middle: Counts of positively stained infiltrative neutrophils in the adjacent muscle to the fracture site comparing neutrophil depletion with anti-Ly6G antibody versus IgG control. Below:*

Representative sections showing local infiltration of neutrophils in the adjacent muscle stained using anti-Ly6G (left) and anti-neutrophil elastase (right) primary antibodies at Day 1 and Day 7 post-fracture following neutrophil depletion using anti-Ly6G antibody (lower row) or IgG control (upper row).

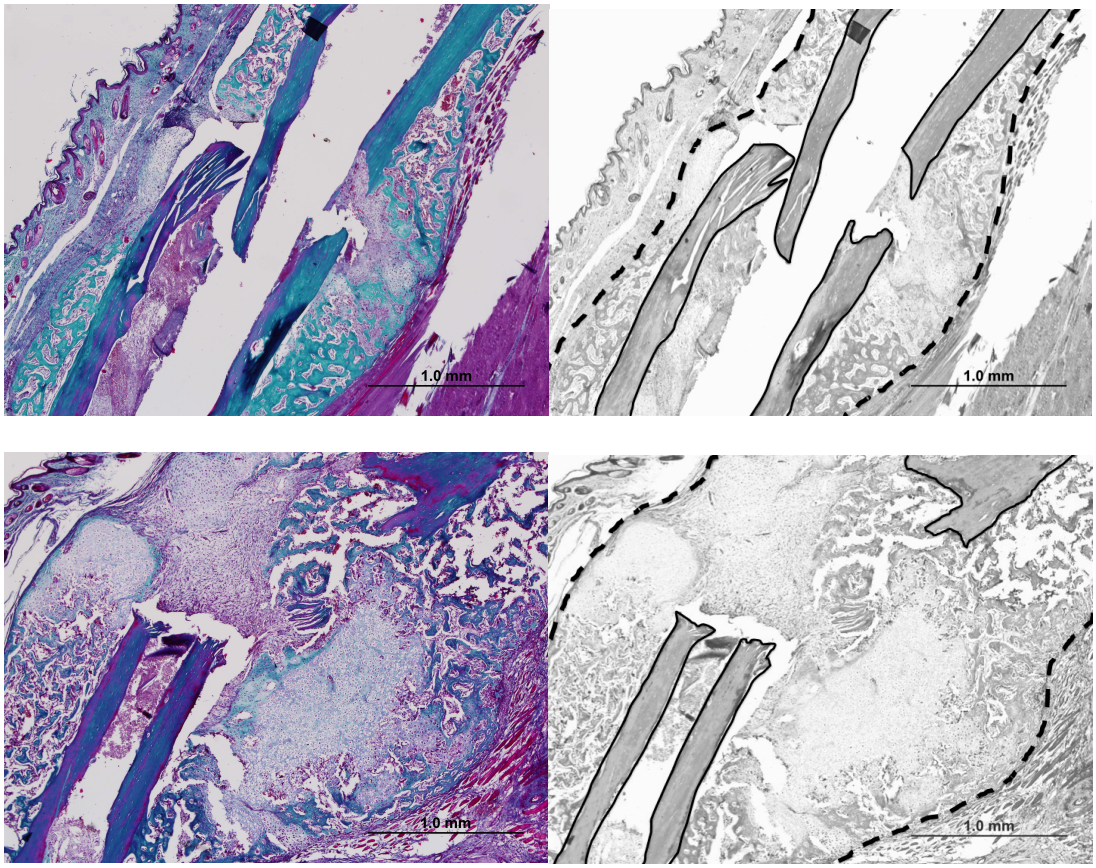


***Fig 4.12**. Depletion of neutrophil using anti-Ly6G led to impaired fracture healing. Representative section stained with Masson's Trichrome at day 14 comparing fracture healing in mouse treated with isotype control versus anti-Ly6G. Control section shows advanced mineralized callus while treatment section shows a large immature unmineralized callus. Muscle fibres – red; collagen, bone and mineralized callus – green. Dotted line outlines callus, solid line outlines cortical bone.*

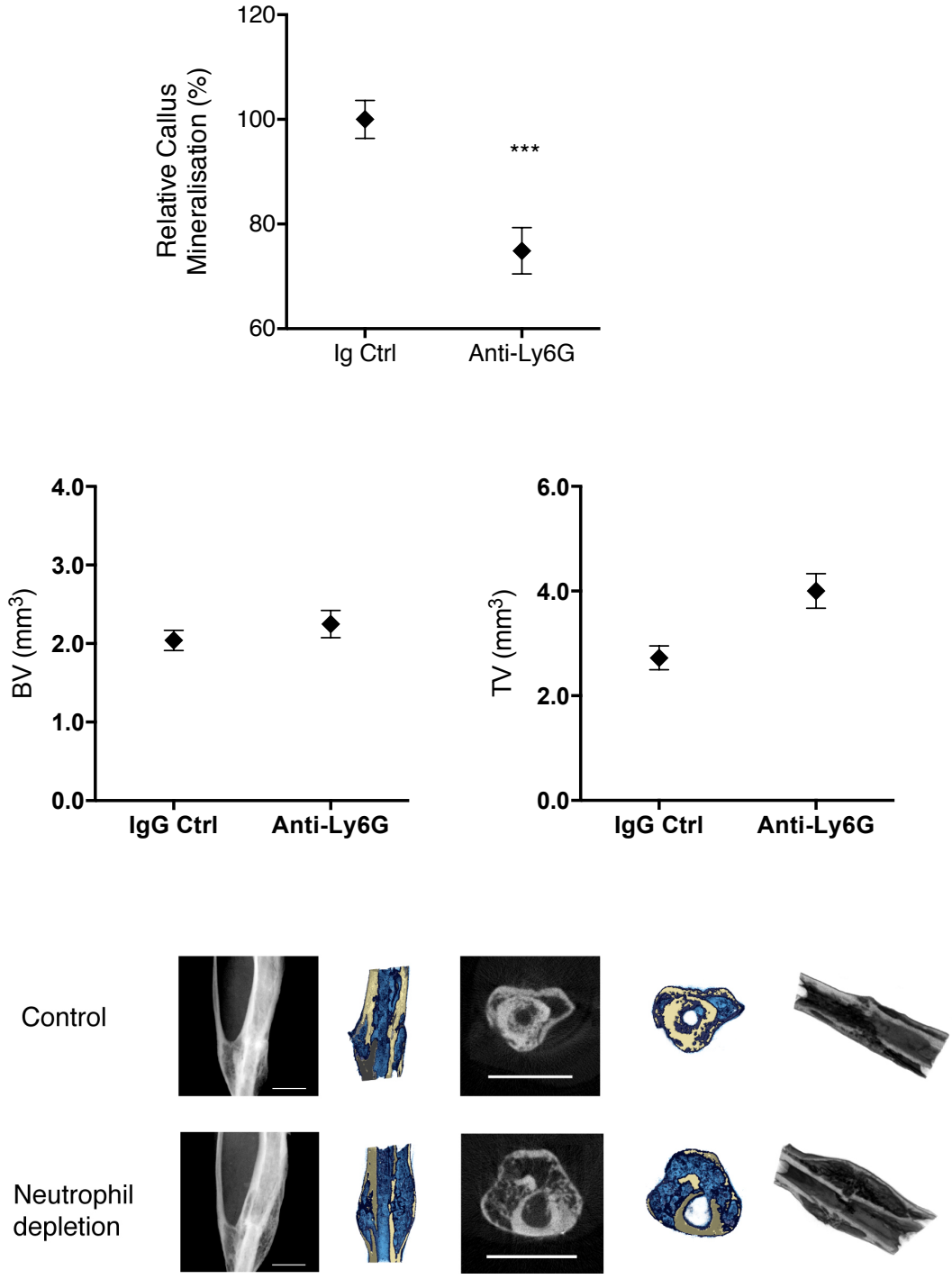


Fig 4.13. *Anti-Ly6G treatment led to impaired fracture healing as shown by the reduced % callus mineralization at day 28 after surgery. At least 6 animals were used per group. Figures represent mean ± SEM. Relative % callus mineralization: IgG Ctrl, 100 ± 3.61 %; Anti-Ly6G 74.90 ± 4.435. *** p ≤ 0.001 by 2 sided t-test. Below: Representative micro-CT images showing anti-Ly6G treatment led to delayed mineralization and remodeling of the fracture callus.*

4.4 Addition of rTNF promotes neutrophil recruitment

4.4.1 The murine air pouch model of inflammation is a suitable technique to investigate the recruitment of inflammatory cells in response to a local inflammatory stimulus.

The analysis of many sections at multiple time points is required to combine the static pictures provided by histology into an appreciation of the dynamics of cell migration in vivo. Moreover, my optimization experiments showed that the decalcification process with EDTA, necessary in order to enable sectioning of the bone, precludes the use of the neutrophil markers, neutrophil elastase and Ly6G. Therefore, the air pouch model of inflammation, which is a widely accepted and validated in vivo model for studying the regulation of the early events of local inflammation, particularly the role of cytokines and cell migration, was used (Romano, Faggioni et al. 1997). Fracture supernatant was injected into the air pouch to emulate the local inflammatory fracture environment and cells and supernatants were collected at 4 hours, which had been previously been found to be a suitable time point for the study of neutrophil and monocyte recruitment (Bertini, Howard et al. 1999).

To verify that the presence of an inflammatory stimulus leads to an influx in inflammatory cells, KC, zymosan and TNF were tested in the air pouch model. KC is a well known neutrophil chemokine (Ritzman, Hughes-Hanks et al. 2010). It was elevated from 3 hours post-fracture in the circulation (Fig 4.3). Inhibition of

its main receptor CXCR2 in vivo led to reduced mineralized callus volume although % callus mineralization did not change at day 28 (Fig 4.13). This chemokine would therefore serve as an appropriate positive control to verify the air pouch model as an in vivo assay to investigate recruitment of inflammatory cells. Fig 4.14 shows that a high dose of KC (100 ug or more) in media + 10% FCS was necessary to stimulate the influx of inflammatory cells in the air pouch model. Zymosan is a potent and non-specific immunostimulant. Similar to KC, zymosan stimulated recruitment of inflammatory cells at high doses (100 ug and above) (Fig 4.14). rhTNF has been reported to have a chemotactic effect on neutrophils and monocytes (Figari, Mori et al. 1987; Ming, Bersani et al. 1987; Wang, Walter et al. 1990; Lim, Ryu et al. 2009). Fig 4.15 shows that rhTNF stimulated a small influx of inflammatory cells when 1 ng was injected into the air pouch but this effect was abolished at higher doses. Interestingly, a combination of both rhTNF and zymosan led to an additive effect in inflammatory cell recruitment, suggesting that TNF can promote influx of inflammatory cells in the presence of other inflammatory stimuli (Fig 4.16).

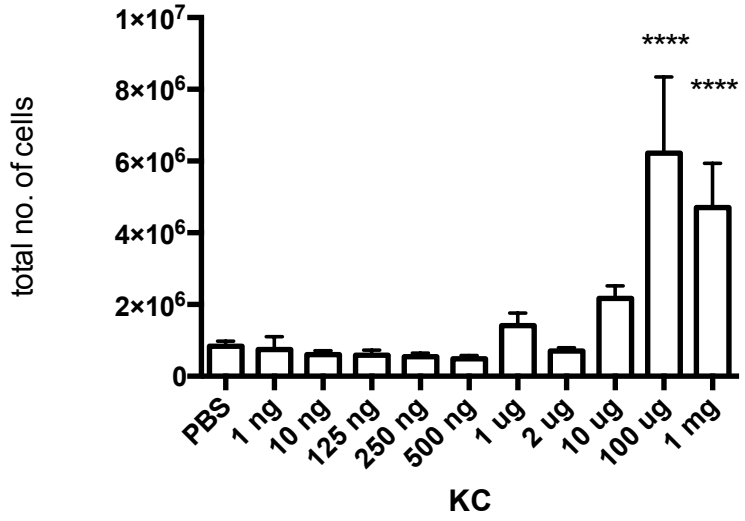


***Fig 4.14**. Immune cell migration into air pouch in response to KC, a known neutrophil chemokine. 6 mice were used per group. Figures represent mean ± SEM. **** p<0.0001, 1-way ANOVA using Bonferroni's post-test correction.*

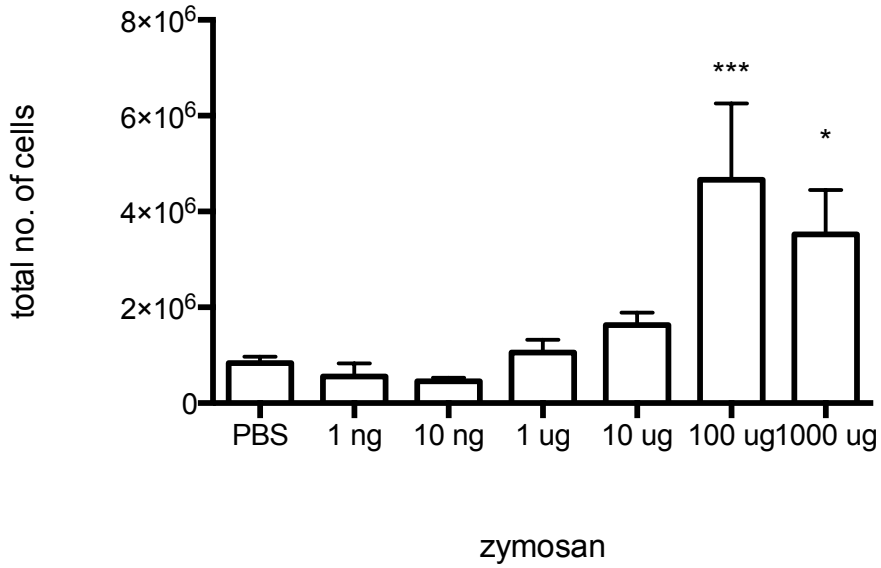


***Fig 4.15**. Immune cell migration into air pouch in response to zymosan, an inflammatory stimulus. 6 mice were used per group. Figures represent mean ± SEM. * p<0.05, *** p<0.001, 1-way ANOVA using Bonferroni's post-test correction.*

4.4.2 Addition of rhTNF promotes the recruitment of neutrophils to the fracture environment.

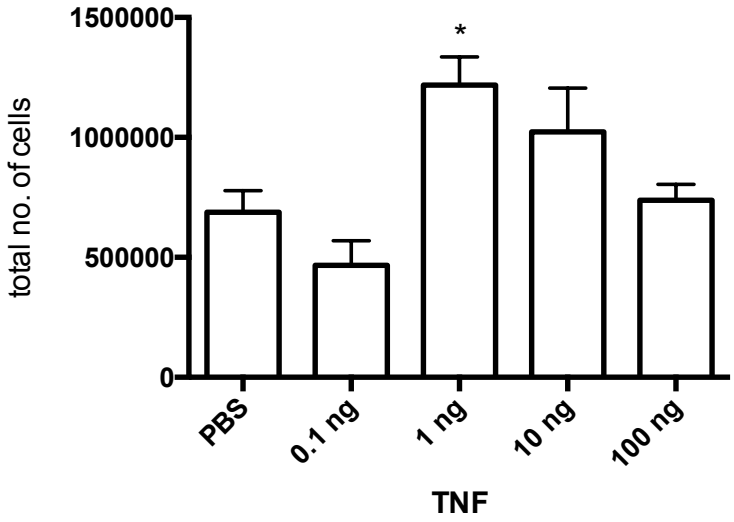


***Fig 4.16**. Immune cell migration into air pouch – dose response with rhTNF. 6 mice were used per group. * p<0.05., 1-way ANOVA using Bonferroni's post-test correction.*

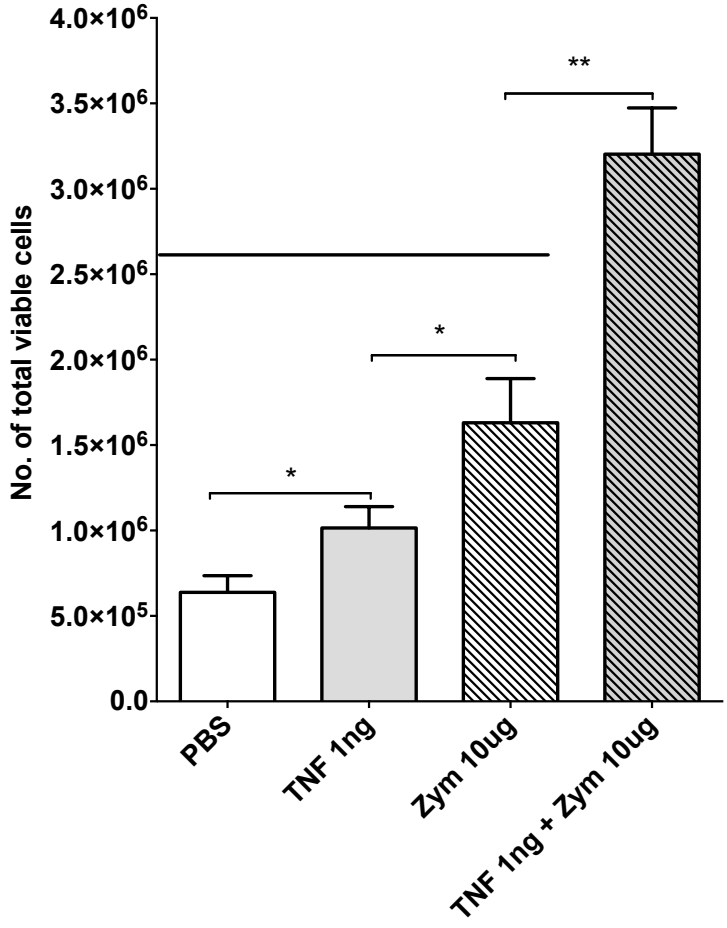
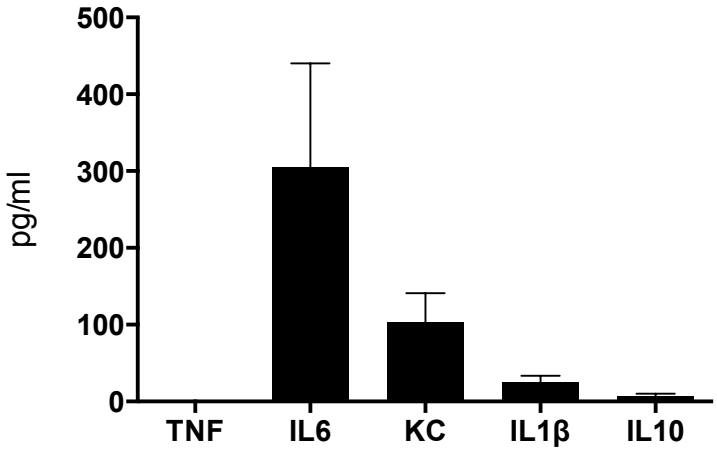


***Fig 4.17**. rhTNF and zymosan have an additive effect in the recruitment of inflammatory cells into the air pouch. 6 animals were used per group. Figures represent mean ± SEM. *p<0.05, **p<0.01. ANOVA using Bonferroni's correction*



As shown in Fig 4.17, the murine fracture supernatants contain low levels of inflammatory cytokines and chemokines. To assess whether the early cytokine environment at the fracture site stimulates leukocyte recruitment, the inflammatory cell recruitment following injection of fracture supernatant (FS) into the air pouch was compared to media only. FS led to a significantly elevated influx of inflammatory cells in the air pouch (Fig 4.18).

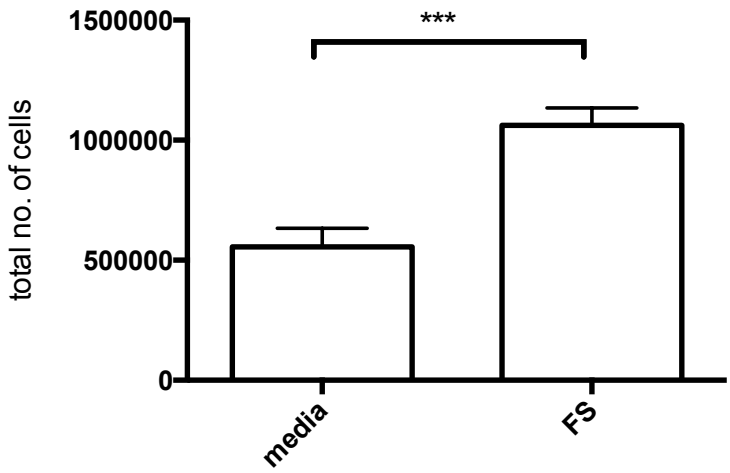
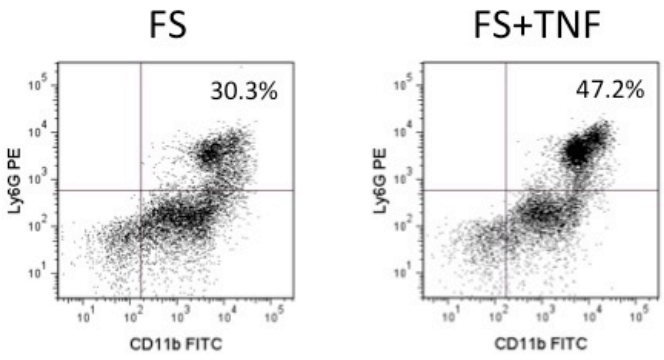


Fig 4.18*. FS provides a cytokine environment that promotes migration of immune cells. T-test (unpaired) ***p<0.001.*

To test whether rhTNF promotes the recruitment of inflammatory cells, rhTNF was added to fracture supernatant (FS+TNF) and compared to fracture supernatant alone, media alone or media + rhTNF. Addition of rhTNF in media promoted total inflammatory cell recruitment compared to media alone. Its addition to fracture supernatant also promoted a significant rise in the number of inflammatory cells in the air pouch exudate compared to fracture supernatant alone. To test the effect on neutrophil influx specifically, FACs was performed to identify Ly6G+CD11b+ cells and the total numbers derived from the percentages measured (Fig 4.19).



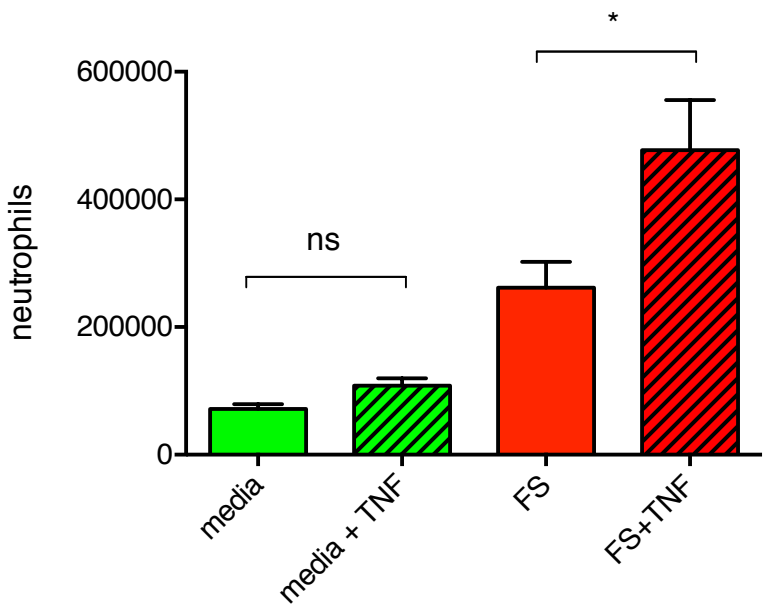


***Fig 4.19**. Addition of TNF promoted influx of neutrophils into air pouch; derived neutrophil numbers (above) and representative FACs plots (below). *p<0.05, 1-way ANOVA using Bonferroni's post-test correction.*

4.5 Discussion

Although it is known that TNF is released at the fracture site, the kinetics of release of this cytokine and its cellular origin(s) have not previously been precisely defined. In their fracture model using TNFR-deficient mice, Gerstenfeld et al found that TNF participates at several functional levels including the recruitment of MSCs, apoptosis of hypertrophic chondrocytes and recruitment of osteoclasts during fracture healing (Gerstenfeld, Cho et al. 2003). However, they did not investigate the role of TNF in initiating the inflammatory and repair phases of fracture healing. The data presented in chapter 3 provided evidence that TNF plays an important role during the early stage, within the first 24 hours, following skeletal injury. This and the following chapter attempt to elucidate the mechanism by which TNF initiates the fracture healing cascade.

Aside from KC, which showed a significant peak at 9 hours, the circulating level of proinflammatory cytokines, including TNF, did not alter significantly over 72 hours following skeletal injury (Fig 4.3). This suggests that an isolated tibial fracture is not sufficient to lead to release of detectable levels of proinflammatory cytokines in the systemic circulation. This would be consistent with the clinical observation that the systemic inflammatory response syndrome is typically seen only in polytrauma patients. To elucidate the timing and cellular origins of endogenous TNF following fracture, a combination of immunohistochemistry and in situ hybridization was used. Consistent with previous reports (Andrew, Andrew et al. 1994; Chung, Cool et al. 2006), I found that neutrophils are recruited first, followed by monocytes/macrophages 3 days

following skeletal injury (Fig 4.7). Of note, although monocytes are known to be major producers of TNF, the cells that produce TNF at the fracture site during the first 24 hours are neutrophils. Neutrophil TNF is thought to be mainly membrane bound and can induce cytokine production, including TNF, by other immune cells through neutrophil TNF release and cell-cell contact (Bennouna and Denkers 2005). The in situ hybridization (ISH) images also suggested that TNF expression co-localized with the endothelium. While endothelial cells have been found to express TNF and have TACE activity (Imaizumi, Itaya et al. 2000), it is also possible that the TNF signal originated from activated immune cells lining the endothelium.

Neutrophils are often associated with impaired healing processes and dysregulated inflammation. For example, they have been found to be important effectors in the pathogenic inflammation seen in rheumatoid arthritis through the formation of NETs and production of IL-17 (Katayama, Ohmura et al. 2013; Khandpur, Carmona-Rivera et al. 2013). Conversely, neutrophil depletion impaired bony repair in a rat model (Chung, Cool et al. 2006). To investigate the role of neutrophils in fracture repair, a series of experiments to manipulate neutrophils in vivo were performed.

In many models of inflammation, PMN mobilization from the bone marrow into the blood and recruitment into tissues is regulated via ELR+CXC chemokines, which act in the mouse via CXCR2 (Furze and Rankin 2008). I found that KC, a CXC chemokine and a functional homologue of human IL-8, was the only proinflammatory cytokine that was significantly upregulated in the systemic

serum within the first 24 hours (Fig 4.3). Furthermore, the number of circulating neutrophils increased post-injury, peaking at 3 hours (Fig 4.8). The murine CXCR2 is able to mediate neutrophil chemotaxis in response to both murine KC and human IL-8 (Singer and Sansonetti 2004; Ritzman, Hughes-Hanks et al. 2010). To further investigate the role of PMNs in fracture healing, mice were treated systemically with a CXCR2 blocking monoclonal antibody. Systemic administration of a neutralizing antibody to CXCR2 effectively inhibited the mobilization of PMNs into the blood stream (Fig 4.9) and was associated with significantly lower callus volumes at the fracture site (Fig 4.10), indicating that neutrophils play an important role in fracture healing. While local administration of KC did not augment callus volume or mineralization, this could be due to a number of reasons, including the short half life of KC in vivo (<10 minutes) (Laterveer, Lindley et al. 1996), KC being a systemic mobiliser rather than local recruiter of neutrophils(Scapini, Lapinet-Vera et al. 2000) and the amount of KC release not being a limiting factor. Administration of systemic KC was not attempted as this is a highly non-specific approach. Furthermore, chemokines are only effective when a concentration gradient is established. Systemic KC would not directly promote local recruitment of neutrophils specifically to the fracture site.

The alternative strategy of neutrophil inhibition using anti-Ly6G antibody demonstrated clearly that neutrophils play an essential role in fracture healing in vivo. Anti-Ly6G treatment significantly reduced systemic mobilization of neutrophils and recruitment at the fracture site (Fig 4.11) and led to significantly impaired fracture healing (Fig 4.12, 4.13). However, it remains

unclear why anti-CXCR2 and anti-Ly6G treatment, both of which essentially depleted systemic neutrophil mobilization led to different effects on fracture healing. A number of factors may account for this difference, including redundancy of the neutrophil chemokine network which features multiple chemokines including C5a, N-formyl-methionyl-leucyl, phenylalanine (fMLP), and leukotriene B4 (LTB4) as well as multiple chemokine receptors including CXCR1 and CXCR2(Yoshimura 2007). Other possibilities include different pharmacokinetics and pharmacodynamics of these reagents as well as differing mechanisms of action of the antibodies including depletion, blockade and cell lysis.

During inflammation, neutrophils are recruited to the damaged tissues and with time, the cells apoptose and are cleared by macrophages and dendritic cells. Humans and mice differ in their numbers of circulating neutrophils. In humans, 50-70% of circulating leukocytes are neutrophils whereas in mice only 10-25% are neutrophils (Mestas and Hughes 2004). It may therefore be reasonable to postulate that neutrophils serve a role that is at least as important in the human as it is in the mouse.

TNF has also been found to be a neutrophil chemokine both in vitro, using migration assays, and in vivo (Ming, Bersani et al. 1987; Cybulsky, McComb et al. 1989; Newman and Wilkinson 1989; Zhang, Ramos et al. 1992). Recent data suggest that neutrophil functions extend beyond the induction and persistence of inflammation. Neutrophils play a key role in cellular wound debridement by removing dead cells and pathogens to set the stage for healing. Indeed, removal

of neutrophils from an inflamed tissue exacerbates tissue damage, including following sterile injury (Fournier and Parkos 2012). Neutrophils express many proteases that may be important for tissue repair, for example matrix metalloprotease-9 (MMP-9), which digests extracellular matrix and degrades intracellular matrix components including actin, tubulin, annexin and HMGB1 (Cauwe, Martens et al. 2009). This provides a potential mechanism by which alarmins may be removed to downregulate and control inflammation during resolution and the latter phases of healing. MMP9 also activates vascular endothelial growth factor (VEGF) to promote revascularization at an injured site (Christoffersson, Vagesjo et al. 2012) and neutrophils release angiogenic factors such as VEGF-A (Christoffersson, Henriksnas et al. 2010; Gong and Koh 2010). Neutrophils also promote the recruitment of other cells types including monocytes and macrophages into the inflamed tissues. The recruited monocytes and macrophages phagocytose apoptotic neutrophils and other dying cells. Therefore, by promoting their own removal, neutrophils contribute to the resolution of inflammation and promote healing and tissue repair (Canturk, Esen et al. 2001). However, the role of the early neutrophil-derived TNF in the inflammatory response remains unknown (Bennouna, Bliss et al. 2003; Tsuda, Takahashi et al. 2004; van Gisbergen, Sanchez-Hernandez et al. 2005) and is the subject of the next chapter.

References

Andrew, J. G., S. M. Andrew, et al. (1994). "Inflammatory cells in normal human fracture healing." Acta Orthop Scand **65**(4): 462-466.

Bastian, O., J. Pillay, et al. (2011). "Systemic inflammation and fracture healing." J Leukoc Biol **89**(5): 669-673.

Bennouna, S., S. K. Bliss, et al. (2003). "Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection." J Immunol **171**(11): 6052-6058.

Bennouna, S. and E. Y. Denkers (2005). "Microbial antigen triggers rapid mobilization of TNF-alpha to the surface of mouse neutrophils transforming them into inducers of high-level dendritic cell TNF-alpha production." J Immunol **174**(8): 4845-4851.

Bertini, R., O. M. Howard, et al. (1999). "Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells." J Exp Med **189**(11): 1783-1789.

Canturk, N. Z., N. Esen, et al. (2001). "The relationship between neutrophils and incisional wound healing." Skin Pharmacol Appl Skin Physiol **14**(2): 108-116.

Cauwe, B., E. Martens, et al. (2009). "Multidimensional degradomics identifies systemic autoantigens and intracellular matrix proteins as novel gelatinase B/MMP-9 substrates." Integr Biol (Camb) **1**(5-6): 404-426.

Christoffersson, G., J. Henriksnas, et al. (2010). "Clinical and experimental pancreatic islet transplantation to striated muscle: establishment of a vascular system similar to that in native islets." Diabetes **59**(10): 2569-2578.

Christoffersson, G., E. Vagesjo, et al. (2012). "VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue." Blood **120**(23): 4653-4662.

Chung, R., J. C. Cool, et al. (2006). "Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats." J Leukoc Biol **80**(6): 1272-1280.

Cybulsky, M. I., D. J. McComb, et al. (1989). "Protein synthesis dependent and independent mechanisms of neutrophil emigration. Different mechanisms of inflammation in rabbits induced by interleukin-1, tumor necrosis factor alpha or endotoxin versus leukocyte chemoattractants." Am J Pathol **135**(1): 227-237.

Daley, J. M., A. A. Thomay, et al. (2008). "Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice." J Leukoc Biol **83**(1): 64-70.

Figari, I. S., N. A. Mori, et al. (1987). "Regulation of neutrophil migration and superoxide production by recombinant tumor necrosis factors-alpha and -beta: comparison to recombinant interferon-gamma and interleukin-1 alpha." Blood **70**(4): 979-984.

Fournier, B. M. and C. A. Parkos (2012). "The role of neutrophils during intestinal inflammation." Mucosal Immunol **5**(4): 354-366.

Furze, R. C. and S. M. Rankin (2008). "Neutrophil mobilization and clearance in the bone marrow." Immunology **125**(3): 281-288.

Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A **108**(4): 1585-1590.

Gong, Y. and D. R. Koh (2010). "Neutrophils promote inflammatory angiogenesis via release of preformed VEGF in an in vivo corneal model." Cell Tissue Res **339**(2): 437-448.

Imaizumi, T., H. Itaya, et al. (2000). "Expression of tumor necrosis factor-alpha in cultured human endothelial cells stimulated with lipopolysaccharide or interleukin-1alpha." Arterioscler Thromb Vasc Biol **20**(2): 410-415.

Katayama, M., K. Ohmura, et al. (2013). "Neutrophils are essential as a source of IL-17 in the effector phase of arthritis." PLoS One **8**(5): e62231.

Khandpur, R., C. Carmona-Rivera, et al. (2013). "NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis." Sci Transl Med **5**(178): 178ra140.

Kon, T., T. J. Cho, et al. (2001). "Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing." J Bone Miner Res **16**(6): 1004-1014.

Kong, H. L., K. O. Lee, et al. (1992). "Medical treatment of Cushing's syndrome with aminoglutethimide and ketoconazole." Singapore Med J **33**(5): 523-524.
Laterveer, L., I. J. Lindley, et al. (1996). "Rapid mobilization of hematopoietic progenitor cells in rhesus monkeys by a single intravenous injection of interleukin-8." Blood **87**(2): 781-788.
Lekstrom-Himes, J. A. and J. I. Gallin (2000). "Immunodeficiency diseases caused by defects in phagocytes." N Engl J Med **343**(23): 1703-1714.
Lim, S., J. Ryu, et al. (2009). "Tumor necrosis factor-alpha potentiates RhoA-mediated monocyte transmigratory activity in vivo at a picomolar level." Arterioscler Thromb Vasc Biol **29**(12): 2138-2145.
Mestas, J. and C. C. Hughes (2004). "Of mice and not men: differences between mouse and human immunology." J Immunol **172**(5): 2731-2738.
Ming, W. J., L. Bersani, et al. (1987). "Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes." J Immunol **138**(5): 1469-1474.
MSD. from http://www.mesoscale.com/CatalogSystemWeb/WebRoot/index.aspx.
Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nat Rev Immunol **6**(3): 173-182.
Newman, I. and P. C. Wilkinson (1989). "Chemotactic activity of lymphotoxin and tumour necrosis factor alpha for human neutrophils." Immunology **66**(2): 318-320.
Rico, R. M., R. Ripamonti, et al. (2002). "The effect of sepsis on wound healing." J Surg Res **102**(2): 193-197.
Ritzman, A. M., J. M. Hughes-Hanks, et al. (2010). "The chemokine receptor CXCR2 ligand KC (CXCL1) mediates neutrophil recruitment and is critical for development of experimental Lyme arthritis and carditis." Infect Immun **78**(11): 4593-4600.
Romano, M., R. Faggioni, et al. (1997). "Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor." Mediators Inflamm **6**(1): 32-38.
Roos, D. and S. K. Law (2001). "Hematologically important mutations: leukocyte adhesion deficiency." Blood Cells Mol Dis **27**(6): 1000-1004.
Scapini, P., J. A. Lapinet-Vera, et al. (2000). "The neutrophil as a cellular source of chemokines." Immunol Rev **177**: 195-203.
Singer, M. and P. J. Sansonetti (2004). "IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis." J Immunol **173**(6): 4197-4206.
Soehnlein, O. and L. Lindbom (2010). "Phagocyte partnership during the onset and resolution of inflammation." Nat Rev Immunol **10**(6): 427-439.
Tessier, P. A., P. H. Naccache, et al. (1997). "Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-alpha." J Immunol **159**(7): 3595-3602.
Tsuda, Y., H. Takahashi, et al. (2004). "Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus." Immunity **21**(2): 215-226.
van Gisbergen, K. P., M. Sanchez-Hernandez, et al. (2005). "Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN." J Exp Med **201**(8): 1281-1292.
Wang, J. M., S. Walter, et al. (1990). "Re-evaluation of the chemotactic activity of tumour necrosis factor for monocytes." Immunology **71**(3): 364-367.
Weiss, S. J. (1989). "Tissue destruction by neutrophils." N Engl J Med **320**(6): 365-376.
Yoshimura, T. (2007). Chemokine Receptors.
Zhang, Y., B. F. Ramos, et al. (1992). "Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis." Science **258**(5090): 1957-1959.

Chapter 5

TNF initiates fracture healing by promoting monocyte/macrophage recruitment via the CCL2/CCR2 axis.

Chapter 5
TNF initiates fracture healing by promoting monocyte/macrophage recruitment via the CCL2/CCR2 axis.

5.1 Introduction

Based on the finding that TNF is expressed at the fracture site within minutes of the injury by infiltrating leukocytes, I next sought to elucidate the early mechanisms by which exogenous TNF accelerates fracture healing. TNF has been shown to activate and promote the recruitment and survival of inflammatory cells, including neutrophils and monocytes, and TNF inhibition reduces leukocyte infiltration. Indeed, local expression of TNF is essential for the recruitment of leukocytes to extravascular sites (Qin, Qiu et al. 2003). Furthermore, the effect of TNF on PMNs is dose dependent. Whilst low levels of TNF are associated with neutrophil survival and lead to further neutrophil recruitment, higher levels enhance neutrophil apoptosis and hence termination of the inflammatory response (van den Berg, Weyer et al. 2001).

In vitro, TNF promotes the release of chemokines, including CCL3 (MIP-1α) and sIL-6R, the main neutrophil-derived chemokines responsible for monocyte recruitment (Soehnlein and Lindbom 2010), as well as CCL2 (MCP-1) (Yamashiro, Kamohara et al. 2000). These drive the local recruitment of other inflammatory cells, including monocytes and macrophages (Pliyev 2008), which are critical for bone repair (Alexander, Chang et al. 2011; Wythe, Nicolaidou et al. 2014). Although compared to monocytes or macrophages, each neutrophil produces lower levels of a given cytokine, neutrophils typically represent the earliest cell type present and outnumber other phagocytes at inflammatory sites by 1 to 2 orders of magnitude. Hence, they are an important source of cytokines, including TNF, at a critical juncture of the healing response.

The chemokine CCL2 and its receptor CCR2 are responsible for monocyte trafficking in the body. In mice that lack the CCR2 gene, the local recruitment of macrophages to sites of injury is impaired (Kuziel, Morgan et al. 1997; Ma, Wei et al. 2002; Schober, Zernecke et al. 2004). The CCL2/CCR2 signaling axis has been shown to control the migration of monocytes from the bone marrow into the bloodstream and also from the circulation to sites of traumatic inflammation (Tsou, Peters et al. 2007).

It was therefore hypothesized that exogenous TNF at the fracture site during the early inflammatory phase leads to earlier recruitment of neutrophils and in turn monocytes/macrophages.

5.2 Anti-Ly6G antibody depletes local recruitment of monocytes/macrophages at the fracture site

Monocytes/macrophages are the predominant immune cell population at the fracture site at 7 days post-fracture (Fig 4.7c). They are the key cells that coordinate repair processes in many types of tissues including bone (Mosser and Edwards 2008; Wythe, Nicolaidou et al. 2014). Using immunohistochemistry for F4/80, a marker for monocytes/macrophages, I found that in addition to neutrophil depletion (Fig 4.10), anti-Ly6G treatment also led to reduced monocyte/macrophage recruitment to the local fracture site, including at day 7 post-fracture.

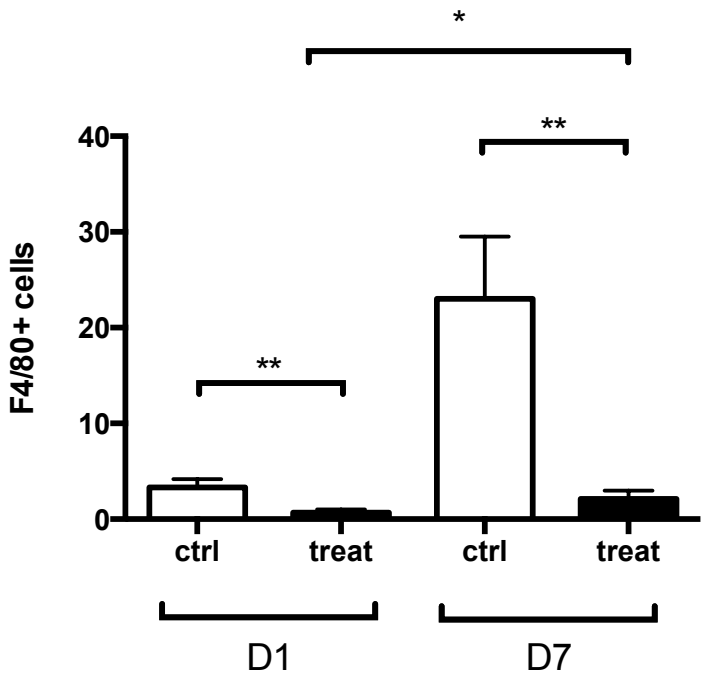


Fig 5.1 *Anti-Ly6G antibody depletes local monocytes/macrophages at the fracture site. Counts of positively stained infiltrative neutrophils in the adjacent muscle to the fracture site comparing neutrophil depletion with anti-Ly6G antibody versus IgG control. 3 animals were used per treatment group at each time point. *p<0.05; **p<0.01, 1-way ANOVA with Bonferonni's post-test correction.*

5.3 Investigation of rhTNF on the recruitment of monocytes/macrophages

5.3.1 rhTNF alone does not promote transmigration of human monocytes across chemotaxis well

To test whether rhTNF directly promotes chemotaxis of monocytes, freshly isolated human monocytes were exposed to a dose range of rhTNF in a transmigration assay. Briefly, the monocytes were prepared in RPMI media with 10% FCS at 1 million cells/ml and placed into the upper chamber of a chemotaxis well. The lower chamber contained serum with 10% FCS with a dose range of rhTNF from 0 to 100 ng/ml. The cells were incubated at 37^{o}C 5% CO2 for 14 hours. Migratory monocytes in the lower chamber were counted under the light microscope and expressed as cells per field of vision. There was no difference in the number of monocytes compared to control over the dose range tested (Fig 5.2).

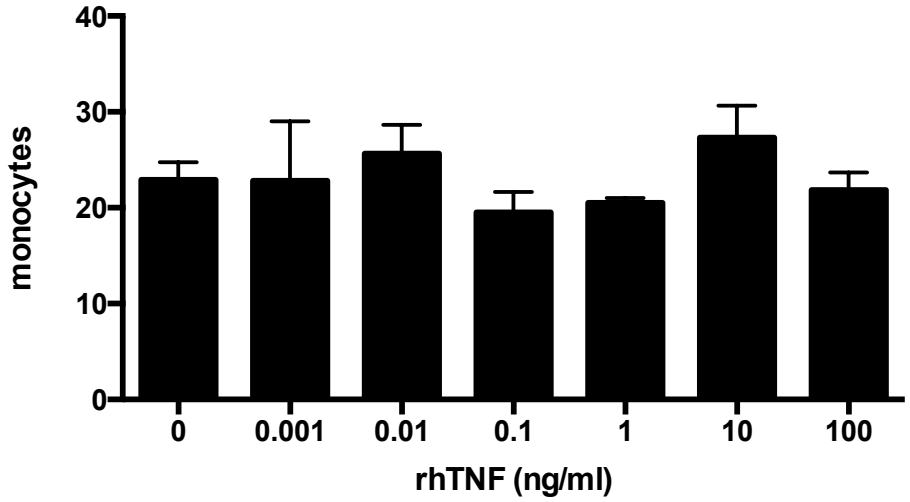


Fig 5.2 *Human monocyte count in the lower chamber of chemotaxis transwell assay across a dose range of rhTNF. The experiment was repeated 3 times. Figures represent the means*

from all 3 experiments ± SEM. No significant change was observed using 1-way ANOVA with Bonferroni's post-test correction.

5.3.2 Addition of rhTNF to the fracture environment promotes the recruitment of neutrophils and monocytes.

Since rhTNF was only effective when given in the first 24 hours following injury and the major inflammatory cell infiltrate during this period consisted primarily of TNF-expressing neutrophils, I investigated whether exogenous rhTNF which led to accelerated fracture repair in vivo acted via neutrophils. We hypothesized that additional rhTNF at the fracture site would enhance the innate immune response to promote the recruitment of neutrophils, which in turn attract monocytes that have been shown to be associated with fracture healing (Alexander, Chang et al. 2011) or orchestrate wound healing in other tissues (Scapini, Lapinet-Vera et al. 2000; Nathan 2006; Soehnlein and Lindbom 2010). Using the murine air pouch model (Lawrence, Gilroy et al. 2001), I set out to determine the dynamics of neutrophil and monocyte chemoattraction to the complex local fracture cytokine milieu. Media or murine fracture supernatants prepared as described above were injected into the air pouch either alone or in combination with 1 ng rhTNF, and the cellular infiltrates assessed at 4 hours. Addition of rhTNF resulted in an increase in both the number of neutrophils (Ly6G+, CD11b+ cells) (Fig 4.18) and monocytes/macrophages (Ly6G(-), CD11b+, CD115+ cells) in the air pouch (Fig 5.3).

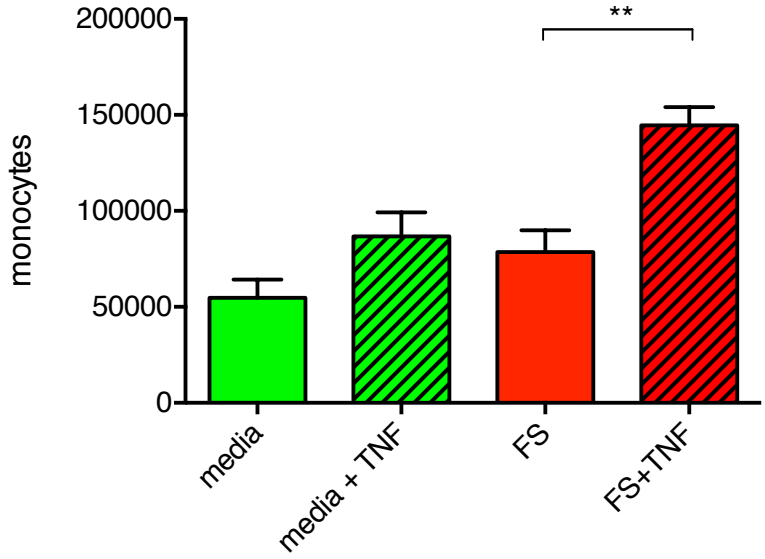


Fig 5.3 *Addition of rhTNF (1 ng) to fracture supernatant promoted recruitment of monocytes/macrophages (Ly6G-, CD11+, CD115+ cells) into air pouch exudate. A minimum of 6 animals were used per treatment group. Figures represent the means ± SEM. ** p ≤ 0.01, 1-way ANOVA with Bonferroni's post-test correction.*

5.4 Mechanism by which rhTNF recruits monocytes/macrophages

5.3.1 Addition of rhTNF to fracture supernatant induced chemokine CCL2 expression.

As monocytes are largely recruited by chemokines derived from neutrophils during the inflammatory response (Nathan 2006; Soehnlein and Lindbom 2010), we examined the production of the key neutrophil-derived monocyte chemokines CCL2 (MCP-1), CCL3 (MIP-1α) and sIL6R. Addition of 1 ng of rhTNF in the air pouches also led to an increase in CCL2 levels as determined by ELISA whilst levels of CCL3 and sIL-6R, levels were unaffected (Fig 5.4). The enhanced recruitment of both neutrophils and monocytes/macrophages in the air pouch model by TNF was abrogated by the addition of anti-CCL2 neutralizing antibody (Figs 5.5, 5.6). Representative FACS plots are also shown.

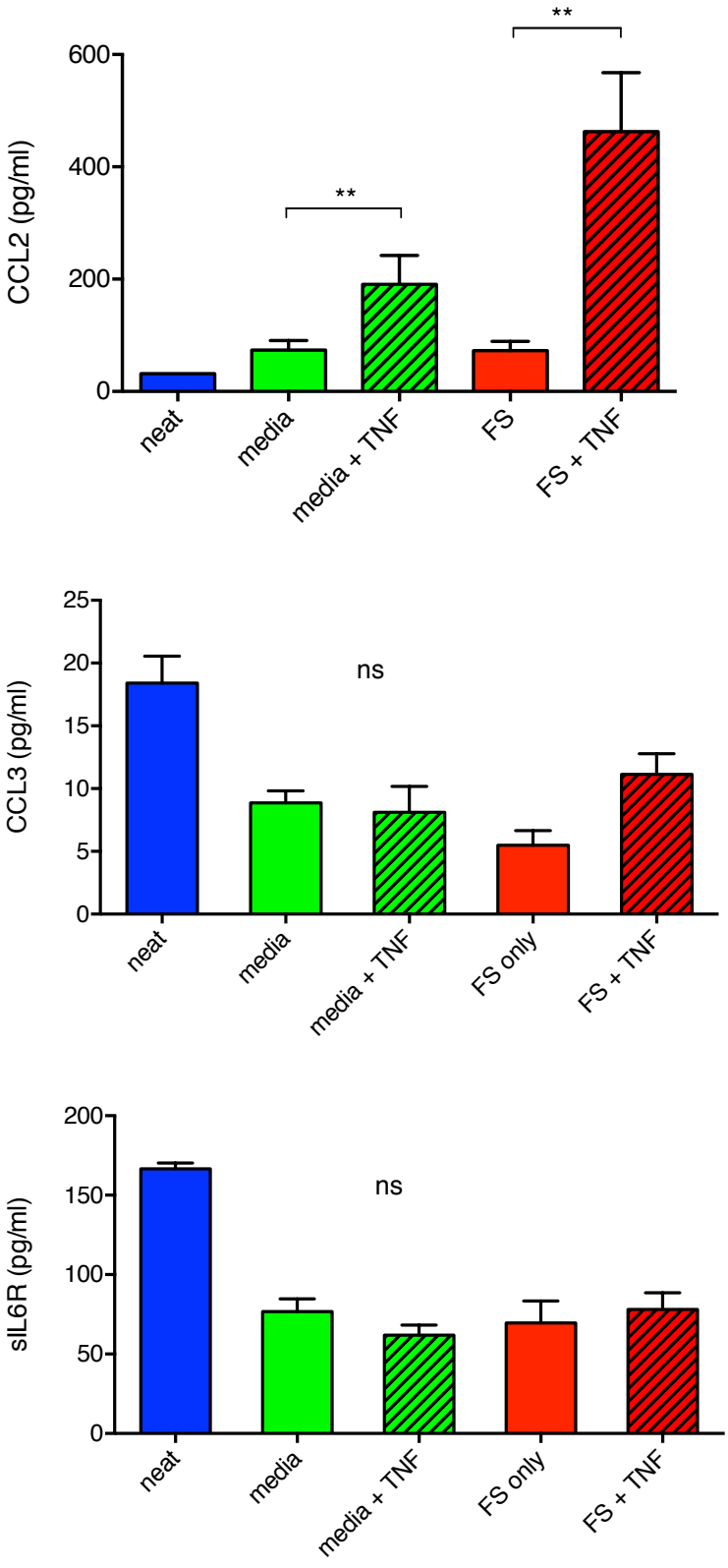


Fig 5.4 *Levels of the major neutrophil-derived monocyte chemokines measured by ELISA in air pouch exudates. At least 6 animals were used per treatment group. Figures represent mean ± SEM. ** p ≤ 0.01, 1-way ANOVA with Bonferroni's post-test correction. No significance differences were detected for CCL3 or sIL6R.*

5.3.2 Anti-CCL2 abrogates the promotion of recruitment of neutrophils as well as monocytes/macrophages.

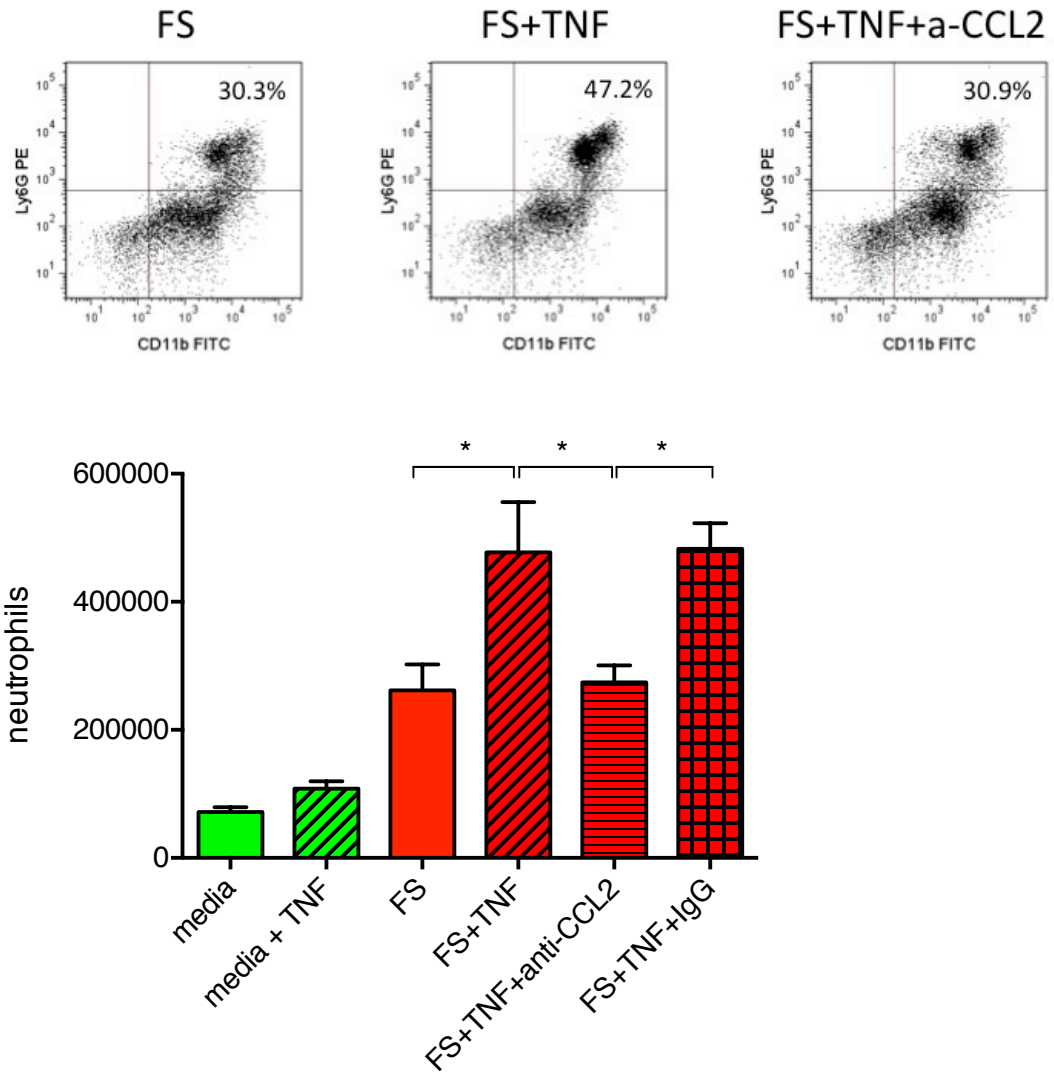


Fig 5.5 *rhTNF-induced promotion of neutrophil influx is abolished by addition of neutralizing antibody to CCL-2. Above: representative FACS plots with percentage of cells that are neutrophils (Ly6G+, CD11b+ cells). Below: derived cell counts. At least 6 animals were used per treatment group. Figures represent means ± SEM. *p<0.05, 1-way ANOVA with Bonferroni's post-test correction.*

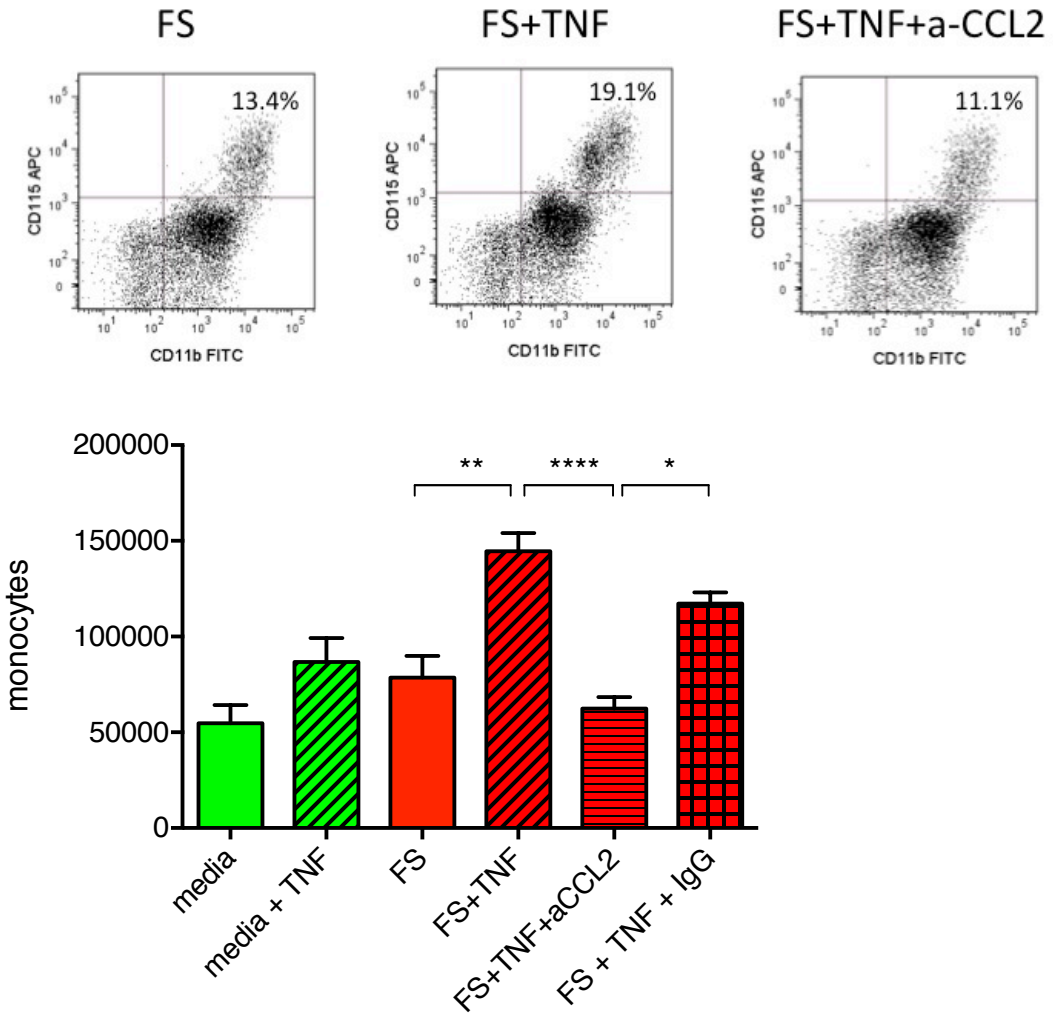


Fig 5.6 *rhTNF-induced promotion of neutrophil influx is abolished by addition of neutralizing antibody to CCL-2. Above: representative FACS plots with percentage of cells that are monocytes/macrophages (Ly6G-, CD11b+, CD115+ cells). Below: derived cell counts. At least 6 animals were used per treatment group. Figures represent means ± SEM. *p<0.05, ***p<0.001, 1-way ANOVA with Bonferroni's post-test correction.*

5.4.3 rhTNF promotes CCL2 expression by neutrophils.

To determine whether rhTNF can promote CCL2 expression by murine neutrophils, I used enriched preparations of murine neutrophils derived from the tibiae of C57BL/6 mice (82.7%) (Fig 5.7) and incubated them in fracture supernatant and rhTNF at a range of concentrations in vitro. As shown in Fig 5.8,

addition of 10 pg or more of rhTNF promoted CCL2 expression in neutrophils at 1 hour following exposure to fracture supernatant.

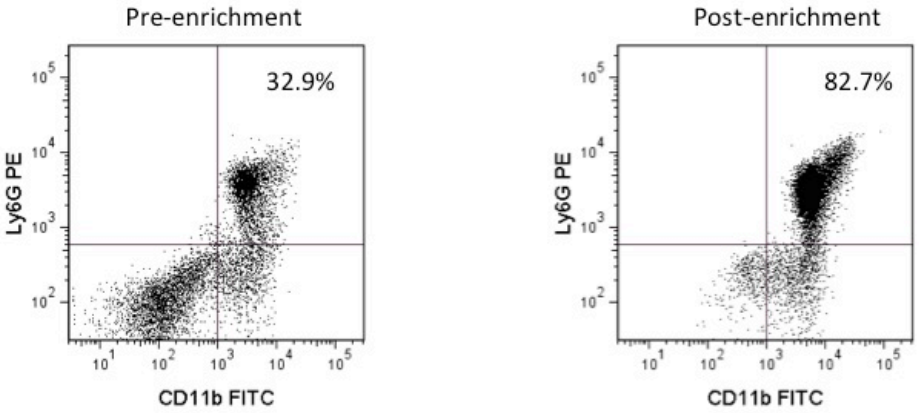


Fig 5.7 *Purity of neutrophils in % using an immunomagnetic negative murine neutrophil enrichment kit (Stem Cell Technology).*

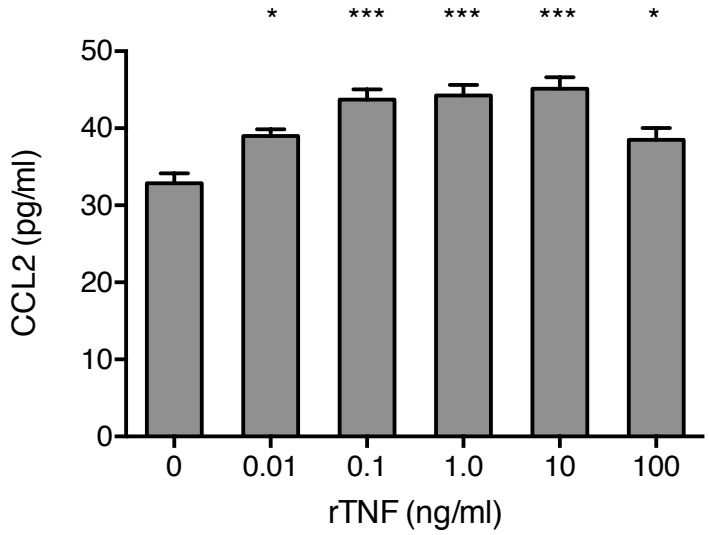


Fig 5.8 *Addition of rhTNF to enriched bone marrow-derived murine neutrophils pre-exposed to fracture supernatant in vitro led to upregulation of CCL production. The experiment was repeated 3 times. The figures represent the means ± SEM. * p<0.05; *** p<0.001, 1-way ANOVA with Bonferroni's post-test correction.*

There is controversy in the literature whether neutrophils are able to produce CCL2. It is possible that the CCL2 production seen in Fig 5.8 may be attributed to

contaminant cells other than neutrophils. To determine the ability of murine neutrophils to express CCL2, I used immunocytochemistry to detect co-expression (merged signal: yellow/orange) of neutrophil elastase(Green) and CCL2 (red). I collected cells from murine whole blood followed by red cell lysis. Neutrophils were identified by the multi-lobulated morphology of their nuclei on DAPI (blue) and the expression of neutrophil elastase. I then exposed the cells to either control media or fracture supernatant for 30 minutes followed by 1 ng rhTNF. As shown in Fig 5.9, murine neutrophils derived from whole blood clearly expressed CCL2 when exposed to a combination of fracture supernatant and TNF but not TNF alone.

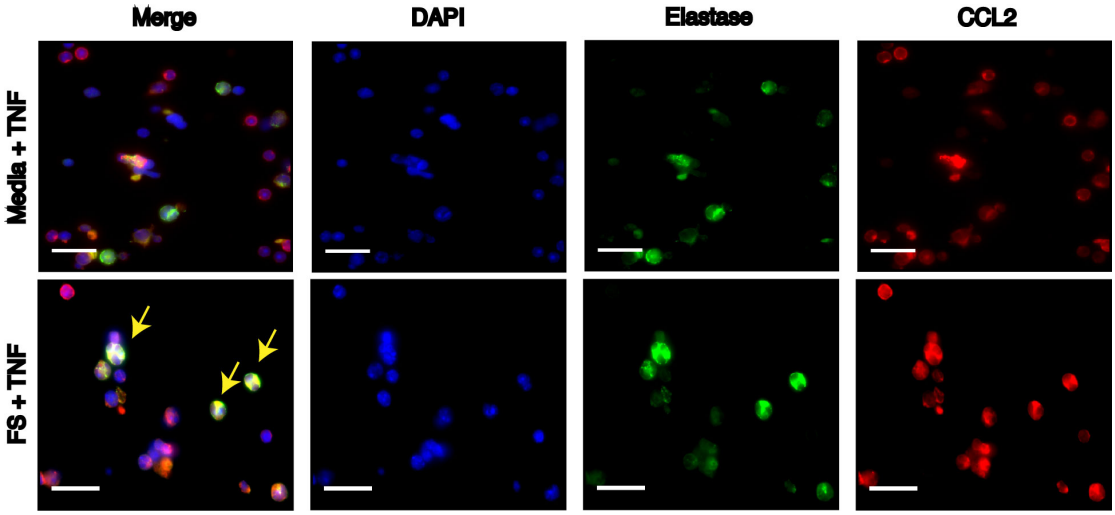


Fig 5.9 *Using immunocytochemistry, CCL2 expression was detected in neutrophils pre-exposed to fracture supernatant and TNF (examples indicated by yellow arrows). The presence of CCL2 immunoreactivity is seen by red fluorescent and co-localization with neutrophil elastase expression (green) led to a yellow signal in the merged images. The polymorphonuclear morphology of the neutrophils was observed in the DAPI (blue) channel. (Performed in collaboration in Dr. Ana Espirito Santo, post-doctoral scientist, Kennedy Institute of Rheumatology).*

5.5 Manipulating the CCL2/CCR2 axis in fracture repair in vivo

5.5.1 Local addition of rCCL2 did not enhance fracture repair.

The CCL2/CCR2 axis has been found to be important in the healing response of other types of tissue, including skeletal muscle (Weber, Nelson et al. 1999; Surgeons 2007; Martinez, McHale et al. 2010; Lu, Huang et al. 2011; Arefieva, Sokolov et al. 2012; Chan, Moore et al. 2012; Chen, Liu et al. 2012; Liang, Jung et al. 2012; Suresh, Yu et al. 2012; Wan, Li et al. 2012; Kostarnoy, Gancheva et al. 2013). Local administration of rmCCL2 at 10 ng and 100 ng at the fracture site on days 0 and 1 did not affect fracture repair at day 28 on microCT analysis (Fig 5.10), probably due the short half-life of the protein (Ruggiero, Flati et al. 2003).

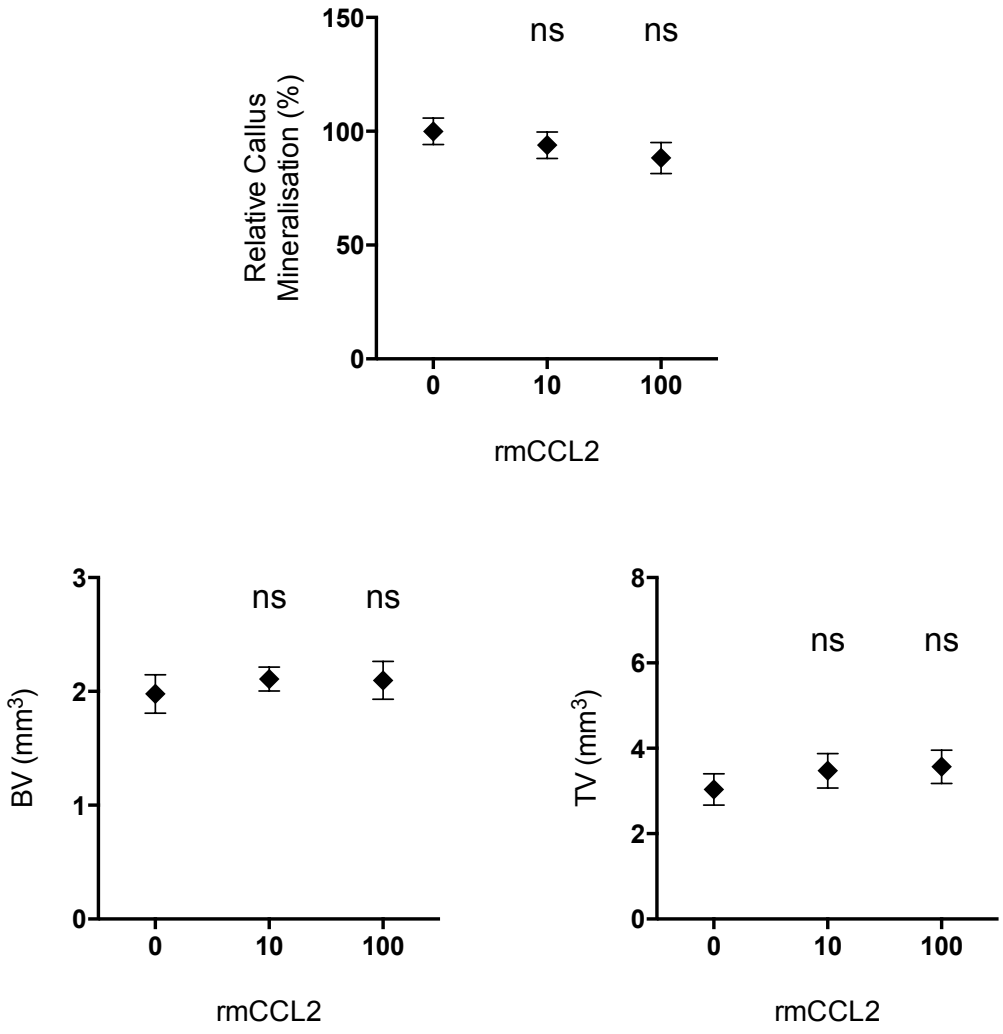


Fig 5.10 *Local addition of rmCCL2 at the fracture site immediately after the operation and again at 24 hours did not affect fracture healing in vivo. 6 animals were used per treatment group. Figures represent mean ± SEM. Relative % callus mineralization: PBS control 100 + 5.84 %; rmCCL2 10 ng 93.89 + 5.85 %; rm CCL2 100 ng 88.3 + 6.82 %. BV: PBS control 1.98 ± 0.17 mm³; rmCCL2 10 ng 2.11 ± 0.11 mm³; rmCCL2 100 ng 2.10 ± 0.17 mm³. No significant changes detected, 1-way ANOVA with Bonferroni's post-test correction.*

5.5.2 Inhibition of the CCL2 did not impair fracture healing.

To test the role of CCL2 in fracture healing in vivo, neutralizing antibody to CCL2 was administered systemically before and after the osteotomy. 5 μg anti-CCL2 was injected intraperitoneally from 2 hours pre-operatively and repeated daily for 14 days inclusive. The animals were sacrificed at day 28 and assessed using microCT (Fig 5.11). No difference in fracture healing was noted.

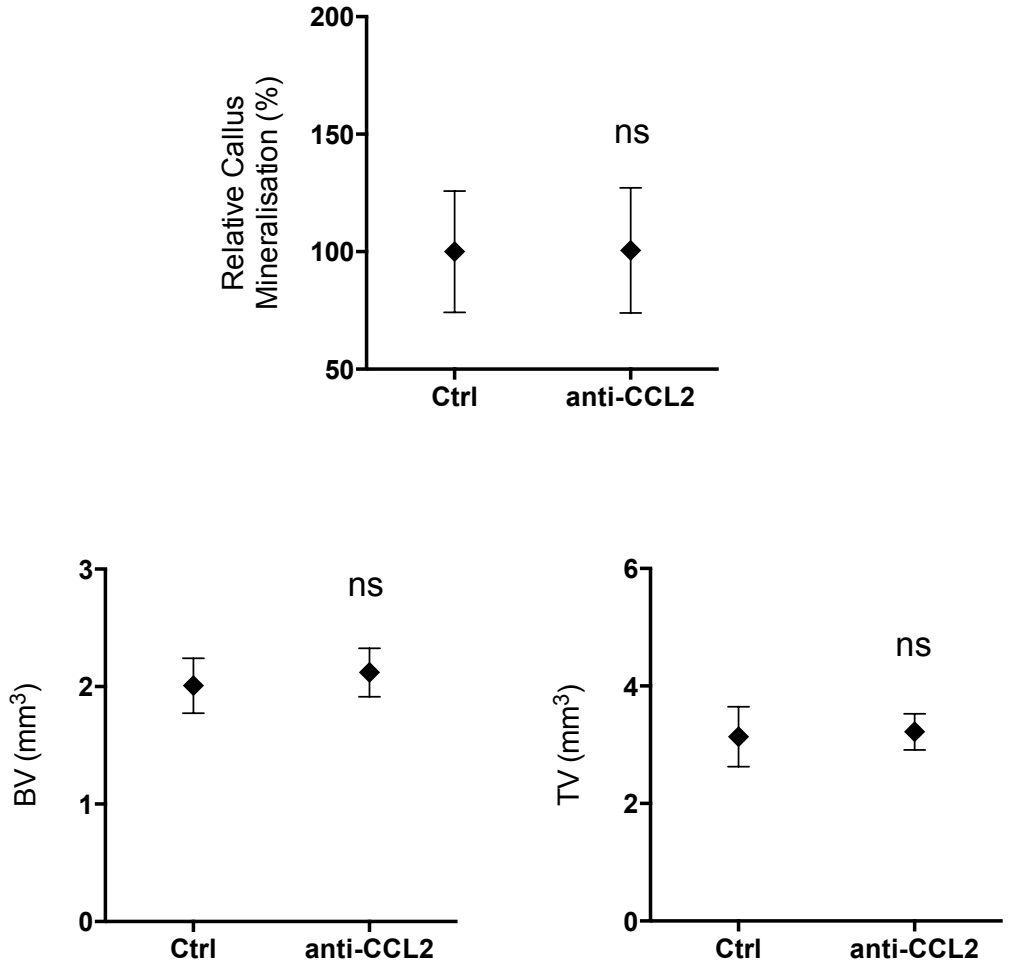


Fig 5.11 *Systemic administration of anti-CCL2 did not affect fracture repair in vivo. 6 animals were used per treatment group. Figures represent mean ± SEM. Relative callus % mineralization: IgG control 66.6 ± 3.02 %; anti-CCL2 66.7 ± 4.83 %. BV: IgG control 2.01 ± 0.23 mm³; anti-CCL2 2.12 ± 0.21 mm³. TV: IgG control 3.14 ± 0.51 mm³; anti-CCL2 2.33 ± 0.31 mm³. No significant changes detected, 2-sided t-test.*

5.5.3 Inhibition of the chemokine receptor for CCL2, CCR2, impaired fracture healing.

In vivo inhibition of CCR2, the G-protein-coupled receptor for CCL2, using small molecule inhibitor INCB3344 as previously described (Brodmerkel, Huber et al. 2005; Shin, Baribaud et al. 2009; Xue, Wang et al. 2010) significantly impaired endochondral fracture healing compared to vehicle control at day 28 after surgery (Fig 5.12). The CT reconstructions show that CCR2 inhibition led to non-union of the fractures with very poor fracture callus formation.

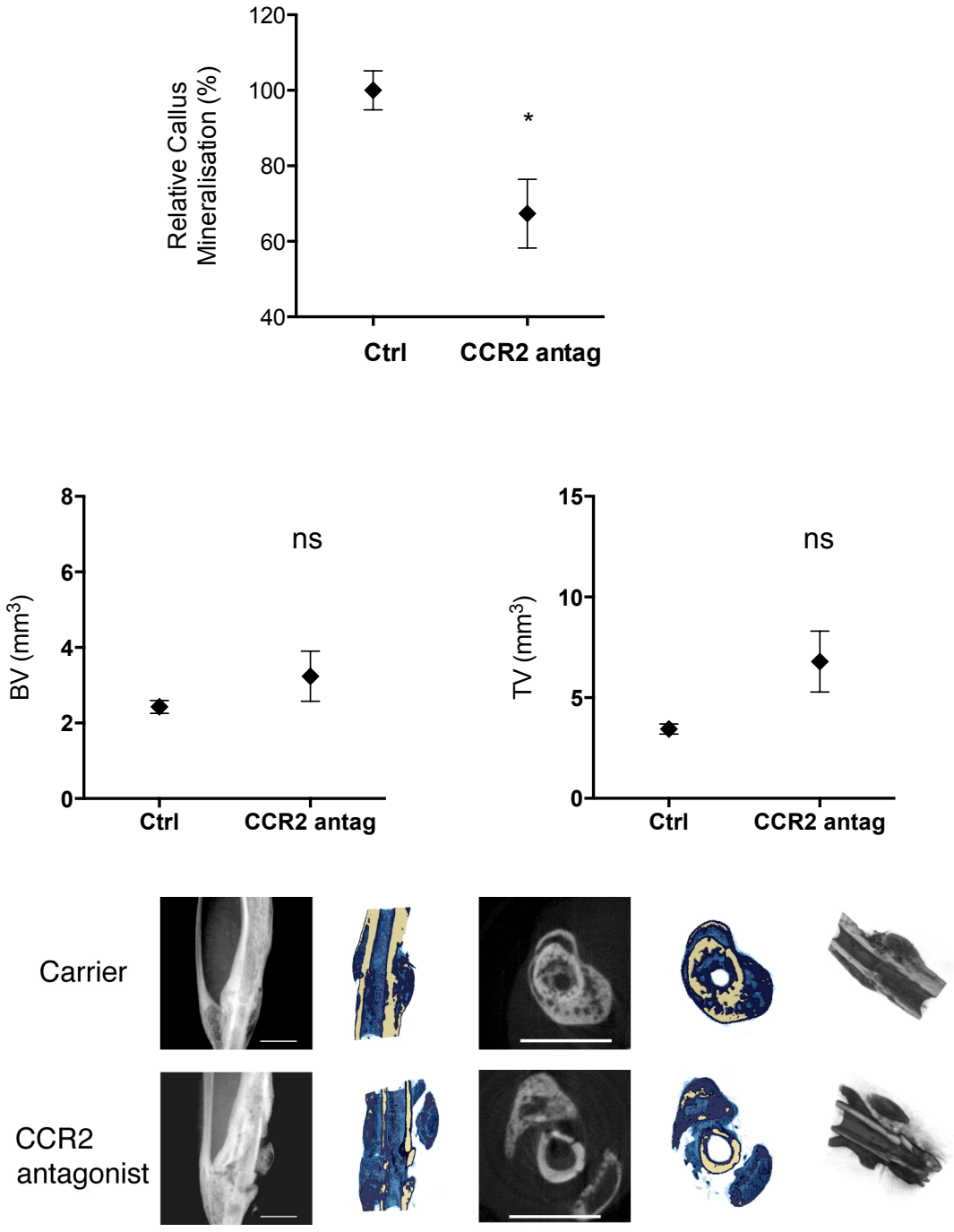


***Fig 5.12**. CCR2 inhibition impairs fracture repair in vivo, with poor fracture callus formation compared to carrier control. 6 animals were used per treatment group. Figures represent mean ± SEM. Relative callus % mineralization: Vehicle control Control, 100 + 5.15 %; CCR2 antagonist 67.34 + 9.11 %. BV: Vehicle control 2.43 ± 0.17 mm³; CCR2 antagonist 3.24 ± 0.66 mm³. TV: Vehicle control 3.44 ± 0.25 mm³; CCR2 antagonist 6.79 ± 1.51 mm³. *p<0.05, 2-sided t-test. Below: representative micro-CT images showing poor fracture callus formation at day 28 post-fracture.*

5.6 Discussion

The mechanism by which TNF promotes fracture healing is unknown. TNF is an inflammatory cytokine that is known to promote chemotaxis of monocytes and macrophage, which accumulate at sites of bone injury and bone remodeling and play an important role in bone repair (Marcus 1987; Williams, Jiang et al. 1992; Rahimi, Wang et al. 1995; Alexander, Chang et al. 2011; Nicolaidou, Wong et al. 2012). Therefore, it was hypothesized that additional TNF promotes the recruitment of monocytes and macrophages to the site of inflammation.

Previous reports on whether TNF is a chemokine for monocytes are conflicting (Ming, Bersani et al. 1987; Newman and Wilkinson 1989; Wang, Walter et al. 1990). I found that TNF alone did not directly promote transmigration across a chemotaxis well in vitro.

TNF is one of the key inflammatory cytokines modulating immune and inflammatory responses at the site of local inflammation. It regulates genes responsible for the recruitment of monocytes including VCAM-1 and E-selectin (Neumann, Machleidt et al. 1996) and CCL2 by endothelial cells (Matsushima and Oppenheim 1989). In a skin wound model, genetic disruption of TNFR1 in vivo resulted in less severe local infiltration of inflammatory leukocytes (Mori, Kondo et al. 2002). Unfortunately, the landmark study by Gerstenfeld et al, which showed impaired early fracture healing in TNFR1/TNFR2-deficient mice, did not describe the impact of TNFR deficiency on leukocyte infiltration at the fracture site(Gerstenfeld, Cho et al. 2003).

It is possible that TNF promotes monocyte or macrophage recruitment indirectly, for example, through the expression of chemokines by other cells (Matsushima and Oppenheim 1989). TNF can also prime the endothelium to express adhesion molecules that promote the recruitment of monocytes and macrophages(Neumann, Machleidt et al. 1996). In order to investigate the recruitment of monocytes/macrophages in response to TNF in the fracture environment, an in vivo approach was used. Quantifying the kinetics of monocyte infiltration into sites of inflammation is challenging in part due to the relatively low numbers of blood monocytes in most experimental animal species, including mice: 0.7%-1% compared to 2%-10% in humans (Laboratory 2007) and it requires large numbers of animals. Furthermore, F4/80 staining does not enable distinction between the resident macrophage populations and the incoming monocytes as a response to the skeletal injury with and without TNF. Therefore, the alternative approach of using the air pouch model of inflammation(Romano, Faggioni et al. 1997; Lawrence, Gilroy et al. 2001), which is a widely used and accepted model to test immune cell recruitment in vivo, was employed.

Neutrophils facilitate the recruitment of monocytes into inflamed tissues via a number of other means. Proteins e.g. azurocidin, LL37 and cathepsin G released from neutrophil granules can promote monocyte recruitment via activation of their formyl-peptide receptors (FPRs) (Kolaczkowska and Kubes 2013). Neutrophils shed sIL-6R which complexes with IL-6 to activate endothelial cells via gp130 to express adhesion molecules. IL-6 trans-signaling also plays a pivotal

role in the recruitment of monocytes at an inflammatory site by synthesizing monocyte-targeted chemokines, including CCL2(Hurst, Wilkinson et al. 2001; Soehnlein and Lindbom 2010). IL-6 acts on cells through interaction with IL-6Rα, a low-affinity receptor, and a transducing protein, gp130 (Taga, Hibi et al. 1989). While gp130 expression is ubiquitous, IL-6Rα expression is limited to a number of cell types (Yamasaki, Taga et al. 1988). However, IL-6Rα can be released from cells in a soluble form, sIL-6Rα, which associates with IL-6. The resultant IL-6/sIL-6Rα complex can then bind gp130 on cell membranes to form the high affinity IL-6 receptor, a mechanism known as 'trans-signaling'. This enables a wide variety of cells that only express gp130 to respond to IL-6. Indeed, IL-6 has been shown to recruit inflammatory cells via this mechanism (Modur, Li et al. 1997; Romano, Sironi et al. 1997; Coletta, Soldo et al. 2000) and IL6/sIL6Rα trans-signaling can induce a switch from neutrophil to monocyte recruitment at the site of inflammation (Hurst, Wilkinson et al. 2001; Marin, Montero-Julian et al. 2001). CCL3 is produced by neutrophils but also other cell types including endothelial cells, monocytes and macrophages (Kasama, Strieter et al. 1993). During the inflammatory phase, it binds to CCR1 on inflammatory monocytes thus promoting their recruitment (Soehnlein, Lindbom et al. 2009). However, sIL-6 and CCL3 levels, unlike CCL2, were not found to be upreguated by addition of TNF in the air pouch model of inflammation and hence does not account for how TNF acts to accelerate fracture healing.

CCL2 has been implicated to be an important chemokine in recruiting monocytes to bone. It is produced by osteoblastic cells in vitro(Williams, Jiang et al. 1992). CCL2 is not expressed in normal bone but is induced during osseous

inflammation and the induction of CCL2 in osseous inflammation correlates with the recruitment of monocytes both spatially and temporally (Rahimi, Wang et al. 1995). During development, CCL2 production is also associated with the recruitment of monocytes to areas of bone formation as well as resorption. Hence CCL2 appears to serve as an important chemotactic factor in bone inflammation and likely plays an important role in orchestrating the repair process.

Although CCL2 was originally named monocyte chemoattractant protein-1 (MCP-1), it is now known to be important for the recruitment of neutrophils (Matsukawa, Hogaboam et al. 1999; Speyer, Gao et al. 2004) as well as monocytes and macrophages(Shi and Pamer 2011), including at the fracture site(Xing, Lu et al. 2010). It is produced by neutrophils (Burn, Petrovick et al. 1994; Yoshimura and Takahashi 2007; Pliyev 2008; Pelletier, Maggi et al. 2010) and monocytes (Yoshimura, Yuhki et al. 1989) as well as a number of other cell types, including endothelial (Hu, Lee et al. 2009), fibroblasts, vascular smooth muscle (Xing, Feng et al. 2007) and bone marrow cells (Cushing, Berliner et al. 1990; Moehle, Bhamidipati et al. 2011). It was detected within the first 24 hours following trauma in an in vitro fracture hematoma model (Hoff, Maschmeyer et al. 2013). Despite reports that mRNA for CCL2 can been detected in neutrophils (Burn, Petrovick et al. 1994; Yamashiro, Kamohara et al. 2000), it is unknown whether human or murine neutrophils express the protein. I found that addition of rhTNF to enriched bone marrow-derived murine neutrophils exposed to fracture supernatant in vitro led to increased levels of CCL2 by ELISA (Fig 5.4). Notably, I was also able to directly visualize murine neutrophils derived from

bone marrow readily expressing CCL2 protein by immunocytochemistry when they were exposed to fracture supernatant and rhTNF but not rhTNF alone (Fig 5.9). My findings are supported by a previous report, which demonstrated that TNF upregulated CCL2 message by human neutrophils in vitro co-stimulated with TLR ligands (Yamashiro, Kamohara et al. 2000; Yoshimura and Takahashi 2007).

The observation that addition of TNF led to enhanced monocyte/macrophage recruitment is notable as this cellular subset has been implicated in osteogenesis (Guihard, Danger et al. 2012; Nicolaidou, Wong et al. 2012) and fracture callus remodeling (Alexander, Chang et al. 2011). Furthermore, we previously found that exposure to rhTNF enhanced CCL2-mediated recruitment of osteogenic precursor cells (Glass, Chan et al. 2011). While the CCL2/CCR2 axis has also been shown to promote healing in various types of tissue, including skeletal muscle, lung, endothelium, as well as bone through the recruitment of monocytes/macrophages and MSCs (Weber, Nelson et al. 1999; Martinez, McHale et al. 2010; Lu, Huang et al. 2011; Arefieva, Sokolov et al. 2012; Chan, Moore et al. 2012; Chen, Liu et al. 2012; Liang, Jung et al. 2012; Suresh, Yu et al. 2012; Wan, Li et al. 2012; Kostarnoy, Gancheva et al. 2013), most of the data have been based on experiments designed to inhibit CCL2/CCR2 interaction using antagonists and inhibitors. Only two of these studies were able to demonstrate a beneficial effect of upregulating this axis. Liang et al used transgenic mice showing that lung-specific overexpression of CCL2 protected mice from bleomycin-induced lung injury (Liang, Jung et al. 2012) while Arefieva et al found that subcutaneous implantation of agarose with the addition of a

peptide fragment of amino acid sequence of CCL-2 improved skin wound healing in rats(Laterveer, Lindley et al. 1996). Although addition of rmCCL2 did not accelerate fracture repair in our in vivo murine fracture model, this may be due to a number of reasons, including the short half-life and low bioavailability of the protein (Ruggiero, Flati et al. 2003). Strategies to prolong the half-life of CCL2 for future investigation include PEGylation, fusion proteins, antibody complexes and mutagenesis.

It was surprising that anti-CCL2 treatment did not affect fracture healing in vivo. However, this may be due to the redundancy in the chemokine networks. CCR2 is a G protein-coupled receptor that binds multiple ligands including CCL2, CCL7, CCL8 and CCL13. Although CCR2 is thought to be the exclusive receptor for CCL2, the relative contribution of each of the other ligands in vivo remains unknown (Zhao 2010).

CCR2-deficient mice have previously been reported to exhibit impaired healing in a closed fracture model (Xing, Lu et al. 2010). However, genetically modified mice often display subtle skeletal phenotypes that may influence the interpretation of data on complex physiological processes such as fracture healing. My finding that CCR2 inhibition using a small molecule inhibitor impairs healing confirms the importance of the CCL2/CCR2 axis. CCR2 has been shown to be crucial in the recruitment of macrophages to sites of injury, and the movement of monocytes from the bone marrow into the bloodstream and site of traumatic injury (Kuziel, Morgan et al. 1997; Ma, Wei et al. 2002; Schober, Zernecke et al. 2004; Tsou, Peters et al. 2007). However, they may also

contribute to other aspects of fracture healing including the expression of osteogenic cytokines such as TNF and IL-6 (Yang, Ricciardi et al. 2007; Mountziaris, Tzouanas et al. 2010; Glass, Chan et al. 2011; Lu, Wang et al. 2012), osteoinductive growth factors such as BMP-2(Champagne, Takebe et al. 2002) as well as osteoprogenitor differentiation, osteoclast differentiation and vasculogenesis (Xing, Lu et al. 2010). Therefore it is possible that the drastic impairment of fracture repair is a consequence of the disruption of multiple CCR2-dependent processes.

CCR2 is also involved in osteoclast differentiation. CCR2 mutant mice are osteosclerotic and the reduced number and function of osteoclasts protect these mice from ovariectomy-induced osteoporosis (Binder, Niederreiter et al. 2009). However, the finding that CCR2 inhibition led to the appearance of delayed or non-union suggest that while CCR2 may be involved in osteoclastogenesis, which is important during the remodeling phase, CCR2 is crucial during the earlier phase of cellular recruitment and bone formation.

Apoptotic neutrophils release 'find me' signals that attract monocytes (Soehnlein and Lindbom 2010). The observation that anti-CCL2 reduced influx of neutrophils as well as monocytes in the air pouch model is of interest. It is possible that CCL2 also serves as a neutrophil chemokine even though it was originally discovered as a monocyte chemokine. Indeed, a recent study found using a mouse arthritis model found that CCR2-positive monocytes are key regulators of neutrophil extravasation during arthritis initiation (Wang, Zinselmeyer et al. 2012). It is therefore possible that inhibition of CCR2 impairs

fracture healing through depletion of neutrophils in addition to, or rather than through, its effect on monocytes/macrophages. Further studies to test the role of monocytes in fracture healing include assessing fracture healing in mice following monocyte depletion with clodronate liposomes, and more specifically, depletion of CCR2-positive monocytes with monoclonal antibody MC-21 (Wang, Zinselmeyer et al. 2012).

References

Alexander, K. A., M. K. Chang, et al. (2011). "Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model." J Bone Miner Res **26**(7): 1517-1532.

Arefieva, T. I., V. O. Sokolov, et al. (2012). "Peptide fragment 29-40 of amino acid sequence of monocyte chemoattractant protein-1 (MCP-1) stimulates monocyte migration in vivo and facilitates wound healing." Dokl Biol Sci **446**: 327-330.

Binder, N. B., B. Niederreiter, et al. (2009). "Estrogen-dependent and C-C chemokine receptor-2-dependent pathways determine osteoclast behavior in osteoporosis." Nat Med **15**(4): 417-424.

Brodmerkel, C. M., R. Huber, et al. (2005). "Discovery and pharmacological characterization of a novel rodent-active CCR2 antagonist, INCB3344." J Immunol **175**(8): 5370-5378.

Burn, T. C., M. S. Petrovick, et al. (1994). "Monocyte chemoattractant protein-1 gene is expressed in activated neutrophils and retinoic acid-induced human myeloid cell lines." Blood **84**(8): 2776-2783.

Champagne, C. M., J. Takebe, et al. (2002). "Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2." Bone **30**(1): 26-31.

Chan, C. T., J. P. Moore, et al. (2012). "Reversal of vascular macrophage accumulation and hypertension by a CCR2 antagonist in deoxycorticosterone/salt-treated mice." Hypertension **60**(5): 1207-1212.

Chen, X., Y. Liu, et al. (2012). "Topical insulin application improves healing by regulating the wound inflammatory response." Wound Repair Regen **20**(3): 425-434.

Coletta, I., L. Soldo, et al. (2000). "Selective induction of MCP-1 in human mesangial cells by the IL-6/sIL-6R complex." Exp Nephrol **8**(1): 37-43.

Cushing, S. D., J. A. Berliner, et al. (1990). "Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells." Proc Natl Acad Sci U S A **87**(13): 5134-5138.

Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A **108**(4): 1585-1590.

Green, D. P. (1984). "Diagnostic and therapeutic value of carpal tunnel injection." J Hand Surg Am **9**(6): 850-854.

Guihard, P., Y. Danger, et al. (2012). "Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling." Stem Cells **30**(4): 762-772.

Hoff, P., P. Maschmeyer, et al. (2013). "Human immune cells' behavior and survival under bioenergetically restricted conditions in an in vitro fracture hematoma model." Cell Mol Immunol **10**(2): 151-158.

Hu, C. J., Y. L. Lee, et al. (2009). "Reduction of monocyte chemoattractant protein-1 and interleukin-8 levels by ticlopidine in TNF-alpha stimulated human umbilical vein endothelial cells." J Biomed Biotechnol **2009**: 917837.

Hurst, S. M., T. S. Wilkinson, et al. (2001). "Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation." Immunity **14**(6): 705-714.

Kasama, T., R. M. Strieter, et al. (1993). "Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha." J Exp Med **178**(1): 63-72.

Kolaczkowska, E. and P. Kubes (2013). "Neutrophil recruitment and function in health and inflammation." Nat Rev Immunol **13**(3): 159-175.

Kostarnoy, A. V., P. G. Gancheva, et al. (2013). "Topical Bacterial Lipopolysaccharide Application Affects Inflammatory Response and Promotes Wound Healing." J Interferon Cytokine Res.

Kuziel, W. A., S. J. Morgan, et al. (1997). "Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2." Proc Natl Acad Sci U S A **94**(22): 12053-12058.

Laboratory, T. J. (2007). "Physiological Data Summary - C57BL/6J." from http://jaxmice.jax.org/support/phenotyping/B6data000664.pdf.

Laterveer, L., I. J. Lindley, et al. (1996). "Rapid mobilization of hematopoietic progenitor cells in rhesus monkeys by a single intravenous injection of interleukin-8." Blood **87**(2): 781-788.

Lawrence, T., D. W. Gilroy, et al. (2001). "Possible new role for NF-kappaB in the resolution of inflammation." Nat Med **7**(12): 1291-1297.
Liang, J., Y. Jung, et al. (2012). "A macrophage subpopulation recruited by CC chemokine ligand-2 clears apoptotic cells in noninfectious lung injury." Am J Physiol Lung Cell Mol Physiol **302**(9): L933-940.
Lu, H., D. Huang, et al. (2011). "Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair." FASEB J **25**(10): 3344-3355.
Lu, Z., G. Wang, et al. (2012). "Short-term exposure to tumor necrosis factor-alpha enables human osteoblasts to direct adipose tissue-derived mesenchymal stem cells into osteogenic differentiation." Stem Cells Dev **21**(13): 2420-2429.
Ma, M., T. Wei, et al. (2002). "Monocyte recruitment and myelin removal are delayed following spinal cord injury in mice with CCR2 chemokine receptor deletion." J Neurosci Res **68**(6): 691-702.
Marcus, R. (1987). "Normal and abnormal bone remodeling in man." Annu Rev Med **38**: 129-141.
Marin, V., F. A. Montero-Julian, et al. (2001). "The IL-6-soluble IL-6Ralpha autocrine loop of endothelial activation as an intermediate between acute and chronic inflammation: an experimental model involving thrombin." J Immunol **167**(6): 3435-3442.
Martinez, C. O., M. J. McHale, et al. (2010). "Regulation of skeletal muscle regeneration by CCR2-activating chemokines is directly related to macrophage recruitment." Am J Physiol Regul Integr Comp Physiol **299**(3): R832-842.
Matsukawa, A., C. M. Hogaboam, et al. (1999). "Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4." J Immunol **163**(11): 6148-6154.
Matsushima, K. and J. J. Oppenheim (1989). "Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF." Cytokine **1**(1): 2-13.
Ming, W. J., L. Bersani, et al. (1987). "Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes." J Immunol **138**(5): 1469-1474.
Modur, V., Y. Li, et al. (1997). "Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor alpha." J Clin Invest **100**(11): 2752-2756.
Moehle, C. W., C. M. Bhamidipati, et al. (2011). "Bone marrow-derived MCP1 required for experimental aortic aneurysm formation and smooth muscle phenotypic modulation." J Thorac Cardiovasc Surg **142**(6): 1567-1574.
Mori, R., T. Kondo, et al. (2002). "Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration." FASEB J **16**(9): 963-974.
Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-969.
Mountziaris, P. M., S. N. Tzouanas, et al. (2010). "Dose effect of tumor necrosis factor-alpha on in vitro osteogenic differentiation of mesenchymal stem cells on biodegradable polymeric microfiber scaffolds." Biomaterials **31**(7): 1666-1675.
Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nat Rev Immunol **6**(3): 173-182.
Neumann, B., T. Machleidt, et al. (1996). "Crucial role of 55-kilodalton TNF receptor in TNF-induced adhesion molecule expression and leukocyte organ infiltration." J Immunol **156**(4): 1587-1593.
Newman, I. and P. C. Wilkinson (1989). "Chemotactic activity of lymphotoxin and tumour necrosis factor alpha for human neutrophils." Immunology **66**(2): 318-320.
Nicolaidou, V., M. M. Wong, et al. (2012). "Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation." PLoS One **7**(7): e39871.
Pelletier, M., L. Maggi, et al. (2010). "Evidence for a cross-talk between human neutrophils and Th17 cells." Blood **115**(2): 335-343.
Pliyev, B. K. (2008). "Chemotactically active proteins of neutrophils." Biochemistry (Mosc) **73**(9): 970-984.
Qin, L., P. Qiu, et al. (2003). "Gene expression profiles and transcription factors involved in parathyroid hormone signaling in osteoblasts revealed by microarray and bioinformatics." J Biol Chem **278**(22): 19723-19731.
Rahimi, P., C. Y. Wang, et al. (1995). "Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse." Endocrinology **136**(6): 2752-2759.
Romano, M., R. Faggioni, et al. (1997). "Carrageenan-induced acute inflammation in the mouse air pouch synovial model. Role of tumour necrosis factor." Mediators Inflamm **6**(1): 32-38.
Romano, M., M. Sironi, et al. (1997). "Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment." Immunity **6**(3): 315-325.

Ruggiero, P., S. Flati, et al. (2003). "Glycosylation enhances functional stability of the chemotactic cytokine CCL2." Eur Cytokine Netw **14**(2): 91-96.
Scapini, P., J. A. Lapinet-Vera, et al. (2000). "The neutrophil as a cellular source of chemokines." Immunol Rev **177**: 195-203.
Schober, A., A. Zernecke, et al. (2004). "Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets." Circ Res **95**(11): 1125-1133.
Shi, C. and E. G. Pamer (2011). "Monocyte recruitment during infection and inflammation." Nat Rev Immunol **11**(11): 762-774.
Shin, N., F. Baribaud, et al. (2009). "Pharmacological characterization of INCB3344, a small molecule antagonist of human CCR2." Biochem Biophys Res Commun **387**(2): 251-255.
Soehnlein, O. and L. Lindbom (2010). "Phagocyte partnership during the onset and resolution of inflammation." Nat Rev Immunol **10**(6): 427-439.
Soehnlein, O., L. Lindbom, et al. (2009). "Mechanisms underlying neutrophil-mediated monocyte recruitment." Blood **114**(21): 4613-4623.
Speyer, C. L., H. Gao, et al. (2004). "Novel chemokine responsiveness and mobilization of neutrophils during sepsis." Am J Pathol **165**(6): 2187-2196.
Suresh, M. V., B. Yu, et al. (2012). "Role of macrophage chemoattractant protein-1 in acute inflammation after lung contusion." Am J Respir Cell Mol Biol **46**(6): 797-806.
Surgeons, A. A. o. O. (2007). Diagnosis of carpal tunnel syndrome: evidence report. Rosemont, IL, AAOS.
Taga, T., M. Hibi, et al. (1989). "Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130." Cell **58**(3): 573-581.
Tsou, C. L., W. Peters, et al. (2007). "Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites." J Clin Invest **117**(4): 902-909.
van den Berg, J. M., S. Weyer, et al. (2001). "Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils." J Leukoc Biol **69**(3): 467-473.
Wan, M., C. Li, et al. (2012). "Injury-activated transforming growth factor beta controls mobilization of mesenchymal stem cells for tissue remodeling." Stem Cells **30**(11): 2498-2511.
Wang, B., B. H. Zinselmeyer, et al. (2012). "In vivo imaging implicates CCR2(+) monocytes as regulators of neutrophil recruitment during arthritis." Cell Immunol **278**(1-2): 103-112.
Wang, J. M., S. Walter, et al. (1990). "Re-evaluation of the chemotactic activity of tumour necrosis factor for monocytes." Immunology **71**(3): 364-367.
Weber, K. S., P. J. Nelson, et al. (1999). "Expression of CCR2 by endothelial cells : implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium." Arterioscler Thromb Vasc Biol **19**(9): 2085-2093.
Williams, S. R., Y. Jiang, et al. (1992). "Regulated expression of monocyte chemoattractant protein-1 in normal human osteoblastic cells." Am J Physiol **263**(1 Pt 1): C194-199.
Wythe, S. E., V. Nicolaidou, et al. (2014). "Cells of the immune system orchestrate changes in bone cell function." Calcif Tissue Int **94**(1): 98-111.
Xing, D., W. Feng, et al. (2007). "Estrogen modulates TNF-alpha-induced inflammatory responses in rat aortic smooth muscle cells through estrogen receptor-beta activation." Am J Physiol Heart Circ Physiol **292**(6): H2607-2612.
Xing, Z., C. Lu, et al. (2010). "Multiple roles for CCR2 during fracture healing." Dis Model Mech **3**(7-8): 451-458.
Xue, C. B., A. Wang, et al. (2010). "Discovery of INCB3344, a potent, selective and orally bioavailable antagonist of human and murine CCR2." Bioorg Med Chem Lett **20**(24): 7473-7478.
Yamasaki, K., T. Taga, et al. (1988). "Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor." Science **241**(4867): 825-828.
Yamashiro, S., H. Kamohara, et al. (2000). "Alteration in the responsiveness to tumour necrosis factor-alpha is crucial for maximal expression of monocyte chemoattractant protein-1 in human neutrophils." Immunology **101**(1): 97-103.
Yang, X., B. F. Ricciardi, et al. (2007). "Callus mineralization and maturation are delayed during fracture healing in interleukin-6 knockout mice." Bone **41**(6): 928-936.
Yoshimura, T. and M. Takahashi (2007). "IFN-gamma-mediated survival enables human neutrophils to produce MCP-1/CCL2 in response to activation by TLR ligands." J Immunol **179**(3): 1942-1949.

Yoshimura, T., N. Yuhki, et al. (1989). "Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE." FEBS Lett **244**(2): 487-493.
Zhao, Q. (2010). "Dual targeting of CCR2 and CCR5: therapeutic potential for immunologic and cardiovascular diseases." J Leukoc Biol **88**(1): 41-55.

Chapter 6

Role of alarmins in fracture repair:

Experimental plan and preliminary data

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6.1 Introduction

The ideal biological therapy for accelerating fracture healing would be administered at the time of fracture fixation. The preceding chapters demonstrate the critical role of the early inflammatory response in determining the outcome of fracture healing. Here, preliminary data are presented on the role of endogenous molecules called alarmins, which are released by injured and necrotic cells as a result of trauma and initiate the inflammatory and healing responses.

The alarmin family comprises structurally diverse and evolutionarily unrelated multifunctional endogenous molecules that are released passively from necrotic cells upon infection or tissue injury, or are rapidly secreted by stimulated leukocytes and epithelia (Chan, Roth et al. 2012). In the absence of injury or infection, alarmins play important intracellular roles. However, once released extracellularly, alarmins promote activation of innate immune cells and recruitment and activation of antigen-presenting cells engaged in host defense and tissue repair through pattern recognition receptors such as the toll-like receptors (TLRs), many of which have a key role in the detection of pathogens (Matzinger 2002; Oppenheim and Yang 2005; Bianchi and Manfredi 2009). In health, inflammation is self-limiting and a vital part of the innate host defense. It occurs in response to sterile injury or infection and involves the recruitment of phagocytes to remove cell debris and microbes. This is followed by resolution, with the recruitment of other cell types, including stem and endothelial cells, to restore tissue homeostasis. As potent mediators of inflammation, alarmins play

a fundamental role in the pathogenesis of a wide range of sterile or infection-induced immune and inflammatory disorders (Ehrchen, Sunderkotter et al. 2009; Zhu, Li et al. 2010; Andersson and Tracey 2011). Crucially, their ability to enhance the adaptive immune response through their effects on antigen-presenting cells, including dendritic cells (DCs), makes them a critical link between the innate and adaptive arms of the immune response (Bianchi and Manfredi 2007). Hence, the alarmin family represents an intriguing therapeutic target, not only to dampen excessive inflammation but also to uncouple the innate and adaptive immune responses in chronic pathologies, including autoimmune disorders. Furthermore, alarmins may serve as useful diagnostic and prognostic biomarkers in inflammatory disorders.

Intriguingly alarmins have also been implicated in wound healing. The regenerative role of extracellular HMGB1 is largely mediated by its chemoattractant effects (Degryse, Bonaldi et al. 2001; Palumbo, Sampaolesi et al. 2004; Rouhiainen, Kuja-Panula et al. 2004; Schlueter, Weber et al. 2005; Mitola, Belleri et al. 2006) as well as its ability to promote cell proliferation (Palumbo, Sampaolesi et al. 2004; Limana, Germani et al. 2005) and neo-angiogenesis (Palumbo, Sampaolesi et al. 2004; Schlueter, Weber et al. 2005; Mitola, Belleri et al. 2006). The β-defensin family and cathelicidins also exhibit pro-angiogenic (Conejo-Garcia, Benencia et al. 2004), chemotactic and proliferative properties (De, Chen et al. 2000; Koczulla, von Degenfeld et al. 2003). Currently, research on alarmins as regenerative therapy is limited to preclinical studies, the greatest challenge being to understand how to enhance the regenerative processes in

postnatal human tissues where most cells are terminally differentiated and tissues heal with fibrosis following injury.

Currently there is circumstantial evidence that two of the best-characterized alarmins, HMGB1 and S100A8/9, are associated with bone repair. The archetypal alarmin, HMGB1, is a chromatin protein that can be either actively secreted by stimulated cells or passively released by injured or necrotic cells (Wang, Bloom et al. 1999; Andersson, Wang et al. 2000). Release by damaged osteocytes has been shown to trigger bone regeneration(Bidwell, Yang et al. 2008; Yang, Shah et al. 2008) and HMGB1 is a chemoattractant and proliferative signal for murine bone marrow-derived MSCs (Taniguchi, Yoshida et al. 2007; Yang, Shah et al. 2008). It also regulates endochondral ossification (Taniguchi, Yoshida et al. 2007) and osteoclastogenesis (Yamoah, Brebene et al. 2008; Zhou, Han et al. 2008). However, it is difficult to study the role of this protein as the activity of highly purified HMGB1 is dependent on conjugation with other TLR ligands (Sims, Rowe et al. 2010) and also on the redox state(Venereau, Casalgrandi et al. 2012). This may be overcome by using well-validated in vivo models such as the murine air pouch model (Lawrence, Gilroy et al. 2001) to study its chemotactic activity and our murine fracture model (Glass, Chan et al. 2011) to assess effects on fracture repair. S100A8/A9 is a heterodimeric calcium binding protein that makes up 40% of neutrophil cytoplasmic content(Nacken, Sopalla et al. 2000). It is released on neutrophil death and is a potent chemoattractant for immune and stem cells. S100A8 has been associated with osteoblast differentiation and regulates bone resorption (Zreiqat, Howlett et al. 2007; Grevers, de Vries et al. 2011). Whilst the published data indicate that

HMGB1 and S100A8 are involved in bone repair, the precise role of these alarmins is not known. My hypothesis is that controlled upregulation of HMGB1 or S100A8 can promote fracture healing.

6.2 Trauma induces alarmin release.

6.2.1 Human: HMGB1 and S100A8 are released at the fracture site.

The levels of the alarmins HMGB1 and S100A8 released from bone fragments were quantified by generating human supernatants as described previously (Glass, Chan et al. 2011). Briefly, fracture and control supernatants were generated by incubating fracture fragments or surgically sliced bone from amputated limbs, respectively, from human patients in media for 12 hours and filter-sterilized. The group has previously found that the fracture supernatants contain factors that promoted both the chemotaxis and osteogenic differentiation of muscle-derived MSCs compared to control supernatants (Glass, Chan et al. 2011). Human fracture supernatants contain higher levels of both HMGB1 and S100A8 compared to control supernatants generated from surgically sliced bone from amputated limbs. While the level of HMGB1 showed a positive correlation with the osteogenic potential of the supernatants, this was not seen for S100A8/9 (Figs 6.1, 6.2). These results suggest that HMGB1 and S100A8 are released as a result of the trauma and may be involved in osteogenic differentiation either directly or indirectly. More patient samples will be collected and analyzed to provide greater confidence in these observations and

enable testing of any correlation between alarmin levels and the chemotactic and osteogenic activity of the supernatants.

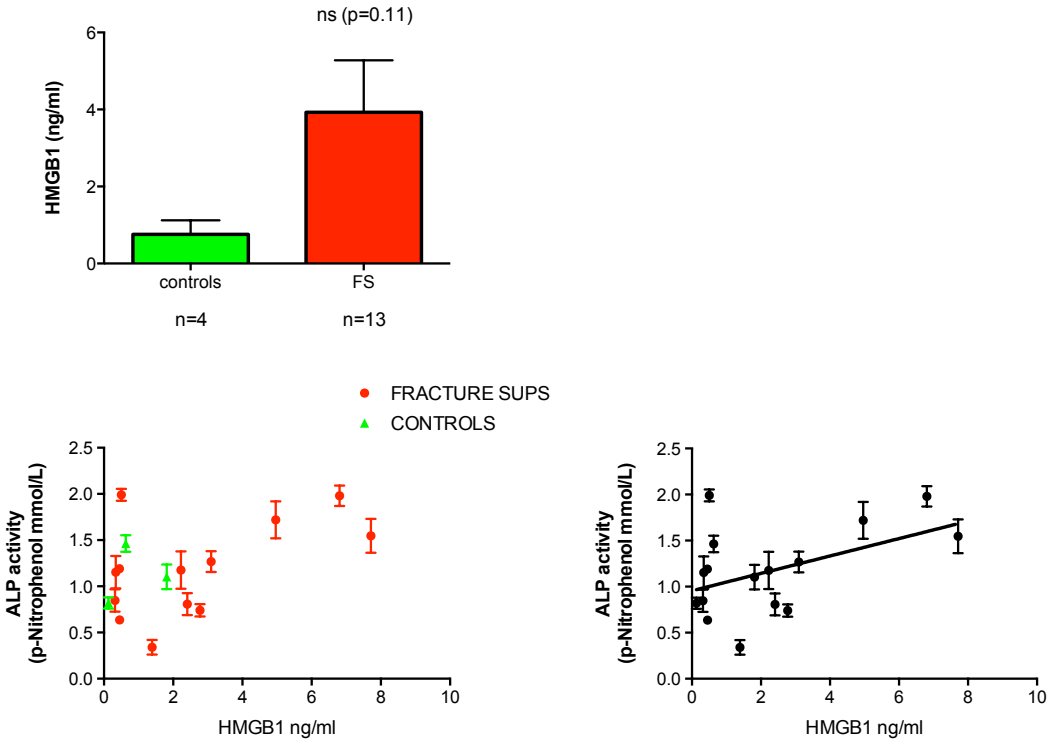


Fig 6.1. *The levels of HMGB1 in the human fracture supernatant compared to control supernatant. The level of HMGB1 in the fracture supernatant appears to correlate with osteogenic activity of the fracture supernatant on human MSCs using ALP activity as a surrogate marker of osteogenesis (Pearson r=0.486, significant correlation).*

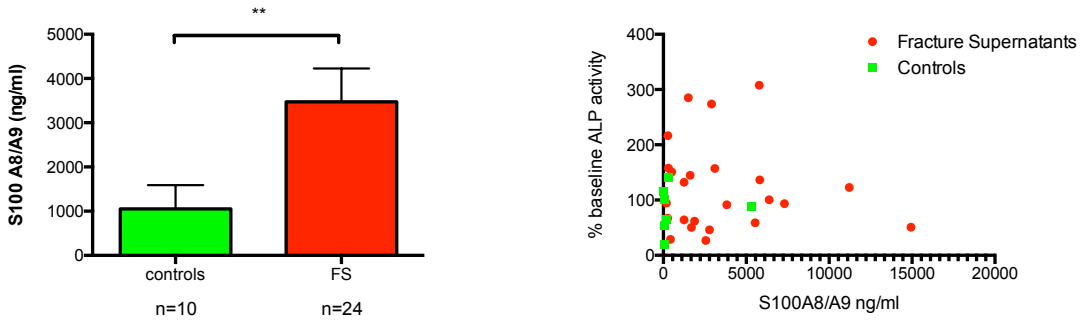


Fig 6.2. *The level of S100A8/9 is higher in the human fracture supernatant compared to control supernatant. The level of S100A8/9 in the fracture supernatant does not correlate with osteogenic activity of the fracture supernatant on human MSCs using ALP activity as a surrogate marker of osteogenesis (Pearson r=0.003, no significant correlation).*

6.2.2. Mouse: HMGB1 and S100A8 are released systemically following fracture.

Following creation of the fracture injury, there were biphasic peaks of serum S100A8/9 levels at 15 minutes and 24 hours (n=6 per time point) (Fig 6.3). For HMGB1 levels, while there also appeared to be biphasic release at 3 and 72 hours, there were not enough replicates at these time points (n=2) to enable meaningful interpretation of results; hence statistical analysis was not performed for this data set. The levels of HMGB1 seen in this experiment are as expected much lower than those previously published by Levy et al., who detected levels of over 100 ng/ml following bilateral closed midshaft femoral fractures (Levy, Mollen et al. 2007). With the tibial fracture model, S100A8/9 levels peaked at around 300 ng/ml following fracture. This is comparable to the levels found in a murine model of seronegative arthritis, where S100A8/9 is thought to be involved in the pathogenesis (Geven, Abdollahi-Roodsaz et al. 2014).

For future work, in order to study the kinetics of alarmin release in vivo, levels of HMGB1 and S100A8 in murine fracture supernatants obtained following a standardized injury will be determined by ELISA. I will also measure the local expression of S100A8 and HMGB1 in the surrounding soft tissues at the murine fracture site compared to no injury controls using RT-PCR.

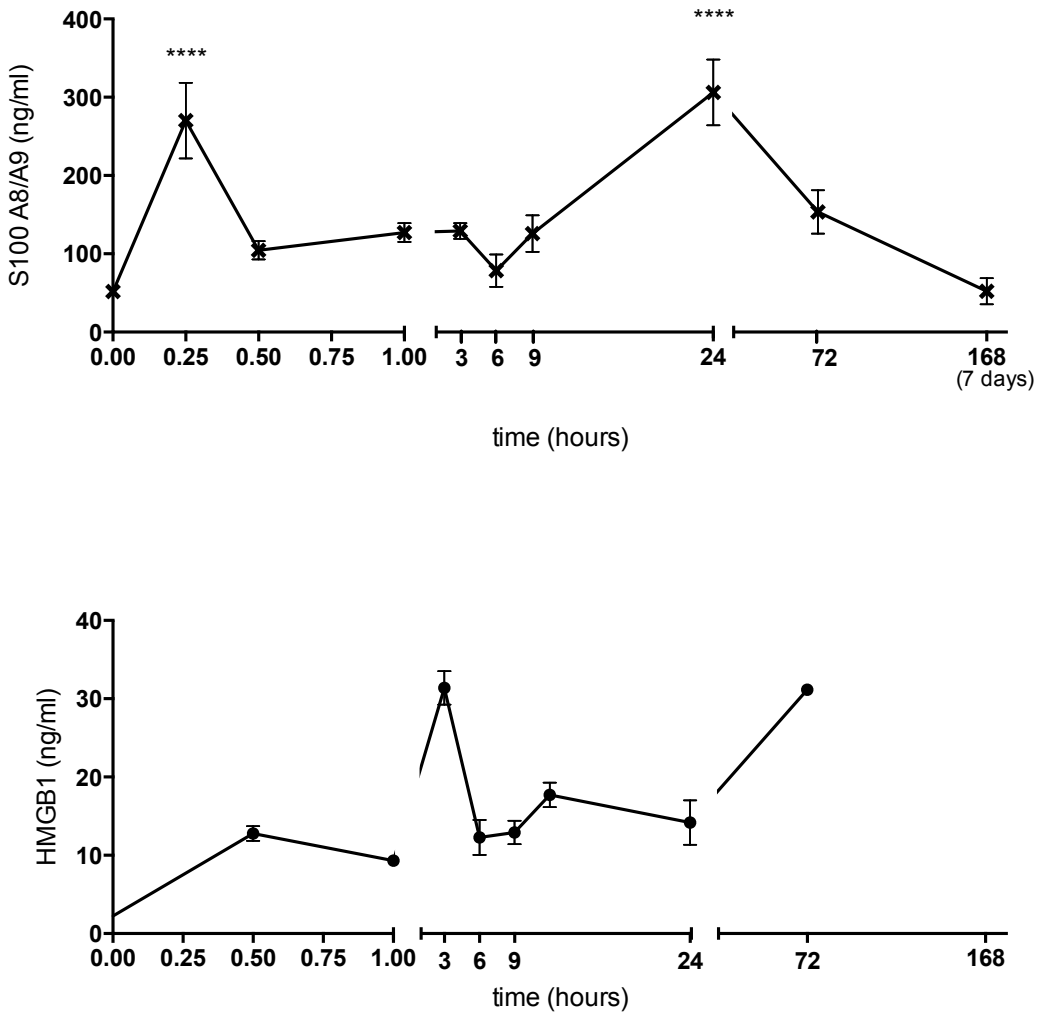
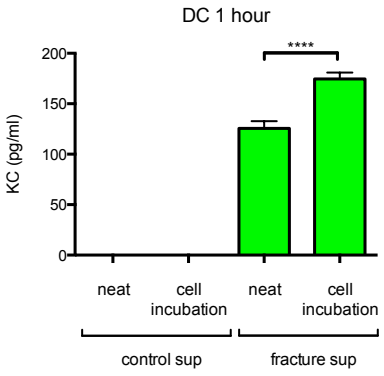


Fig 6.3*. The serum levels of HMGB1 and S100A8/9 post-fracture. 6 mice were used per time point but there was insufficient volumes to test HMGB1 at 15 minutes, 3 hours, 24 hours and 7 days post-fracture. These will be repeated. Each serum sample was repeated in duplicate for the ELISAs. Figures represent mean ± SEM of all experiments. ****p<0.0001, 1-way ANOVA using Bonferonni's post-test analysis compared to no injury control (time-point 0.00). No statistical analysis was performed on HMGB1 data as there were insufficient numbers.*

6.2.3. Tissue resident immune cells produce neutrophil chemokines IL-8 and IL-6 in response to trauma.

Tissue resident immune cells comprise dendritic cells and macrophages that exhibit the alternatively activated M2 phenotype. These cells can be differentiated in vitro by exposing murine bone-marrow derived monocytes to GM-CSF (20 ng/ml) or M-CSF (100 ng/ml), respectively, over 8 days(Krausgruber, Blazek et al. 2011). Exposure of these cells to murine fracture supernatant led to the release of KC (IL-8) and IL-6, key chemokines that act on neutrophils (Fig 6.4). I will go on to confirm the role HMGB1 and S100A8 on chemokine release by adding the HMGB1 inhibitor Box A or a neutralizing antibody to HMGB1 (gifted Kevin Tracey, NY) to the fracture or control (surgically sliced bone) supernatants. There is currently no commercially available inhibitor for S100A8. Therefore, I will assess the efficacy of fracture supernatants generated from S100A9$^{-/-}$ mice (also functionally deficient for S100A8 (Manitz, Horst et al. 2003)). Our laboratory has already established a colony of S100A9$^{-/-}$ animals (gifted T Vogl, Münster). Finally, I will stimulate dendritic cells or M2 macrophages from these mice with rHMGB1 or rmS100A8 to ascertain whether these alarmins also lead to the release of KC and IL-6.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KC (pg/ml)
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100
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0
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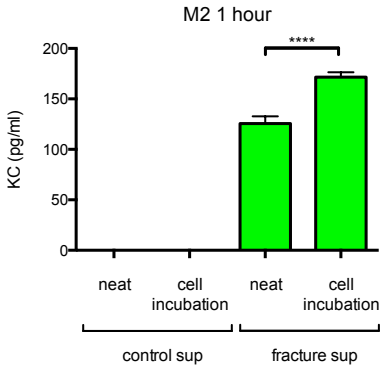
cell incubation
neat
cell incubation
control sup
fracture sup


M2 1 hour
KC (pg/ml)
200
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100
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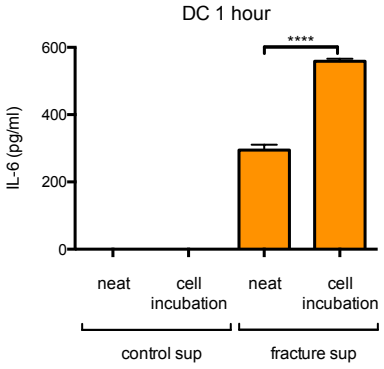
cell incubation
control sup
fracture sup


DC 1 hour
IL-6 (pg/ml)
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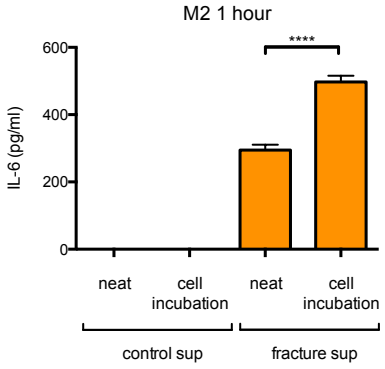
cell incubation
control sup
fracture sup


M2 1 hour
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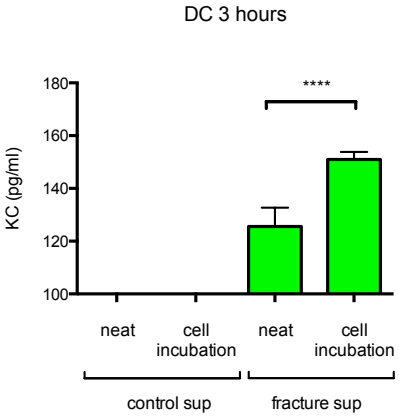
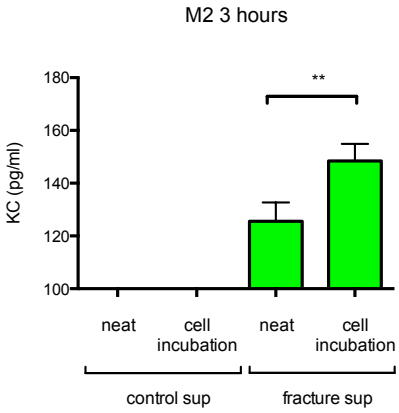
control sup
fracture sup


DC 3 hours
KC (pg/ml)
180
160
140
120
100
****
neat
cell incubation
neat
cell incubation
control sup
fracture sup


M2 3 hours
KC (pg/ml)
180
160
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120
100
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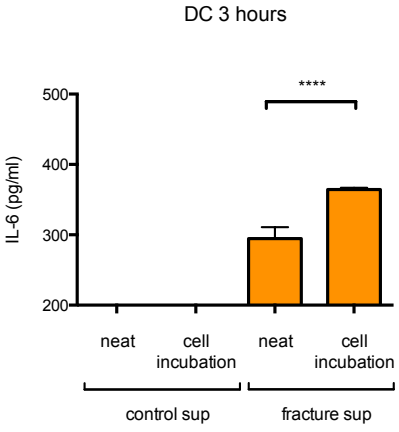
neat
cell incubation
neat
cell incubation
control sup
fracture sup


DC 3 hours
IL-6 (pg/ml)
500
400
300
200
****
neat
cell incubation
neat
cell incubation
control sup
fracture sup

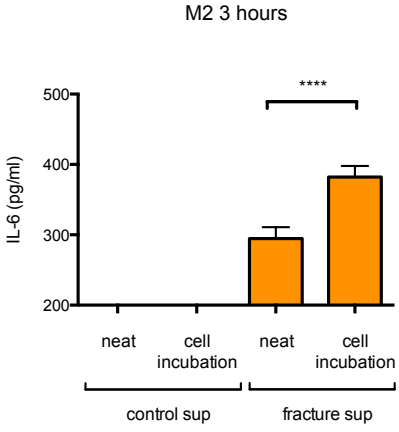

M2 3 hours
IL-6 (pg/ml)
500
400
300
200
****
neat
cell incubation
neat
cell incubation
control sup
fracture sup

***Fig 6.4**. Exposure of murine dendritic cells and M2 macrophages to fracture supernatants, but not control supernatants, led to release of the neutrophil chemoattractants KC and IL-6 at 1 and 3 hours. 'Neat' indicates the amount of KC/IL6 in the supernatants before incubation with cells. The experiment was repeated 3 times and each supernatant was repeated in duplicate on the multiplex chemiluminescence plates. Figures represent mean ± SEM of all experiments. *p<0.05, ****p<0.0001, 2 sided t-test.*

6.2.4. Role of HMGB1 and S100A8 in recruiting cells of the innate immune system to the fracture environment in vivo

In chapter 5, I showed that CCL2 produced by neutrophils is essential for the recruitment of monocytes to the fracture environment. CCL2 levels and monocyte recruitment were enhanced by the addition of rTNF into the air pouch. HMGB1 and S100A8/9 are reported to recruit inflammatory cells to damaged tissues (Ryckman, Vandal et al. 2003; Schiraldi, Raucci et al. 2012). Using the murine air pouch model, rmS100A8 alone at 10 ug led to increased inflammatory cell influx, although this did not reach statistical significance (Fig 6.5). I will repeat this with higher doses of rmS100A8. Next I will repeat this with murine fracture supernatant and analyse the cellular subsets by cell sorting. I will confirm the role of HMGB1 in recruiting neutrophils and monocytic cells to the fracture environment in vivo by adding neutralising antibody or the inhibitor Box A to murine fracture supernatants in the air pouch model. I will also examine the efficacy of fracture supernatants generated from the bones of S100A9-deficient animals in recruiting immune cells. The effect of

HMGB1 inhibition or S100A8/9-deficiency on CCL2 levels will be assessed using the air pouch model and ELISA.

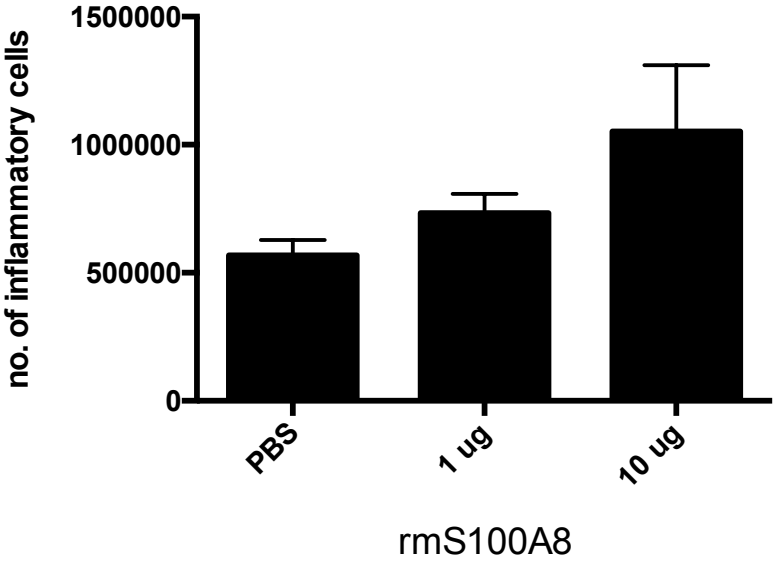


***Fig 6.5**. Investigating the effect of rmS100A8 on immune cell recruitment using the murine air pouch model of inflammation. 6 animals were used per group. No significant difference was detected between groups, 1-way ANOVA with Bonferroni's post-test correction.*

6.2.5. Role of HMGB1 and S100A8 in recruiting MSCs in vitro

Alarmins serve as potent chemoattractants for innate immune cells as well as stem cells (Chan, Roth et al. 2012). The chemotactic property of HMGB1 on MSCs has already been reported (Meng, Guo et al. 2008). I will test the chemotactic effect of rhS100A8 on primary human MSCs derived from our patient samples using a Transwell migration assay. I will also add anti-TLR4, anti-TLR2 or soluble RAGE (the decoy receptor) to test which receptor S100A8 acts through. I will verify the activity of a commercially available preparation of HMGB1 that is

chemotactically active and assess the effect of anti-HMGB1 or BoxA inhibitor on the chemotactic activity of murine fracture supernatants.

6.2.6. Osteoinductive role of S100A8

My preliminary data show that there is an association between the levels of HMGB1 and S100A8/9 and the osteogenic potential of human fracture supernants. I also found that primary human MSCs undergo osteogenic differentiation in a dose-dependent manner on stimulation with supernatant from monocytes that have been exposed to rhS100A8 in vitro (Fig 6.6). Alkaline phosphatase activity is a surrogate marker of osteogenic activity and I will also perform bone nodule formation assays to confirm osteogenic activity. By contrast, human MSCs do not undergo osteogenic differentiation on direct exposure to rhS100A8 alone (Fig 6.7). My preliminary data show that rhS100A8 stimulated human monocytes to produce TNF (Fig 6.6). This effect is inhibited by anti-TLR4 but not anti-TLR2, or inhibitors of RAGE signaling (Fig 6.8). Whilst LPS also leads to the release of these pro-inflammatory cytokines, I found that LPS inhibited osteogenic differentiation of human MSCs in vitro (Fig 6.9) and that local addition of LPS to the fracture site in our murine fracture model led to impairment of fracture healing (Fig 6.10). This would be consistent with the clinical observation that infection with Gram-negative bacteria at the fracture site is associated with impaired healing[38].

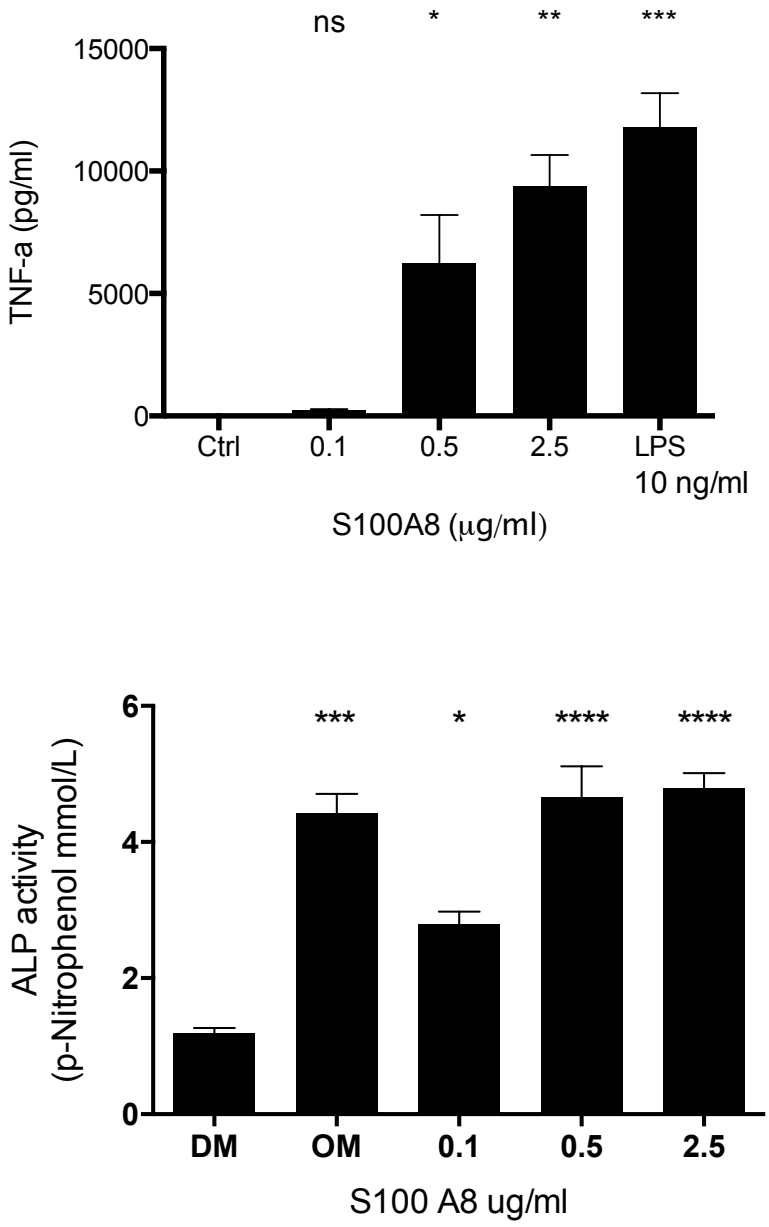


Fig 6.6*. Above: rhS100A8 stimulates human monocytes (n=3) to produce TNF in a dose dependent manner. Supernatants from rhS100A8 stimulated human monocytes (n=3) promoted osteogenesis in a dose-dependent manner. DM = DMEM media without rhS100A8 (negative control); OM = osteogenic media without rhS100A8 (positive control).*

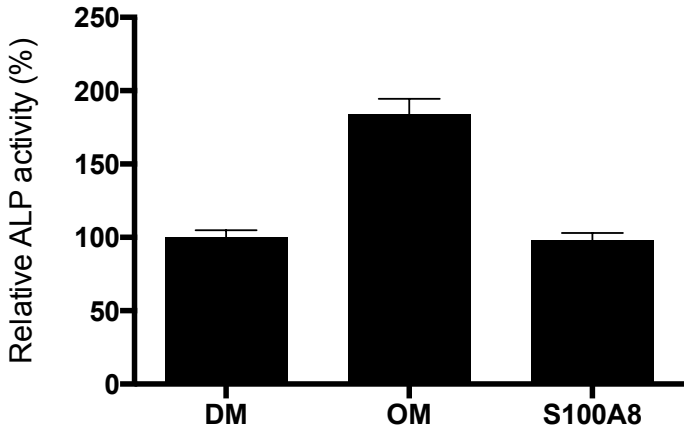


***Fig 6.7**. Addition of S100A8 (0.5 μg/ml) directly onto MSCs (n=4) did not affect ALP activity.*

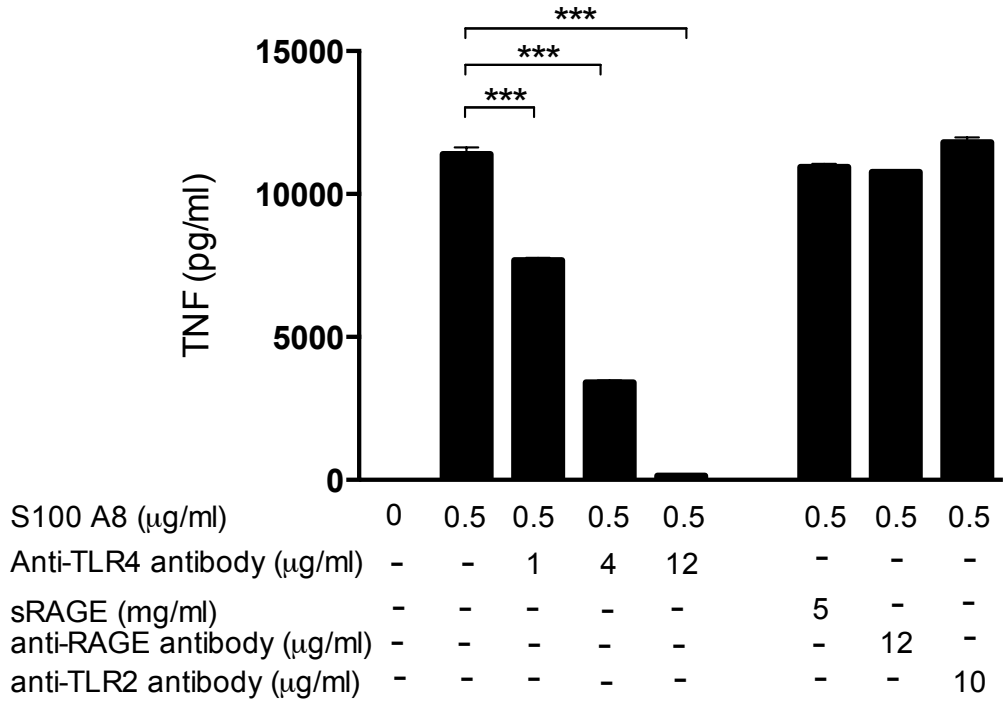


***Fig 6.8**. The production of TNF by S100A8-stimulated monocyte supernatant is inhibited by anti-TLR4 in a dose-dependent manner but not anti-TLR2, soluble RAGE (decoy receptor) or anti-RAGE (n=1, ELISA performed in triplicates).*

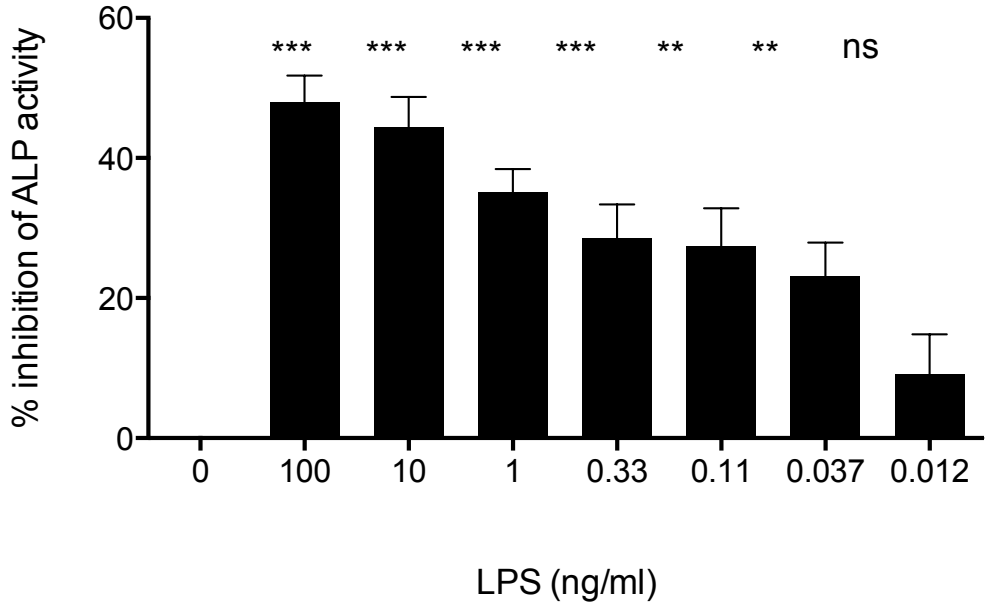


Fig 6.9*. LPS leads to inhibition of osteogenic differentiation of hMSCs (n=2) in a dose-dependent manner.*

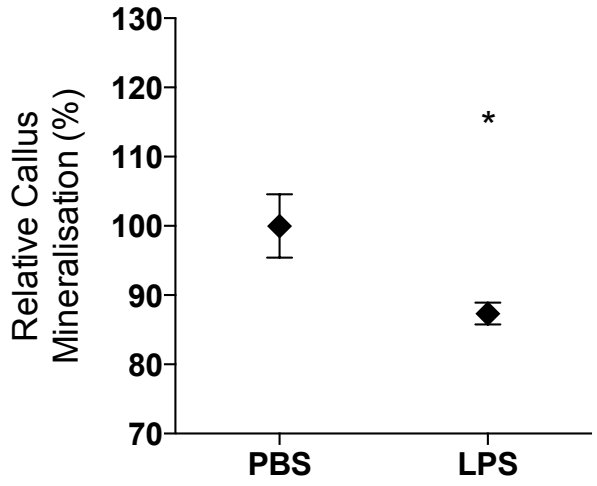


Fig 6.10*. Addition of LPS (10 ng on immediately after fracture and at 24 hours) impaired fracture repair at day 28. 6 mice were used per group. Figures represent mean ± SEM. Relative % mineralization PBS control: 100.0 ± 4.58%; LPS: 87.35 ± 1.57%. *p<0.05, 2-sided t-test.*

To establish which receptor is responsible for the S100A8-induced osteogenic differentiation, I will repeat these experiments and test the osteogenic activity, using ALP quantification and bone nodule formation assays, of the supernatants derived from human monocytes exposed to rhS100A8 and inhibitors of the receptors TLR-4, TLR-2 and RAGE. I will also assess the effects of these inhibitors on the osteogenic activity of the human fracture supernatants. Our preliminary data also show that rmS100A8 stimulated immune cells derived from the bone marrow of wild-type C57BL6 mice to produce TNF. This response was greatly reduced and absent in cells from $TLR4^{-/-}$ and $MyD88^{-/-}$ animals, respectively, suggesting that TNF production by murine inflammatory cells is at least in part dependent on the TLR-4/MyD88 signaling pathway (Fig 6.11).

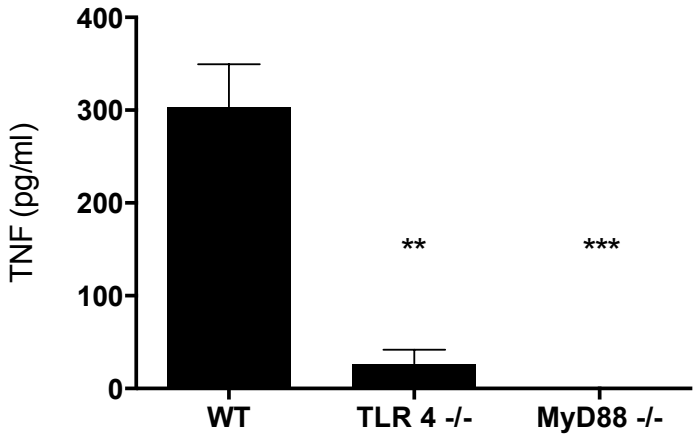


Fig 6.11*. rmS100A8 (5µg/ml) stimulates murine bone marrow cells to produce TNF; this was reduced and absent in cells from TLR4-/- and MyD88-/- mice (n=1 per group, ELISA performed in triplicates).*

I will repeat this experiment but test the osteogenic activity of the supernatants on murine MSCs to verify that the S100A8-induced osteogenic differentiation

and that molecular pathway by which this occurs also pertains to the murine system. I will also repeat these experiments to assess the role of HMGB1 in osteogenic differentiation. Finally, I will assess fracture healing in TLR-4$^{-/-}$ and TLR-2$^{-/-}$ mice; RAGE and MyD88 deficient animals are known to have a bone phenotype(Zhou, Immel et al. 2006).

6.2.7. Role of HMGB1 and S100A8 in fracture repair in vivo

I will examine the role of HMGB1 and S100A8 in fracture healing in vivo by assessing fracture repair in wild type C57BL6 animals following treatment with rHMGB1, anti-HMGB1, Box A inhibitor and rmS100A8, as well as in S100A9$^{-/-}$ animals that are functionally deficient for S100A8 (Manitz, Horst et al. 2003); S100A8 deletion is embryonically lethal(Passey, Williams et al. 1999). A previous member of the group (GG) found that local injection of HMGB1, 1 μg of HMGB1 immediately and at 24 hours post-fracture, at the fracture site led to greater percentage callus mineralization at day 28 (unpublished). I plan to repeat verify this finding and perform dose and time response experiments. Fracture healing will be assessed by microCT and histomorphometry at 2 and 4 weeks post-fracture.

6.3 Discussion

Exogenous application of alarmins may represent a therapeutic approach in promoting reparative responsnses in adult tissues. For instance, it has shown promise in cutaneous wounds. The antimicrobial alarmins are particularly attractive for cutaneous wound healing due to their additional antimicrobial activities. The pre-form hCAP18 is upregulated in human skin upon wounding but its levels are low in chronic ulcers. Antibodies against LL-37 inhibited re-epithelialization (Heilborn, Nilsson et al. 2003). Skin wound repair is problematic in diabetes mellitus due to a dysregulated inflammatory response compounded by an increased microbial load, excessive protease activity and vascular compromise (Straino, Di Carlo et al. 2008). Human β-defensin-3 expression through viral transfection led to accelerated wound closure in Staphylococcus aureus infected diabetic wounds in a pig model (Hirsch, Spielmann et al. 2009). HMGB1 expression is reduced in diabetic skin (Straino, Di Carlo et al. 2008). Topical application of HMGB1 to wounds accelerated healing in diabetic mice but not in normoglycemic mice, whereas topical BoxA impaired wound healing in normoglycemic mice, suggesting the latter may already have optimal levels of HMGB1 (Straino, Di Carlo et al. 2008). S100A8 and S100A9 also appear to promote skin wound healing(Wu and Davidson 2004) and wound fluid from non-diabetic patients with non-healing venous leg ulcers showed that S100A8/S100A9 levels were significantly reduced (Trostrup, Lundquist et al. 2011).

The use of exogenous alarmins to recruit and induce proliferation and differentiation of resident stem cells to enhance wound healing was demonstrated initially in a murine model of myocardial infarction (Limana, Germani et al. 2005; Germani, Limana et al. 2007). Local administration of HMGB1 led to improved structural and functional outcomes post infarction (Limana, Esposito et al. 2011). Furthermore, cardiac-specific overexpression of HMGB1 conferred significant protection against tissue damage and was associated with improved cardiac function (Kitahara, Takeishi et al. 2008) while anti-HMGB1 antibodies exacerbated injury (Oozawa, Mori et al. 2008).

Despite the evidence for alarmins promoting tissue homeostasis, there are also data that suggest the contrary. For instance, although HMGB1 is a potent neurotrophic mediator, it also contributes to neuronal cell death in cerebral ischemia (Muhammad, Barakat et al. 2008) and down-regulation conferred significant protection (Liu, Mori et al. 2007; Muhammad, Barakat et al. 2008; Maroso, Balosso et al. 2010). Activation of proinflammatory pathways by HMGB1 also exacerbated myocardial injury. Serum HMGB1 levels are elevated in patients with myocardial infarction and correlate with poor clinical outcomes (Goldstein, Gallowitsch-Puerta et al. 2006). Treatment with Box A significantly reduced infarct size and tissue damage in an ischemia/reperfusion injury model of the murine heart and systemically administered rHMGB1 worsened the severity of damage (Andrassy, Volz et al. 2008). That these findings conflict with a study that showed exogenous HMGB1 promoted cardiac regeneration (Andrassy, Volz et al. 2008) may be explained by the low dose HMGB1 being

administered during a critical time window when its expression was low (Limana, Germani et al. 2005).

The effects of alarmins, whether beneficial or detrimental, appear to depend on timing of release, dose and context. Excessive and chronic presence of alarmins and unremitting alarmin-induced events exacerbate injury, but when expressed in a transient and self-limited manner upon injury and acute inflammation they mediate repair (Glaros, Larsen et al. 2009). This dual role is exemplified by the pro-inflammatory cytokine TNFα where sustained up-regulation has a destructive role in many inflammatory conditions whilst it also acts as a growth factor for myelin-producing cells (Arnett, Mason et al. 2001), differentiation factor for mesenchymal stem cells (Hess, Ushmorov et al. 2009) and potential therapeutic in the infarcted myocardium (Kim, Park et al. 2009) or bone fractures (Glass, Chan et al. 2011).

The harmful role of alarmins is particularly evident in chronic conditions. For example, activated macrophages promote destruction and impair regeneration via secretion of S100A8 and S100A9 in inflammatory muscle diseases (Seeliger, Vogl et al. 2003) and blockade of RAGE restores effective cutaneous wound healing in diabetic mice (Goova, Li et al. 2001). Comparison of acute and chronic wounds in humans identified elevated levels of S100A8 and S100A9 from the exudate of non-healing wounds (Eming, Koch et al. 2010). However, this may again be attributed to a dose-dependent effect: for example low doses of S100B have been found to promote neurite outgrowth whereas high doses led to apoptosis (Huttunen, Kuja-Panula et al. 2000; Donato, Sorci et al. 2009).

An understanding of the early signaling pathways using an osteoimmunological approach may result in a strategy that can be clinically translated to accelerate healing of fractures. My preliminary data support the hypothesis that alarmins are the most upstream mediators that initiate the crucial innate inflammatory response following bone fracture. By activating the upstream signaling pathways involved in fracture healing, it may be possible to create a more complex, physiological environment that promotes fracture healing. Indeed, the direct addition of S100A8 to MSC did not induce osteogenic differentiation. However, stimulation of monocytes with S100A8 led to the production of a supernatant, which included TNF, that strongly promoted osteogenic differentiation of MSC. Such an osteoimmunological approach may circumvent the current issues of recombinant BMPs, which have not been able to induce the complex pattern of cytokine and growth factor production required for optimal fracture healing. Intriguingly, while S100A8 and LPS seem to function predominantly via the common TLR4 and promote TNF production, their effect on monocyte activation is divergent as the supernatant from the S100A8-stimulated monocytes promoted osteogenic differentiation while the supernatant from LPS-stimulated monocytes inhibited osteogenic differentiation. There are a number of possibilities for this observation. S100A8 may lead to the expression of an unidentified factor(s) that promotes osteogenic differentiation, and conversely, LPS may lead to the expression of factors that inhibit osteogenic differentiation. Alternatively, it may be the relative ratios of soluble factors released by the different ligands that either promote or inhibit ostegenic differentiation. Mechanistically, these TLR4 agonists may activate

other receptors. For example, LPS has recently been shown to signal via TLR-independent mechanisms to activate caspase-11 which induces pyroptosis, a form of programmed cell death (Hagar, Powell et al. 2013; Kayagaki, Wong et al. 2013). However, these pathways remain elusive. Furthermore, the signal transduction pathways and hence cellular effect of a TLR4 ligand is dependent on the compartment within which the receptor is ligated. Future studies using DNA microarray technology, comparing the genetic expression of monocytes following stimulation with S100A8 and LPS would help to identify these putative factors.

Following injury, alarmins are released and activate PRRs, which are expressed by a number of cell types, including macrophages and mast cells. These cells reside in tissues and serve as sentinel cells to initiate neutrophil recruitment following injury or infection. They control and induce a number of processes, including increasing vascular permeability and release of chemokines. Pharmacological or genetic ablation of either cell population has been shown to significantly decrease neutrophil infiltration into inflamed tissues. However, other cells types, including parenchymal cells, such as epithelial cells and fibroblasts, and particularly endothelial cells also possess pathogen detection mechanisms and likely contribute to neutrophil recruitment and the overall inflammatory response (Kolaczkowska and Kubes 2013; Mai, Virtue et al. 2013).

Inflammation occurs in response to PAMPs during infection or alarmins following sterile injury. PAMPs include LPS, CpG (unmethylated cytosine phosphate guanosine motifs) and other pathogenic products whereas alarmins

are host derived intracellular molecules including ATP, HMGB1 or structurally or proteolytically altered host molecules such as fragmented hyaluronan or collagen. There appears to be a remarkable redundancy in the danger recognition system with distinct alarmins and PAMPs activating the same PRRs. However, there is also evidence that neutrophils are able to distinguish between infectious and sterile inflammation by activating different intracellular pathways, leading to pathogen elimination versus debris clearance (Ye, Boulay et al. 2009). The preliminary data presented here suggest that S100A8, an alarmin, and LPS, a DAMP, have divergent effects on osteogenesis. The observation that addition of HMGB1 at the fracture site during the first 24 hours post-fracture accelerated fracture healing is particularly promising.

In summary, these preliminary data provide some evidence that alarmins play an important role during fracture healing and elucidation of their mechanisms of action in promoting inflammation and repair may lead to identification of new therapeutic targets.

References

Andersson, U. and K. J. Tracey (2011). "HMGB1 is a therapeutic target for sterile inflammation and infection." Annu Rev Immunol **29**: 139-162.

Andersson, U., H. Wang, et al. (2000). "High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes." J Exp Med **192**(4): 565-570.

Andrassy, M., H. C. Volz, et al. (2008). "High-mobility group box-1 in ischemia-reperfusion injury of the heart." Circulation **117**(25): 3216-3226.

Arnett, H. A., J. Mason, et al. (2001). "TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination." Nat Neurosci **4**(11): 1116-1122.

Bianchi, M. E. and A. A. Manfredi (2007). "High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity." Immunol Rev **220**: 35-46.

Bianchi, M. E. and A. A. Manfredi (2009). "Immunology. Dangers in and out." Science **323**(5922): 1683-1684.

Bidwell, J. P., J. Yang, et al. (2008). "Is HMGB1 an osteocyte alarmin?" J Cell Biochem **103**(6): 1671-1680.

Chan, J. K., J. Roth, et al. (2012). "Alarmins: awaiting a clinical response." J Clin Invest **122**(8): 2711-2719.

Conejo-Garcia, J. R., F. Benencia, et al. (2004). "Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A." Nat Med **10**(9): 950-958.

De, Y., Q. Chen, et al. (2000). "LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells." J Exp Med **192**(7): 1069-1074.

Degryse, B., T. Bonaldi, et al. (2001). "The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells." J Cell Biol **152**(6): 1197-1206.

Donato, R., G. Sorci, et al. (2009). "S100B's double life: intracellular regulator and extracellular signal." Biochim Biophys Acta **1793**(6): 1008-1022.

Ehrchen, J. M., C. Sunderkotter, et al. (2009). "The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer." J Leukoc Biol **86**(3): 557-566.

Eming, S. A., M. Koch, et al. (2010). "Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds." J Proteome Res **9**(9): 4758-4766.

Germani, A., F. Limana, et al. (2007). "Pivotal advances: high-mobility group box 1 protein--a cytokine with a role in cardiac repair." J Leukoc Biol **81**(1): 41-45.

Geven, E. J., S. Abdollahi-Roodsaz, et al. (2014). "A5.13 Serum levels of S100A8/A9 complex and corticosterone correlate to synovial inflammation and cartilage/bone damage in IL-1RA-/-MICE, a model system for seronegative arthritis." Ann Rheum Dis **73 Suppl 1**: A68.

Glaros, T., M. Larsen, et al. (2009). "Macrophages and fibroblasts during inflammation, tissue damage and organ injury." Front Biosci **14**: 3988-3993.

Glass, G. E., J. K. Chan, et al. (2011). "TNF-{alpha} promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A.

Goldstein, R. S., M. Gallowitsch-Puerta, et al. (2006). "Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia." Shock **25**(6): 571-574.

Goova, M. T., J. Li, et al. (2001). "Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice." Am J Pathol **159**(2): 513-525.

Grevers, L. C., T. J. de Vries, et al. (2011). "S100A8 enhances osteoclastic bone resorption in vitro through activation of Toll-like receptor 4: implications for bone destruction in murine antigen-induced arthritis." Arthritis Rheum **63**(5): 1365-1375.

Hagar, J. A., D. A. Powell, et al. (2013). "Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock." Science **341**(6151): 1250-1253.

Heilborn, J. D., M. F. Nilsson, et al. (2003). "The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium." J Invest Dermatol **120**(3): 379-389.

Hess, K., A. Ushmorov, et al. (2009). "TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway." Bone **45**(2): 367-376.

Hirsch, T., M. Spielmann, et al. (2009). "Human beta-defensin-3 promotes wound healing in infected diabetic wounds." J Gene Med **11**(3): 220-228.

Huttunen, H. J., J. Kuja-Panula, et al. (2000). "Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation." J Biol Chem **275**(51): 40096-40105.

Kayagaki, N., M. T. Wong, et al. (2013). "Noncanonical inflammasome activation by intracellular LPS independent of TLR4." Science **341**(6151): 1246-1249.

Kim, Y. S., H. J. Park, et al. (2009). "TNF-alpha enhances engraftment of mesenchymal stem cells into infarcted myocardium." Front Biosci **14**: 2845-2856.

Kitahara, T., Y. Takeishi, et al. (2008). "High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice." Cardiovasc Res **80**(1): 40-46.

Koczulla, R., G. von Degenfeld, et al. (2003). "An angiogenic role for the human peptide antibiotic LL-37/hCAP-18." J Clin Invest **111**(11): 1665-1672.

Kolaczkowska, E. and P. Kubes (2013). "Neutrophil recruitment and function in health and inflammation." Nat Rev Immunol **13**(3): 159-175.

Krausgruber, T., K. Blazek, et al. (2011). "IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses." Nat Immunol **12**(3): 231-238.

Lawrence, T., D. W. Gilroy, et al. (2001). "Possible new role for NF-kappaB in the resolution of inflammation." Nat Med **7**(12): 1291-1297.

Levy, R. M., K. P. Mollen, et al. (2007). "Systemic inflammation and remote organ injury following trauma require HMGB1." Am J Physiol Regul Integr Comp Physiol **293**(4): R1538-1544.
Limana, F., G. Esposito, et al. (2011). "HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3." PLoS One **6**(6): e19845.
Limana, F., A. Germani, et al. (2005). "Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation." Circ Res **97**(8): e73-83.
Liu, K., S. Mori, et al. (2007). "Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats." FASEB J **21**(14): 3904-3916.
Mai, J., A. Virtue, et al. (2013). "An evolving new paradigm: endothelial cells--conditional innate immune cells." J Hematol Oncol **6**: 61.
Manitz, M. P., B. Horst, et al. (2003). "Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro." Mol Cell Biol **23**(3): 1034-1043.
Maroso, M., S. Balosso, et al. (2010). "Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures." Nat Med **16**(4): 413-419.
Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.
Meng, E., Z. Guo, et al. (2008). "High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway." Stem Cells Dev **17**(4): 805-813.
Mitola, S., M. Belleri, et al. (2006). "Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine." J Immunol **176**(1): 12-15.
Muhammad, S., W. Barakat, et al. (2008). "The HMGB1 receptor RAGE mediates ischemic brain damage." J Neurosci **28**(46): 12023-12031.
Nacken, W., C. Sopalla, et al. (2000). "Biochemical characterization of the murine S100A9 (MRP14) protein suggests that it is functionally equivalent to its human counterpart despite its low degree of sequence homology." Eur J Biochem **267**(2): 560-565.
Oozawa, S., S. Mori, et al. (2008). "Effects of HMGB1 on ischemia-reperfusion injury in the rat heart." Circ J **72**(7): 1178-1184.
Oppenheim, J. J. and D. Yang (2005). "Alarmins: chemotactic activators of immune responses." Curr Opin Immunol **17**(4): 359-365.
Palumbo, R., M. Sampaolesi, et al. (2004). "Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation." J Cell Biol **164**(3): 441-449.
Passey, R. J., E. Williams, et al. (1999). "A null mutation in the inflammation-associated S100 protein S100A8 causes early resorption of the mouse embryo." J Immunol **163**(4): 2209-2216.
Rouhiainen, A., J. Kuja-Panula, et al. (2004). "Regulation of monocyte migration by amphoterin (HMGB1)." Blood **104**(4): 1174-1182.

Ryckman, C., K. Vandal, et al. (2003). "Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion." J Immunol **170**(6): 3233-3242.
Schiraldi, M., A. Raucci, et al. (2012). "HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4." J Exp Med **209**(3): 551-563.
Schlueter, C., H. Weber, et al. (2005). "Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule." Am J Pathol **166**(4): 1259-1263.
Seeliger, S., T. Vogl, et al. (2003). "Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases." Am J Pathol **163**(3): 947-956.
Sims, G. P., D. C. Rowe, et al. (2010). "HMGB1 and RAGE in inflammation and cancer." Annu Rev Immunol **28**: 367-388.
Straino, S., A. Di Carlo, et al. (2008). "High-mobility group box 1 protein in human and murine skin: involvement in wound healing." J Invest Dermatol **128**(6): 1545-1553.
Taniguchi, N., K. Yoshida, et al. (2007). "Stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification." Mol Cell Biol **27**(16): 5650-5663.
Trostrup, H., R. Lundquist, et al. (2011). "S100A8/A9 deficiency in nonhealing venous leg ulcers uncovered by multiplexed antibody microarray profiling." Br J Dermatol **165**(2): 292-301.
Venereau, E., M. Casalgrandi, et al. (2012). "Mutually exclusive redox forms of HMGB1 promote cell recruitment or proinflammatory cytokine release." J Exp Med **209**(9): 1519-1528.
Wang, H., O. Bloom, et al. (1999). "HMG-1 as a late mediator of endotoxin lethality in mice." Science **285**(5425): 248-251.
Wu, N. and J. M. Davidson (2004). "Migration inhibitory factor-related protein (MRP)8 and MRP14 are differentially expressed in free-electron laser and scalpel incisions." Wound Repair Regen **12**(3): 327-336.
Yamoah, K., A. Brebene, et al. (2008). "High-mobility group box proteins modulate tumor necrosis factor-alpha expression in osteoclastogenesis via a novel deoxyribonucleic acid sequence." Mol Endocrinol **22**(5): 1141-1153.
Yang, J., R. Shah, et al. (2008). "HMGB1 is a bone-active cytokine." J Cell Physiol **214**(3): 730-739.
Ye, R. D., F. Boulay, et al. (2009). "International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family." Pharmacol Rev **61**(2): 119-161.
Zhou, Z., J. Y. Han, et al. (2008). "HMGB1 regulates RANKL-induced osteoclastogenesis in a manner dependent on RAGE." J Bone Miner Res **23**(7): 1084-1096.
Zhou, Z., D. Immel, et al. (2006). "Regulation of osteoclast function and bone mass by RAGE." J Exp Med **203**(4): 1067-1080.
Zhu, S., W. Li, et al. (2010). "High mobility group box 1 protein as a potential drug target for infection- and injury-elicited inflammation." Inflamm Allergy Drug Targets **9**(1): 60-72.

Zreiqat, H., C. R. Howlett, et al. (2007). "S100A8/S100A9 and their association with cartilage and bone." J Mol Histol **38**(5): 381-391.

Chapter 7: Discussion

Chapter 7: Discussion

7.1 General Discussion

7.1.1 Targeting the innate inflammatory response in fracture repair

Fracture healing involves the coordination of a number of molecular pathways, which are initiated by the acute inflammatory response to the skeletal injury. Inflammation occurs as a result of the release of alarmins and the recruitment of leukocytes including neutrophils, monocytes and macrophages, which remove debris and microorganisms as well as release osteoinductive and other growth factors that initiate reparative process. Over time, the fracture haematoma is replaced by a fracture callus, which bridges the fracture gap. Mineralization of the cartilaginous callus leads to the formation of woven, low-density bone that subsequently undergoes remodeling to lamellar cortical bone, which possesses the structural and functional integrity of the pre-fractured bone. The dynamic molecular and biomechanical environment throughout this process determines the ultimate outcome of the fracture healing process.

The data presented in this thesis support the hypothesis that the early inflammatory response is crucial to achieve optimal fracture healing. These data also show that fracture healing may be enhanced or accelerated by targeting the innate immune response. The effects of various growth factors and cytokines have already been characterized by observational studies of fracture repair in animal studies involving genetically altered mice, providing valuable information on the molecular regulation of fracture healing (Gerstenfeld, Cho et al. 2003; Chen, Zhao et al. 2004; Xing, Lu et al. 2010; Wallace, Cooney et al. 2011).

However, such studies are limited. There is considerable redundancy in the regulation of fracture repair and the predicted phenotype may be masked by the expression of genes with redundant functions. Hence TNF may have a more profound effect on fracture repair in humans than the mere delay observed during the early phase of healing in TNFR1/TNFR2-deficient mice (Gerstenfeld, Cho et al. 2003). Furthermore, a number of the genes of interest, for example HMGB1, cannot be compensated for and lead to embryonic or perinatal death and therefore cannot be studied in this way. Conditional knockout mice that express the gene of interest using a system such as tamoxifen or tetracycline-related regulation will be likely prove invaluable in the investigation of fracture healing. The approach of using recombinant proteins and inhibitors described in this thesis aimed to circumvent these limitations and provide further data to support the relevance of the innate immune response in fracture repair.

Our finding that addition of rhTNF promoted the recruitment of monocyte/macrophages to the fracture environment and that CCR2 blockade impaired fracture healing suggest that the recruitment of monocytes/macrophages is critical to fracture repair. Macrophages have been found during fracture healing and were attributed to the early inflammatory events following bone injury (Andrew, Andrew et al. 1994; Einhorn, Majeska et al. 1995; Hankemeier, Grassel et al. 2001; Gerstenfeld, Cullinane et al. 2003). More recently, F4/80+ osteomacs have been shown to be critical to intramembranous bone formation in a murine model of bone injury. Depletion of F4/80+ cells in a transgenic murine model or by clodronate liposome administration suppressed woven bone deposition and mineralization during

bone healing, suggesting that F4/80+ cells are critical to the repair process in vivo (Alexander, Chang et al. 2011). F4/80+ osteomacs are distinct from osteoclasts, which are F4/80 quiescent (Mizoguchi, Muto et al. 2009; Alexander, Chang et al. 2011). While osteoclasts are important in the remodeling of the fracture callus during the later phases of healing, they are not required for bone formation (Alexander, Chang et al. 2011). Depletion of osteomac/macrophages, either at the time of surgery or later, significantly suppressed new bone formation suggesting that F4/80+ cells are essential during all phases of fracture healing (Alexander, Chang et al. 2011).

7.1.2 The role of TNF in fracture healing – revisited

Previously, TNFR1/TNFR2-deficient mice reported to exhibit delayed fracture healing (Gerstenfeld, Cho et al. 2003). It was reported that TNF acted at several levels to coordinate the fracture healing process, including MSC recruitment, chondrocytic apoptosis and callus resorption through histological analyses and RNA profiling of the callus tissue. Although TNF signaling leads to the activation of NFκB, which has been shown to be essential in early limb bud formation (Kanegae, Tavares et al. 1998), the authors found that TNFR1/TNFR2-deficient animals showed no overt anomalies in the embryological or postnatal aspects of the development of their skeletons (Gerstenfeld, Cho et al. 2003). The authors thus challenged the paradigm that endochondral progression during postnatal fracture repair recapitulates the processes that occur during embryological skeletal development, concluding that the postnatal tissue environment is not comparable to that of embryological development (Ferguson, Alpern et al. 1999;

Gerstenfeld, Cullinane et al. 2003). However, their findings also illustrate the redundancy of the fracture repair process, which can confound observations in experiments that use knockout animals. Other in vitro studies using avian tissues also support the role of TNF in inducing apoptosis of hypertrophic and permanent cartilage chondrocytes (Aizawa, Kon et al. 2001). Chondrocyte apoptosis is an important component in the sequential progression of endochondral bone formation during fracture healing as the mineralized cartilaginous callus must be removed and remodeled to allow formation of primary trabecular bone (Gibson, Kohler et al. 1995; Roach, Erenpreisa et al. 1995). Our group and others have also published findings that TNF can promote osteogenic differentiation and chemotaxis of precursor cells (Hess, Ushmorov et al. 2009; Glass, Chan et al. 2011; Lu, Wang et al. 2013). Fukui et al found that TNF induces BMP-2 in chondrocytes (Fukui, Ikeda et al. 2006).

TNF is also a key component of the innate inflammatory response following sterile injury during which neutrophils are activated to exert their biological activity. Priming by pro-inflammatory cytokines such as TNF and IL-1b or through contact with activated endothelial cells has been shown to be necessary for the greatest level of neutrophil degranulation and activation of the NADPH oxidase pathway (Guthrie, McPhail et al. 1984; Summers, Rankin et al. 2010). My data support the role of TNF during the early inflammatory phase of fracture healing as its depletion during this early phase impaired fracture healing while augmentation promoted repair in vivo. I found that rhTNF promoted the recruitment of monocytes/macrophages to the fracture environment via the upregulation of CCL2.

Hence, it is likely that TNF plays multiple roles at different stages during fracture healing. It is released by neutrophils and promotes the recruitment of monocytes/macrophages via CCL2 (Fig 7.1). It also induces the expression of growth factors including BMPs, promotes the osteogenic differentiation and recruitment of osteoprogenitor cells and induces apoptosis of hypertrophic chondrocytes of the fracture callus. The data presented in this thesis suggest that modulation of TNF at the early inflammatory phase affects the final outcome of fracture healing in vivo.

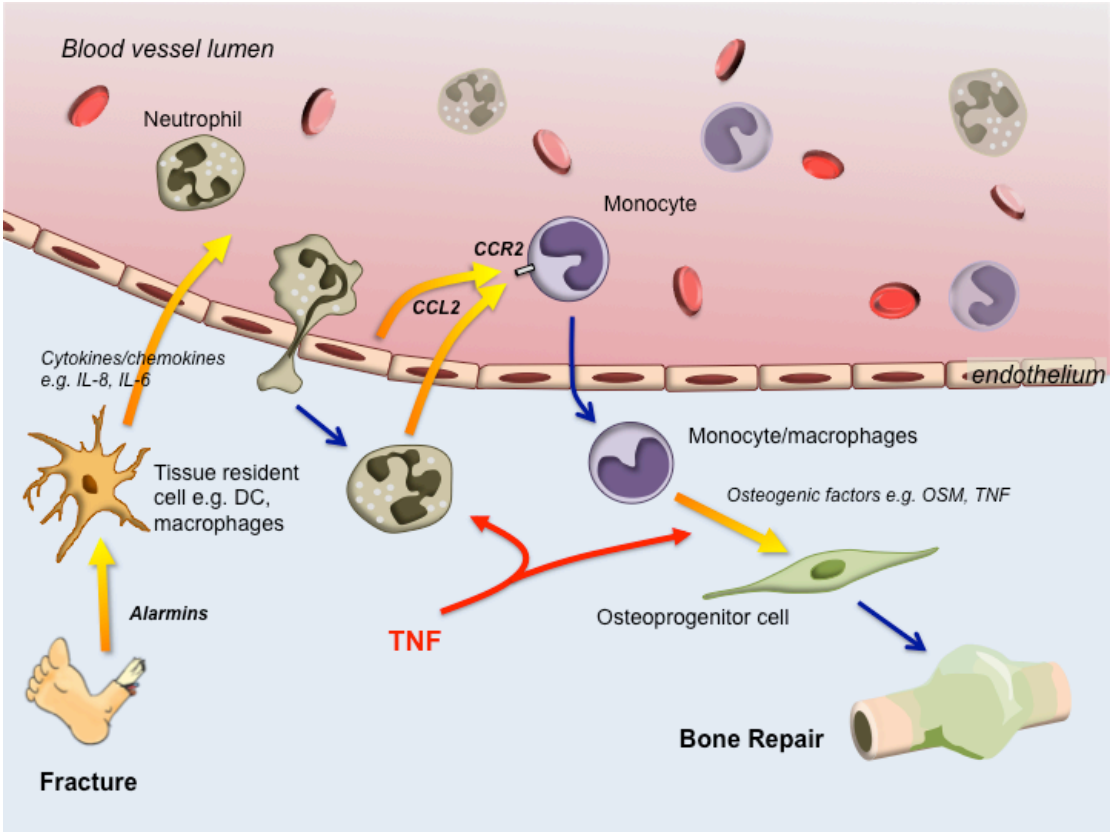


Fig 7.1: Schematic detailing the role of the early inflammatory pathway in fracture healing. Addition of local rhTNF promotes the recruitment of neutrophils and monocytes via upregulation of CCL2, leading to enhancement of fracture repair.

7.1.3 Clinical Relevance

The data presented in this thesis is of clinical relevance as it demonstrates that the early inflammatory response is a viable therapeutic target for the promotion of fracture healing. One approach is the local delivery of recombinant TNF.

The murine fracture model used was designed to reproduce as closely as possible the fracture healing process in the human adult long bone, which occurs by endochondral ossification. It involves the creation of an open tibial osteotomy. Therefore the data described in this thesis would pertain to high energy open tibial injuries in humans. This subset of patients suffers a high rate of complication including delayed and non-union. Whilst there is a requirement for therapies to accelerate fracture healing in patients with high-energy open fractures, the greatest need is for the rapidly growing number of individuals who sustain fragility fractures. Osteoporosis is characterized by low bone mass and weakened bone structure, leading to 1.5 million fragility fractures in the USA every year (Holroyd, Cooper et al. 2008). Half of all patients who sustain fragility fractures involving the femoral neck are permanently disabled and the mortality rate is 21-36% within the first year (Eisman, Bogoch et al. 2012). Hence, there remains a pressing need to develop effective strategies to accelerate healing of fragility fractures. Therefore, I examined whether TNF treatment would also be effective in mice that have been rendered osteoporotic by oophorectomy. I found a 40% improvement in healing at 2 weeks and equivalent mineralization to PBS controls at 4 weeks (Fig 3b). Rates of recovery and mobilization in patients with fragility fractures are critically dependent on fracture healing as premature loading leads to implant failure, accounting for the excessive morbidity and

mortality seen in this vulnerable group of patients. Therefore, acceleration of healing during the early phase of fracture repair is particularly relevant in the clinical setting.

7.2 Methodological Limitations and Future Techniques

7.2.1 In vivo fracture model

The fracture model was designed to replicate the endochondral fracture healing process seen in human long bones. The periosteum is stripped to avoid a disproportionate amount of intramembranous healing as the murine periosteum is highly active. The outcome measures included microCT, histology and weight bearing. The small size of the mouse tibia means that the fracture morphology is heterogeneous in spite of the use of precise and sharp osteotomy pliers. This limits the detection of the effect size and it is possible that biological effects of reagents including rmCCL2 were not identified using this system. Bend testing was attempted but found to be inconsistent and subject to wide variability for fractured bones although this was not problematic for intact bones. As biomechanical testing is considered a gold standard for the evaluation of fracture healing, it would be ideal to validate our findings in larger animals such as rats, dogs or sheep in which the larger sizes of the bones may enable more consistent fracture morphology as well as placement of bones on a bend-testing jig. Compared to three-point loading, four-point loading has the advantage of producing pure bending between the two upper loading points, which ensures

that transverse shear stresses are negated. However, four-point bending also requires that the force at each loading point is equal, which can only be achieved if the test object is a perfect cylinder. As the mouse tibia is highly irregular in shape, consistent placement at all loading points was not possible.

An effective way to minimize the number of animals is the use of an in vivo microCT, which enables each mouse to be followed longitudinally over time. Additionally, the greatest advantage would be that each mouse will serve as its own control so that analyses can be matched to provide more reliable and consistent data. The Kennedy Institute of Rheumatology has now obtained this facility and future experiments will make use of it.

7.2.2 Gait analysis

In clinical trials of fracture healing, functional outcomes are as important as structural parameters of bone healing (Goldhahn, Mitlak et al. 2008). Therefore, longitudinal gait analysis on each mouse over the course of fracture healing would provide valuable data in conjunction with radiographic analysis to determine the clinical outcome of fracture healing. Our laboratory has now obtained an Exergait machine, a treadmill designed to study multiple parameters of gait in mice.

7.2.3 Cell-tracking imaging techniques

In vivo imaging enables non-invasive tracking of inflammatory cells including flurorescently-labelled neutrophils or macrophages in a dynamic and

quantifiable manner (Eisenblatter, Ehrchen et al. 2009). This technique provides a real time alternative to histological techniques to verify the temporal and spatial dynamics of the innate immune cells at the fracture site in response to different candidate factors in future gain and loss of function experiments.

7.2.4 Drug delivery system

Half-lives of recombinant proteins in vivo are typically short (less than 1 hour). This may explain lack of effect of rhTNF if given only once post-fracture or rmCCL2. A number of options including PEGylation and the use of biogels such as pluronic acid, a polyaxamer which is liquid at low temperatures but solidifies at body temperature, or biomaterials such as collagen sponge. When applied at the fracture site, such a preparation will enable improved delivery and localization of the reagent at the fracture site as well as provide a consistent concentration gradient up towards the fracture to enhance the chemotactic effect (Glass, Chan et al. 2011).

7.3 Future directions

To further develop this strategy of controlled upregulation of the early inflammatory pathway in the promotion of fracture healing, the following avenues of investigation will be pursued.

7.3.1 Investigation of macrophage subtypes at the fracture site

After recruitment to the site of inflammation monocytes differentiate into macrophages, which orchestrate the wound healing process. They play a central role in tissue resolution and all stages of wound healing. Their functional phenotype changes during the different phases of healing. Plasticity is a hallmark of these mononuclear phagocytes. Thus, during early inflammation, macrophages exhibit a pro-inflammatory phenotype, including antigen presentation, phagocytosis and production of inflammatory cytokines such as TNF, and growth factors that facilitate the wound healing process. These macrophages are referred to classically activated or M1 phenotype. During the latter phases during which angiogenesis occurs, extracellular matrix is deposited and stem cells undergo proliferation and differentiation, the alternatively activated or M2 macrophages are believed to be responsible for providing the prevailing cytokine microenvironment for these processes to occur. It is likely that these M2 macrophages derive from both the resident macrophages as well as the incoming monocytes. Fully polarized M1 and M2 cells represent extremes of a continuum, and all these subtypes express distinct repertoires of chemokines and chemokine receptors (Mantovani, Sica et al. 2004).

Monocytes migrate from the blood into tissue to replenish tissue-resident macrophages of local tissues including bone (osteomacs and osteoclasts), alveoli (alveolar macrophages) and the central nervous system (microglial cells) (Mosser and Edwards 2008). However, in the blood, monocytes are not a homogenous population of cells. In mice, there are two distinct monocyte populations in the blood that have different phenotypes and biochemical signatures. 'Inflammatory' monocytes are defined as CCR2+, CX_3CR1^{low} and Ly6+ and rapidly exit the blood whereas 'resident' monocytes are CCR2-CX_3CR1^{hi}GR1-. The two populations are approximately equally represented in blood. In humans, monocytes can also be separated into two categories based on cell surface marker expression but they have distinct physiology from that of mouse monocytes. Most human monocytes are either 'classical' $CD14^{hi}$CD16- or 'non-classical' CD14+CD16+. 90% of human monocytes express the classical markers. It is unknown whether specific monocyte populations give rise to specific tissue macrophages. One theory is that monocytes continue to develop and mature in the blood and can be recruited to the tissue at any time, but the point at which they leave defines their function (Mosser and Edwards 2008).

Macrophages can respond to endogenous stimuli or alarmins that are generated following injury. The classically activated macrophages are important players in host defense and key producers of proinflammatory cytokines, including TNF, and can lead to host-tissue damage. Indeed, they are key mediators of the immunopathology that occurs during rheumatoid arthritis and inflammatory bowel disease. The alternatively activated, or 'wound-healing', macrophages are believed to be generated from resident macrophages in response to IL-4

production, a cytokine that is released during tissue injury. They also secrete components of the extracellular matrix.

This thesis shows that addition of recombinant TNF can promote the recruitment of monocytes/macrophages to the fracture environment and that this is associated with enhanced fracture healing. Further studies to elucidate the different subsets of monocytes and macrophages present at the fracture site over a time course by differential immunohistochemical staining should provide further insight into the healing mechanism of bone. However, at present, the methodology to selectively manipulate or deplete macrophage subsets remains challenging.

7.3.2 Comparison of the inflammatory processes in fragility versus non-fragility fractures

There are limited studies that explore the osteoporotic condition at a cellular level. It has been shown that bone marrow derived stromal cells from osteoporotic patients do not show normal proliferation or differentiation patterns in vitro and react differently in response to growth factors when compared to healthy controls (Sterck, Klein-Nulend et al. 1998; Rodriguez, Garat et al. 1999; Rodriguez, Montecinos et al. 2000). More recently, evidence suggests that Th17 cells are a critical modulator in the pathogenesis of oestrogen-deficient osteoporosis and upregulated systemic levels of IL-17 may promote osteoclastogenic cytokines, including TNF (Zhao 2013). Hence it is perhaps surprising that further addition of TNF at the fracture site promoted fracture

healing in this 'pro-inflammatory' condition. Our observation that TNF treatment can accelerate fracture repair in ovariectomised mice suggests that these prevailing factors are not the limiting factors for impaired fracture healing. It would be informative to investigate the effect of rhTNF at the fragility fracture site by histology and using the air pouch model with fracture supernatants from osteoporotic mice to test whether it acts via a similar mechanism as found in the non-ovariectomized mice. One of the most common causes of osteoporosis after post-menopause in women is the long term use of corticosteroids. Therefore, it would also be useful to test the effect of rhTNF in an alternative murine model, glucocorticoid-induced osteoporosis(Weinstein, Jilka et al. 1998), a technique that has recently been established in our laboratory.

7.3.3 Upstream mediators of inflammation and resolution in fracture repair

The ability to enhance healing responses would benefit from a greater understanding of the interplay between MSCs and innate inflammation. MSCs exhibit immunoregulatory and regenerative properties and may be recruited from local tissues or systemically from the bone marrow (Pitchford, Furze et al. 2009). Our group has previously found that proinflammatory cytokines associated with the innate inflammatory response, including TNF, IL-6 and IL-1b, promoted the recruitment and osteogenic differentiation of primary human MSCs (Glass, Chan et al. 2011). This supports the concept that the innate immune response can be targeted to promote regenerative processes following injury or infection. The data presented in this thesis provide further evidence that the

early innate inflammatory response can also be targeted to promote the influx of neutrophils and in turn monocytes and accelerate fracture healing.

Once the dangerous insult has been neutralized and the debris and foreign material removed during inflammation, resolution and tissue repair occur in an attempt to restore pre-injury anatomy and function as closely as possible. Alarmins have a central role in orchestrating this phase of resolution. First, the local microenvironment must be converted from a highly cytotoxic and catabolic state to an anabolic one that is conducive to the recruitment, proliferation and differentiation of precursor cells. Interestingly, recent evidence points to redox regulation of HMGB1 function. High levels of oxidative stress present in the acute inflammatory phase serves as an effective self-limiting mechanism through the oxidation of alarmins. For example, terminal oxidation of HMGB1 abolishes its chemotactic and cytokine-inducing properties, rendering it inactive (Yang, Lundback et al. 2011).

Secondly, the damaged tissues must be replaced either through the recruitment of fibroblasts to form a scar or stem cells to repair and regenerate the local tissues. Of all the alarmins, the regenerative effects of HMGB1 is the best characterized. Indeed, it was first identified as amphoterin due to its neurotropic properties (Merenmies, Pihlaskari et al. 1991). It acts as a chemokine for a variety of cell types, including mesoangioblasts, fibroblasts and myogenic and cardiac precursors (Degryse, Bonaldi et al. 2001; Mitola, Belleri et al. 2006; Palumbo, Galvez et al. 2007), and is able to induce their proliferation (Palumbo, Sampaolesi et al. 2004; Limana, Germani et al. 2005) and differentiation (Sorci,

Riuzzi et al. 2004; Limana, Germani et al. 2005; Germani, Limana et al. 2007; Meng, Guo et al. 2008). It induces neoangiogenesis (Palumbo, Sampaolesi et al. 2004; Mitola, Belleri et al. 2006) by promoting proliferation and migration of mesangioblasts (Degryse, Bonaldi et al. 2001; Palumbo, Sampaolesi et al. 2004; Palumbo, Galvez et al. 2007). Its role in tissue regeneration is best illustrated in a murine model of myocardial infarction, in which exogenous HMGB1 injected locally into the heart led to the recruitment, proliferation and differentiation of myocardial progenitor cells, resulting in improved structural and functional outcomes after infarction (Germani, Limana et al. 2007; Limana, Esposito et al. 2011). Furthermore, cardiac-specific overexpression of HMGB1 reduced tissue damage (Kitahara, Takeishi et al. 2008), leading to improved cardiac function while anti-HMGB1 antibodies exacerbated injury (Oozawa, Mori et al. 2008). HMGB1 has also been shown to contribute to skeletal muscle regeneration by recruiting mesangioblasts, myogenic cells and fibroblasts (De Mori, Straino et al. 2007; Palumbo, Galvez et al. 2007), and myogenic differentiation (Sorci, Riuzzi et al. 2004). Inhibition of HMGB1 with BoxA reduced vessel density and skeletal muscle regeneration, whereas HMGB1 attracted endothelial precursors and stimulated angiogenesis (Schlueter, Weber et al. 2005; Mitola, Belleri et al. 2006; Chavakis, Hain et al. 2007; De Mori, Straino et al. 2007). Topical application of HMGB1 to the wounds of diabetic mice accelerated wound healing (Straino, Di Carlo et al. 2008). HMGB1, together with S100B, also induced neuronal growth, cell survival and migration (Rauvala and Pihlaskari 1987; Daston and Ratner 1991; Hori, Brett et al. 1995; Huttunen, Kuja-Panula et al. 2000); its neurotrophic effect makes it an attractive target to promote neurovascular repair following stroke (Hayakawa, Qiu et al. 2010).

Other alarmins also have regenerative capacities, although their exact modes of action remain to be defined. AMPs including the β-defensins and cathelicidins exhibit pro-angiogenic, chemotactic and proliferative properties (De, Chen et al. 2000; Koczulla, von Degenfeld et al. 2003; Conejo-Garcia, Benencia et al. 2004) and appear to possess regenerative capabilities independent of their antimicrobial activities; they have been shown to play a role in skin wound repair. Overexpression of β-defensin-3 by viral transduction led to improved healing of diabetic wounds (Hirsch, Spielmann et al. 2009) while inhibition of LL-37 with antibodies inhibited re-epithelialization (Heilborn, Nilsson et al. 2003). Moreover, proteomic analysis of MRL/MpJ-Fas^{lpr} mice, which have the ability to regenerate excised ear tissue, revealed distinct up-regulation of members of the S100 protein family, including S100A8 and S100A9(Caldwell, Opalenik et al. 2008). Recombinant IL-33 improved survival in a caecal ligation puncture model of sepsis (Horwitz, Le Blanc et al. 2005).

It remains to be seen how much alarmins can contribute in the tissue homeostasis in adult human tissues, including bone, skin, skeletal muscle, heart and kidney, in which most cells are terminally differentiated and which tend to heal by fibrosis. Nonetheless, the regenerative and repair capacities of alarmins represent a relatively unexplored yet fertile field of tremendous translational interest. It will require better understanding of the functional regulation of alarmins.

As previously discussed, alarmins, like many other signaling molecules, can exhibit both harmful and beneficial effects within the same disease context,

depending on factors including timing and dosage(Chan, Roth et al. 2012). Total abolition of the host defense is detrimental, leaving the patient susceptible to opportunistic infections and tumorigenesis as well as impairing repair and remodeling pathways. Alternatively, the alarmin-signaling axis can also be manipulated to activate transient and self-limited inflammation and pathways that orchestrate tissue homeostasis. This is likely to be achieved by a better understanding of how the microenvironment and dosage contribute to the net effects of alarmins. For example, it has been found that low doses of S100B induce trophic effects in neurites whereas high doses induce apoptosis (Huttunen, Kuja-Panula et al. 2000). Furthermore, we must understand how to clinically modulate the local environment so as to activate the innate protective pathways that initiate regenerative and repair processes whilst down-regulating the self-injurious pathways that inhibit repair and drive excessive and deleterious cytokine release. An area that deserves particular attention is the mechanism by which alarmins mediate the interaction between alarmin-stimulated dendritic cells and tissue-repair macrophages, as this would offer the prospect of a rational, mechanism-based approach to promote wound repair.

7.4 Conclusions

The combined in vivo and in vitro data presented in this thesis show that addition of low dose rhTNF during the early inflammatory response promotes the recruitment of neutrophils and monocytes via upregulation of CCL2 and leads to acceleration of fracture repair in vivo. They also demonstrate that the role of neutrophils is not simply limited to clearance of pathogens and cellular debris. Neutrophils orchestrate the next stage of resolution and regeneration through the recruitment of monocytes. Mechanistically, local administration of a low dose of rhTNF at the fracture site shortly after injury acts on multiple levels. My data shows that TNF is a critical cytokine in the fracture healing pathway and that local addition of TNF potentiates the early innate immune response comprising neutrophils followed by monocyte/macrophage recruitment to accelerate the physiological healing processes through the CCL2/CCR2 axis. Our group and others have also found TNF to promote osteogenic differentiation and recruitment of osteoprogenitors, induction of BMPs and apoptosis of hypertrophic chondrocytes of the fracture callus.

By systematically unraveling the early events in the fracture healing pathway, we have identified the potential of enhancing the early innate immune response following fracture to accelerate fracture repair. This has profound implications in the clinical setting. For example, during surgical treatment of fractures, especially open fractures, surgeons often lavage the wound extensively in order to reduce the risk of infection, but also unintentionally deplete the wound bed of crucial mediators as well as immune and osteoprogenitor cells. Therapeutic up-

regulation of the innate immune system may be especially relevant under these circumstances. However, of greatest potential clinical significance is the efficacy of this novel regenerative therapy in osteoporotic bone, a major unmet clinical need.

References

Aizawa, T., T. Kon, et al. (2001). "Induction of apoptosis in chondrocytes by tumor necrosis factor-alpha." J Orthop Res **19**(5): 785-796.

Alexander, K. A., M. K. Chang, et al. (2011). "Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model." J Bone Miner Res **26**(7): 1517-1532.

Andrew, J. G., S. M. Andrew, et al. (1994). "Inflammatory cells in normal human fracture healing." Acta Orthop Scand **65**(4): 462-466.

Caldwell, R. L., S. R. Opalenik, et al. (2008). "Tissue profiling MALDI mass spectrometry reveals prominent calcium-binding proteins in the proteome of regenerative MRL mouse wounds." Wound Repair Regen **16**(3): 442-449.

Chan, J. K., J. Roth, et al. (2012). "Alarmins: awaiting a clinical response." J Clin Invest **122**(8): 2711-2719.

Chavakis, E., A. Hain, et al. (2007). "High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells." Circ Res **100**(2): 204-212.

Chen, D., M. Zhao, et al. (2004). "Signal transduction and biological functions of bone morphogenetic proteins." Front Biosci **9**: 349-358.

Conejo-Garcia, J. R., F. Benencia, et al. (2004). "Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A." Nat Med **10**(9): 950-958.

Daston, M. M. and N. Ratner (1991). "Expression of P30, a protein with adhesive properties, in Schwann cells and neurons of the developing and regenerating peripheral nerve." J Cell Biol **112**(6): 1229-1239.

De Mori, R., S. Straino, et al. (2007). "Multiple effects of high mobility group box protein 1 in skeletal muscle regeneration." Arterioscler Thromb Vasc Biol **27**(11): 2377-2383.

De, Y., Q. Chen, et al. (2000). "LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells." J Exp Med **192**(7): 1069-1074.

Degryse, B., T. Bonaldi, et al. (2001). "The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells." J Cell Biol **152**(6): 1197-1206.

Einhorn, T. A., R. J. Majeska, et al. (1995). "The expression of cytokine activity by fracture callus." J Bone Miner Res **10**(8): 1272-1281.

Eisenblatter, M., J. Ehrchen, et al. (2009). "In vivo optical imaging of cellular inflammatory response in granuloma formation using fluorescence-labeled macrophages." J Nucl Med **50**(10): 1676-1682.

Eisman, J. A., E. R. Bogoch, et al. (2012). "Making the first fracture the last fracture: ASBMR task force report on secondary fracture prevention." J Bone Miner Res **27**(10): 2039-2046.

Ferguson, C., E. Alpern, et al. (1999). "Does adult fracture repair recapitulate embryonic skeletal formation?" Mech Dev **87**(1-2): 57-66.

Fukui, N., Y. Ikeda, et al. (2006). "Pro-inflammatory cytokine tumor necrosis factor-alpha induces bone morphogenetic protein-2 in chondrocytes via

mRNA stabilization and transcriptional up-regulation." J Biol Chem **281**(37): 27229-27241.
Germani, A., F. Limana, et al. (2007). "Pivotal advances: high-mobility group box 1 protein--a cytokine with a role in cardiac repair." J Leukoc Biol **81**(1): 41-45.
Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.
Gerstenfeld, L. C., D. M. Cullinane, et al. (2003). "Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation." J Cell Biochem **88**(5): 873-884.
Gibson, G. J., W. J. Kohler, et al. (1995). "Chondrocyte apoptosis in endochondral ossification of chick sterna." Dev Dyn **203**(4): 468-476.
Glass, G. E., J. K. Chan, et al. (2011). "TNF-{alpha} promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells." Proc Natl Acad Sci U S A.
Goldhahn, J., B. Mitlak, et al. (2008). "Critical issues in translational and clinical research for the study of new technologies to enhance bone repair." J Bone Joint Surg Am **90 Suppl 1**: 43-47.
Guthrie, L. A., L. C. McPhail, et al. (1984). "Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme." J Exp Med **160**(6): 1656-1671.
Hankemeier, S., S. Grassel, et al. (2001). "Alteration of fracture stability influences chondrogenesis, osteogenesis and immigration of macrophages." J Orthop Res **19**(4): 531-538.
Hayakawa, K., J. Qiu, et al. (2010). "Biphasic actions of HMGB1 signaling in inflammation and recovery after stroke." Ann N Y Acad Sci **1207**: 50-57.
Heilborn, J. D., M. F. Nilsson, et al. (2003). "The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium." J Invest Dermatol **120**(3): 379-389.
Hess, K., A. Ushmorov, et al. (2009). "TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway." Bone **45**(2): 367-376.
Hirsch, T., M. Spielmann, et al. (2009). "Human beta-defensin-3 promotes wound healing in infected diabetic wounds." J Gene Med **11**(3): 220-228.
Holroyd, C., C. Cooper, et al. (2008). "Epidemiology of osteoporosis." Best Pract Res Clin Endocrinol Metab **22**(5): 671-685.
Hori, O., J. Brett, et al. (1995). "The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system." J Biol Chem **270**(43): 25752-25761.
Horwitz, E. M., K. Le Blanc, et al. (2005). "Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement." Cytotherapy **7**(5): 393-395.
Huttunen, H. J., J. Kuja-Panula, et al. (2000). "Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for

advanced glycation end products (RAGE) activation." J Biol Chem **275**(51): 40096-40105.
Kanegae, Y., A. T. Tavares, et al. (1998). "Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb." Nature **392**(6676): 611-614.
Kitahara, T., Y. Takeishi, et al. (2008). "High-mobility group box 1 restores cardiac function after myocardial infarction in transgenic mice." Cardiovasc Res **80**(1): 40-46.
Koczulla, R., G. von Degenfeld, et al. (2003). "An angiogenic role for the human peptide antibiotic LL-37/hCAP-18." J Clin Invest **111**(11): 1665-1672.
Limana, F., G. Esposito, et al. (2011). "HMGB1 attenuates cardiac remodelling in the failing heart via enhanced cardiac regeneration and miR-206-mediated inhibition of TIMP-3." PLoS One **6**(6): e19845.
Limana, F., A. Germani, et al. (2005). "Exogenous high-mobility group box 1 protein induces myocardial regeneration after infarction via enhanced cardiac C-kit+ cell proliferation and differentiation." Circ Res **97**(8): e73-83.
Lu, Z., G. Wang, et al. (2013). "Activation and promotion of adipose stem cells by tumour necrosis factor-alpha preconditioning for bone regeneration." J Cell Physiol **228**(8): 1737-1744.
Mantovani, A., A. Sica, et al. (2004). "The chemokine system in diverse forms of macrophage activation and polarization." Trends Immunol **25**(12): 677-686.
Meng, E., Z. Guo, et al. (2008). "High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway." Stem Cells Dev **17**(4): 805-813.
Merenmies, J., R. Pihlaskari, et al. (1991). "30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane." J Biol Chem **266**(25): 16722-16729.
Mitola, S., M. Belleri, et al. (2006). "Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine." J Immunol **176**(1): 12-15.
Mizoguchi, T., A. Muto, et al. (2009). "Identification of cell cycle-arrested quiescent osteoclast precursors in vivo." J Cell Biol **184**(4): 541-554.
Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-969.
Oozawa, S., S. Mori, et al. (2008). "Effects of HMGB1 on ischemia-reperfusion injury in the rat heart." Circ J **72**(7): 1178-1184.
Palumbo, R., B. G. Galvez, et al. (2007). "Cells migrating to sites of tissue damage in response to the danger signal HMGB1 require NF-kappaB activation." J Cell Biol **179**(1): 33-40.
Palumbo, R., M. Sampaolesi, et al. (2004). "Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation." J Cell Biol **164**(3): 441-449.
Pitchford, S. C., R. C. Furze, et al. (2009). "Differential mobilization of subsets of progenitor cells from the bone marrow." Cell Stem Cell **4**(1): 62-72.

Rauvala, H. and R. Pihlaskari (1987). "Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons." J Biol Chem **262**(34): 16625-16635.
Roach, H. I., J. Erenpreisa, et al. (1995). "Osteogenic differentiation of hypertrophic chondrocytes involves asymmetric cell divisions and apoptosis." J Cell Biol **131**(2): 483-494.
Rodriguez, J. P., S. Garat, et al. (1999). "Abnormal osteogenesis in osteoporotic patients is reflected by altered mesenchymal stem cells dynamics." J Cell Biochem **75**(3): 414-423.
Rodriguez, J. P., L. Montecinos, et al. (2000). "Mesenchymal stem cells from osteoporotic patients produce a type I collagen-deficient extracellular matrix favoring adipogenic differentiation." J Cell Biochem **79**(4): 557-565.
Schlueter, C., H. Weber, et al. (2005). "Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule." Am J Pathol **166**(4): 1259-1263.
Sorci, G., F. Riuzzi, et al. (2004). "Amphoterin stimulates myogenesis and counteracts the antimyogenic factors basic fibroblast growth factor and S100B via RAGE binding." Mol Cell Biol **24**(11): 4880-4894.
Sterck, J. G., J. Klein-Nulend, et al. (1998). "Response of normal and osteoporotic human bone cells to mechanical stress in vitro." Am J Physiol **274**(6 Pt 1): E1113-1120.
Straino, S., A. Di Carlo, et al. (2008). "High-mobility group box 1 protein in human and murine skin: involvement in wound healing." J Invest Dermatol **128**(6): 1545-1553.
Summers, C., S. M. Rankin, et al. (2010). "Neutrophil kinetics in health and disease." Trends Immunol **31**(8): 318-324.
Wallace, A., T. E. Cooney, et al. (2011). "Effects of interleukin-6 ablation on fracture healing in mice." J Orthop Res **29**(9): 1437-1442.
Weinstein, R. S., R. L. Jilka, et al. (1998). "Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone." J Clin Invest **102**(2): 274-282.
Xing, Z., C. Lu, et al. (2010). "Multiple roles for CCR2 during fracture healing." Dis Model Mech **3**(7-8): 451-458.
Yang, H., P. Lundback, et al. (2011). "Redox modification of cysteine residues regulates the cytokine activity of HMGB1." Mol Med.
Zhao, R. (2013). "Immune regulation of bone loss by Th17 cells in oestrogen-deficient osteoporosis." Eur J Clin Invest **43**(11): 1195-1202.

Appendices

Grants and Fellowships

2013	Paton-Masser Memorial Fund – British Association of Plastic, Reconstructive and Aesthetic Surgeons (£5,000)
2012	Small Research Support Grant – Royal College of Surgeons of Edinburgh (£10,000)
2011	Wellcome Clinical Research Training Fellowship (£249,591)
2011	Royal College of Surgeons of England Research Fellowship (£3,500)
2011	MRC Clinical Research Training Fellowship – *awarded, not accepted*
2011	Arthritis Research UK Clinical Fellowship – *awarded, not accepted*

Prizes / Awards

2013	Rosetrees Essay Prize runner up – Royal College of Surgeons of England
2013	Senior Scholarship – Lincoln College, Oxford
2013	European Federation of Immunological Societies Travel Grant
2013	Review article (Chan et al JCI 2012) listed as top article published on the topic according to BioMedLib.
2012	Senior Scholarship – Lincoln College, Oxford
2012	Bone Research Society Best Oral Presentation – Osteoporosis and Bone Conference
2011	Barron Prize – British Association of Plastic, Reconstructive and Aesthetic Surgeons
2011	Winner: 'I'm a Scientist, Get me out of here' – Wellcome Trust-funded science communication and engagement competition
2011	UK-Israel Regenerative Medicine Conference Travel Award

Peer-reviewed publications

1. *Verhoekx JS, Verjee LS, Izadi D,* ***Chan JK****, Nicolaidou V, Davidson D, Midwood KS, Nanchahal J.* Isometric contraction of Dupuytren's Myofibroblasts in inhibited by blocking intercellular junctions.
   Journal of Investigative Dermatology. 2013; epub
2. *Verjee LS, Verhoekx JS,* ***Chan JK****, Grausgruber T, Nicolaidou V, Izadi D, Davidson D, Feldman M, Midwood K, Nanchahal J*. Unravelling the signaling pathways promoting fibrosis in Dupuytren's disease reveals TNF as a novel therapeutic target.
   PNAS. 2013; 110(10): E928-37
3. ***Chan JK****, Shalhoub J, Gardiner MD, Suleman-Verjee L, Nanchahal J*. Strategies to secure surgical research funding: fellowships and grants.
   JRSM Open. 2014; 5(1):2042533313505512
4. ***Chan JK****, Roth J, Oppenheim J, Tracey K, Vogl T, Feldmann M, Horwood N, Nanchahal J*. Alarmins: Awaiting a Clinical Response.
   Journal of Clinical Investigation. *2012*; 122(8): 2711-9
5. ***Chan JK****, Harry L, Williams G, Nanchahal J*. Soft-tissue reconstruction of open fractures of the lower limb: muscle versus fasciocutaneous flaps.
   Plast Reconstr Surg 2012; 130(2): 284e-95e
6. *Glass GE,* ***Chan JK****, Freidin A, Feldmann M, Horwood N, Nanchahal J*. TNF-α promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells.
   PNAS. 2011; 108(4): 1585-90

Abstracts

1. ***Chan JK****, Harry L, Glass G, Horwood N, Nanchahal J.* Acceleration of fracture repair with muscle.
   Osteoporos Int 23(Suppl 5):S537
2. ***Chan JK****, Glass G, Horwood N, Nanchahal J.* TNF-α: A Therapeutic Target in Bone Repair.
   Osteoporos Int 23(Suppl 5): S546

Podium Presentations

International

1. Espirito Santo AI, **Chan JK**, et al. The Role of Alarmins in Fracture Repair. European Calcified Tissue Society, Prague. (Joint 1st authors, awarded ECTS New Investigator Award).
2. ***Chan JK****, Glass GE, Ersek A, Williams GA, Freidin A, Gowers K, Espirito Santo A, Jeffery R, Otto WR, Poulsom R, Feldmann M, Rankin SM, Horwood NJ, Nanchahal J.* 2013. The innate immune response – a potential therapeutic target in the acceleration of fracture repair in normal and osteoporotic fractures. *Joint meeting of the Bone Research Society and the British Orthopaedic Research Society, Oxford*
3. ***Chan JK****, Glass GE, Ersek A, Williams GA, Freidin A, Gowers K, Jeffery R, Otto WR, Poulsom R, Feldmann M, Rankin SM, Horwood NJ, Nanchahal J.* 2013. Strategies to accelerate fracture repair.

Portugese Congress of Internal Medicine, Portugal ***(Invited speaker)***

4. ***Chan JK****, Horwood N, Nanchahal J.* 2012. Regenerative role of inflammation and alarmins.
   4th International Münster Meeting on Danger Signaling in Autoinflammation, Münster, Germany

National

1. ***Chan JK,*** *Harry L, Glass G, Horwood N, Nanchahal J.* 2012. Acceleration of fracture repair with muscle.
   Osteoporosis and Bone Conference, National Osteoporosis Society/Bone Research Society, Manchester
2. ***Chan JK,*** *Glass G, Harry L, Horwood N, Nanchahal J.* 2012. TNF-α: a therapeutic target in bone repair.
   Osteoporosis and Bone Conference, National Osteoporosis Society/Bone Research Society, Manchester **(Best oral presentation)**

Poster Presentations

International

1. Espirito Santo AI, **Chan JK**, et al. The Role of Alarmins in Fracture Repair. 2014. European Society for Clinical Investigation. (Joint 1st authors).
2. Espirito Santo AI, **Chan JK**, et al. Enhancement of Fracture Repair by Upregulation of the Innate Immune Response. 2014. European Society for Clinical Investigation. (Joint 1st authors)
3. Espirito Santo AI, **Chan JK**, et al. Enhanceent of Fracture Repair by Upregulation of the Innate Immune Response. 2014. European Calcified Tissue Society, Prague. (Joint 1st authors)
4. ***Chan JK****, Glass GE, Ersek A, Williams GA, Freidin A, Gowers K, Jeffery R, Otto WR, Poulsom R, Feldmann M, Rankin SM, Horwood NJ, Nanchahal J.* 2013. Harnessing the innate immune system to accelerate fracture healing in osteoporotic mice.
   Annual Scientific Meeting, European Society for Clinical Investigation, Portugal
5. ***Chan JK,*** *Harry L, Glass G, Horwood N, Nanchahal J.* 2011. Acceleration of fracture repair with muscle.
   UK-Israel Regenerative Medicine Conference, BIRAX, Israel
6. ***Chan JK,*** *Glass G, Harry L, Horwood N, Nanchahal J.* 2011. TNF-α: a therapeutic target in fracture repair.
   UK-Israel Regenerative Medicine Conference, BIRAX, Israel
7. ***Chan JK,*** *Harry L, Glass G, Horwood N, Nanchahal J.* 2011. Acceleration of Fracture Repair: muscle v. fasciocutaneous flap.
   Where Science Meets Clinics, AO Foundation, Switzerland
8. ***Chan JK,*** *Glass G, Harry L, Horwood N, Nanchahal J.* 2011. Acceleration of Fracture Repair: TNF-α as a potential therapeutic.
   Where Science Meets Clinics, AO Foundation, Switzerland

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